# Development and Characterisation of an *in vivo* Model of Pulmonary $\beta_2$-Adrenoceptor Desensitisation in the Rat.

**Paul Anthony Finney**

**A Thesis**

**Submitted for the Degree of**

**Doctor of Philosophy**

**in the Faculty of Science**

**of the University of London**

**Thoracic Medicine**

**Imperial College School of Medicine at the National Heart & Lung Institute**

**Dovehouse Street**

**London SW3 6LY**

**October 1999**

BIBL LONDON UNIV

**Declaration**

The analysis of salmeterol-treated rat plasma for metabolites was undertaken by Andy Harris at Glaxo-Wellcome, Ware, Herts.

The GRK phosphorylation assays were under taken by Aaron Chuang at Glaxo-Wellcome, Ware, Herts.

**Acknowledgements.**

I wish to thank Prof. Peter Barnes for providing the opportunity to work on this project and to Glaxo-Wellcome for funding the project. I'd like to place this thesis at the foot of Mark Giembycz and Ian Adcock's office door - without them agreeing to take on a tarnished and slightly dodgy student...who knows? Mark's expertise, advice, and general patience with me deserves a canonisation.

Many thanks to Maria Belvisi for free advice on both scientific and real-life stuff and an unbiased ear on the project, and for sticking up for me when push came to shove (and shove it nearly was!). Thanks to Andy Harris at Glaxo-Wellcome, Stevenage for performing the metabolism experiments for salmeterol, and Aaron Chuang and Carol Scorer for the GRK study and for free advice! Good NHLI stuff! - Rousey - the main man. Thanks to Louise Donnelly for always having something constructive to offer! Other VIP's Dave Stevens for comradeship and being a great laugh. The football posse - McTomdom Gilbey, Max Corden, Micky Salmon. All those others that made life bearable at the institute Hardeep, Linners, Hema, Mike Saunders, Koremu, Pat, Duncan and Oddjob to name a few).

Other great friends - Tony Fawcett, Dave Curley, James Kershaw, Jason Knibbs, David Manning, Elaine Johnstone, Juliew Gough, Louise Struthers, Rik Appleyard, Juliet Wildgoose, Jon Fyfe, and last but not least, Dave "Beno" Beniston, the extended member of my family. All of these people have helped shaped who and what I am.

A mention to my family, the best family in the world - long may we remain best friends - my brothers Andrew and Simon, and my sister Jacqueline (and Nick too!!). Many thanks to my Dad and Nan for their support during my life so far. Finally, a mention for my Mum, the person who most of all has guided and shaped my life, and still does. A person who inspired me with her tenacity at working so hard and enjoying life, her bravery at facing what she did with her dignity and pride intact, and her wonderful, wicked sense of humour which we all loved and miss. This thesis, and my PhD, are dedicated to the memory of Gloria May Finney, my best friend and the person to which I owe the most. I miss you and will never forget you.

**Abstract.**

Inhaled $\beta_2$-adrenoceptor ($\beta_2$AR) agonists are the most effective bronchodilator therapy available for the treatment of asthma. However, many studies have demonstrated tachyphylaxis to the protective effects of $\beta_2$AR agonists in humans, and there have been epidemics of asthma deaths which have been attributed to overuse of this class of drug. Although $\beta_2$AR desensitisation has been studied extensively *in vitro*, relatively little is known of the biochemical and functional consequences of chronic exposure to $\beta_2$AR-agonists *in vivo*. Thus, the aim of the research described in this thesis was to develop a chronic *in vivo* model of $\beta_2$AR desensitisation in the rat and to determine the biochemical basis of this phenomenon.

Subcutaneous infusion of rats with salbutamol and salmeterol for seven days reduced the ability of these $\beta_2$-adrenoceptor agonists, given acutely, to protect against acetylcholine (ACh)-induced bronchoconstriction when compared to rats treated identically with vehicle. The bronchoprotective effect of prostaglandin $E_2$ ($PGE_2$), which relaxes tracheal smooth muscle in the rat by activating $EP_4$-like prostenoid receptors, was also impaired indicating that the desensitisation was heterologous. In contrast, forskolin, which directly activates eight out of the nine isoforms of adenylyl cyclase, protected the airways against ACh-induced bronchoconstriction in vehicle- and salbutamol-treated animals indicating that the primary defect in signalling was upstream of cAMP formation. $\beta_2$AR density (estimated from the binding of [$^{125}$I]iodocyanopindolol in the presence of the $\beta_1$AR antagonist CGP 20712A), but not mRNA expression, was significantly reduced in lung membranes harvested from salbutamol- and salmeterol-treated animals without any change in the affinity of the radioligand. Moreover, in salbutamol-treated rats, this was associated with impaired salbutamol-induced cAMP accumulation and activation of cAMP-dependent protein kinase *ex vivo*. $G_{s\alpha}$, but not $G_\beta$, levels were markedly reduced in the lung and tracheal smooth muscle of salbutamol- and salmeterol-treated rats and, in the case of the former agonist, the capability of cholera toxin, which directly activates $G_s$, to increase cAMP mass in the lung was blunted indicating compromised coupling of $G_S$ to adenylyl cyclase. Chronic treatment of rats with salbutamol also increased the activity of the type 3 and type 4 cAMP phosphodiesterase isoenzyme families, and G protein-coupled receptor kinase-2, but the contribution of these effects to $\beta_2$AR desensitisation was unclear given that forskolin was active in both groups of animals and that desensitisation was heterologous. Collectively, these results indicate that chronic treatment of rats with salbutamol and salmeterol results in heterologous desensitisation of pulmonary $G_s$-coupled receptors with down-regulation of $G_{s\alpha}$ representing a primary molecular aetiology. These results could have significant clinical implications since a reduction in the abundance of $G_{s\alpha}$ could exacerbate pulmonary inflammation by compromising the activity of endogenous anti-inflammatory mediators, such as $PGE_2$, prostacyclin and certain catecholamines, that also act through $G_s$-linked receptors.

# Table of Contents


| | *Page* |
|---|---|
| **Title.** | **1** |
| **Declaration.** | **2** |
| **Acknowledgements.** | **3** |
| **Abstract.** | **4** |
| **Table of Contents.** | **5** |
| **List of Figures.** | **12** |
| **List of Tables.** | **16** |
| **List of Abbreviations.** | **17** |
| **1 Introduction.** | **20** |
| *1.1 The molecular architecture of the β-adrenoceptor.* | **20** |
| 1.1.1 The G protein-coupled receptor superfamily. | **20** |
| 1.1.2 Structural homology among the GPCRs. | **21** |
| 1.1.3 The ligand binding domain. | **24** |
| 1.1.4 The G protein-coupling domain. | **26** |
| *1.2 Coupling of the GPCRs.* | **28** |
| 1.2.1 G protein structure and function. | **28** |

1.2.1.1 The α-subunit. 28

1.2.1.2 The βγ subunits. 30

1.2.2 Adenylyl cyclase. 31

1.2.3 Cyclic adenosine-3', 5'-monophosphate and airway smooth muscle. 35

1.2.4 Non cyclic AMP-mediated relaxation of airway smooth muscle/interaction with ion channels. 36

1.2.5 $\beta_2$-adrenoceptor agonists. 39

1.2.5.1 Endogenous and exogenous agonists. 39

1.2.5.2 Salbutamol and salmeterol. 40

*1.3 Localisation and function of $\beta_2$-adrenoceptors.* 43

1.3.1 Airway smooth muscle. 43

1.3.2 $\beta_2$-adrenoceptor agonists and inflammation. 45

*1.4 Regulation of $\beta_2$-adrenoceptor expression.* 47

1.4.1 Receptor desensitisation and down-regulation: nomenclature. 47

1.4.2 The role of phosphorylation in receptor regulation. 48

1.4.2.1 GPCR kinases. 48

1.4.2.2 Second messenger kinases. 52

1.4.3 Arrestins. 53

1.4.4 Receptor sequestration. 55

1.4.5 Receptor cycling. 56

1.4.6 Down-regulation. 59

1.4.6.1 Receptor degradation. 59

1.4.6.2 Regulation of receptor synthesis. 60

1.4.7 Regulation of signalling downstream from the receptor. 61

1.4.7.1 Regulation at the level of G proteins. 61

1.4.7.2 Possible involvement of PDE up-regulation. 62

1.4.7.3 Regulation of G protein signalling by RGS proteins. 64

*1.5 Adverse effects of $\beta_2$-adrenoceptor agonists.* 65

1.5.1 Tolerance. 65

1.5.2 Other adverse effects. 67

*1.6 Use of animal models.* 69

*1.7 Scope of thesis.* 70

**2 Materials and Methods.** 72

*2.1 Measurement of bronchoconstriction in vivo.* 72

2.1.1 Animals. 72

2.1.2 Animal preparation. 72

2.1.3 Dissection and description of preparation. 73

2.1.4 Measurement of ACh-induced bronchoconstriction. 73

2.1.5 Measurement of bronchoprotection in rats treated chronically with salbutamol, salmeterol or $PGE_2$. 74

*2.2 Radioligand binding studies.* 75

2.2.1 Preparation of lung membranes. 75

2.2.2 Radioligand binding. 75

2.2.3 Data analysis. **76**

*2.3 Northern blot analysis.* **76**

2.3.1 Probes for Northern analyses. **76**

2.3.2 Isolation of mRNA. **76**

2.3.3 Northern blotting. **77**

2.3.4 Prehybridisations and hybridisations. **77**

*2.4 Cyclic AMP experiments.* **78**

2.4.1 Time-response studies. **78**

2.4.2 Concentration-response studies. **78**

2.4.3 Radioimmunoassay of cAMP. **79**

*2.5 Preparation and assay of PKA.* **79**

*2.6 PDE experiments.* **80**

*2.6.1 Measurement of PDE activity.* **80**

*2.7 Western blot analysis.* **81**

2.7.1 Extraction of total, cytoplasmic and membrane protein. **81**

2.7.2 Western blot analysis. **81**

*2.8 Assessment of GRK2 activity.* **82**

2.8.1 Preparation of cytosolic membrane fractions. **82**

2.8.2 Preparation of rod outer segments. **83**

2.8.3 Phosphorylation assay. **83**

*2.9 Protein estimations.* **84**

*2.10 Reagents.* **84**

*2.11 Statistical analyses.* **85**

**3 Results.** **87**

*3.1 Effect of chronic treatment of rats with salbutamol and $PGE_2$ on the acute protective action of salbutamol, $PGE_2$, IBMX, forskolin, and levcromakalim against ACh-induced bronchoconstriction in vivo.* **87**

3.1.1 Introduction. **87**

3.1.2 Dose-response relationship to ACh in anaesthetised rats treated chronically with saline and salbutamol. **88**

3.1.3 Effect of repeated ACh challenge on the magnitude of bronchoconstriction in rats treated chronically with saline and salbutamol. **89**

3.1.4 Effect of chronic treatment of rats with salbutamol on the ability of salbutamol, $PGE_2$, IBMX, forskolin and levcromakalim given acutely to protect against ACh-induced bronchoconstriction. **90**

3.1.5 Effect of chronic treatment of rats with $PGE_2$ on the abiiity of $PGE_2$ and salbutamol given acutely to protect against ACh-induced bronchoconstriction. **98**

3.1.6 Discussion **100**

*3.2 An investigation of the biochemical effects associated with $\beta_2$-adrenoceptor desensitisation in rats treated chronically with salbutamol* **105**

3.2.1 Introduction. **105**

3.2.2 β-Adrenoceptor density and mRNA expression. **107**

3.2.3 Effect of chronic treatment of rats with salbutamol on $G_{s\alpha}$ coupling to the βAR signal transduction cascade. **111**

3.2.4 Effect of chronic treatments of rats with salbutamol on expression of $G_{s\alpha}$ and $G_{\beta}$ proteins. **112**

3.2.5 Effect of chronic treatment of rats with salbutamol on salbutamol- and $PGE_2$-induced cAMP accumulation *ex vivo*. **114**

3.2.6 Effect of chronic treatment of rats with salbutamol on PDE isoenzyme activity. **118**

3.2.7 Effect of chronic treatment of rats with salbutamol on PKA activity. **120**

3.2.8 Effect of chronic treatment of rats with salbutamol on GRK activity and GRK2 protein expression. **120**

3.2.9 Effect of chronic treatment of rats with salbutamol on β-arrestin protein expression. **123**

3.2.10 Effect of chronic treatment of rats with salbutamol on salbutamol-induced cAMP elevation *ex vivo*, and $G_{s\alpha}/G_{\beta}$ protein expression in rat trachea. **124**

3.2.11 Discussion. **126**

*3.3 Effect of chronic treatment of rats with salmeterol on pulmonary $\beta_2$-adrenoceptor-mediated signalling.* **136**

3.3.1 Introduction. **136**

3.3.2 Effect of chronic treatment of rats with salmeterol on the **137**

ability of salmeterol and $PGE_2$, given acutely, to protect against ACh-induced bronchoconstriction.

3.3.3.1 Effect of chronic treatment of rats with salmeterol on the ability of salbutamol, given acutely, to protect against ACh-induced bronchoconstriction. **142**

3.3.3.2 On the mechanism of the short-acting behaviour of salmeterol. **144**

3.3.4 β-Adrenoceptor density and mRNA expression. **144**

3.3.5 Effect of chronic treatment of rats with salmeterol on the expression of $\beta_2$-adrenoceptor protein measured by western blotting. **149**

3.3.6 Effect of chronic treatment of rats with salmeterol on the expression of $G_{s\alpha}$ and $G_{\beta}$. **150**

3.3.7 Effect of chronic treatment of rats with salmeterol on the ability of salmeterol and $PGE_2$ to elevate the intracellular cAMP content in rat lung *ex vivo*. **152**

3.3.8 Effect of chronic treatment of rats with salmeterol on GRK activity and GRK2 expression. **154**

3.3.9 Discussion. **156**

**4 General Discussion.** **164**

**5 References.** **170**

**6 Appendix.** **219**

**7 Appendix 2.** **220**

## List of Figures


| Figure | Title | Page |
|---|---|---|
| 1.1 | Primary structure of the $\beta_2$-adrenoceptor. | **22** |
| 1.2. | Crystallographic structure of a G protein. | **32** |
| 1.3 | Topology of adenylyl cyclase. | **34** |
| 1.4 | cAMP-dependent and independent regulation of high-conductance calcium-activated potassium channels ($BKCa^{2+}$). | **38** |
| 1.5 | Structure of salbutamol and salmeterol. | **39** |
| 1.6 | β-Adrenoceptor signal transduction cascade. | **42** |
| 1.7 | Endocytosis and cycling of $\beta_2$-adrenoceptors after agonist stimulation. | **58** |
| 2.1 | *In vivo* airway resistance instrumentation. | **74** |
| 2.2 | Summary of methodology. | **86** |
| 3.1.1 | Dose-dependence of ACh-induced bronchoconstriction in anaesthetised rats treated chronically with saline and salbutamol. | **89** |
| 3.1.2 | Effect of repeated ACh challenge on the magnitude of bronchoconstriction in rats treated chronically with vehicle and salbutamol. | **90** |
| 3.1.3 | Effect of chronic treatment of rats with salbutamol on the ability of salbutamol, given acutely, to protect against ACh-induced bronchoconstriction. | **91** |
| 3.1.4 | Representative traces of airway resistance experiments. | **92** |
| 3.1.5 | Effect of chronic treatment of rats with salbutamol on the ability of $PGE_2$, given acutely, to protect against ACh-induced bronchoconstriction. | **94** |
| 3.1.6 | Effect of chronic treatment of rats with salbutamol on the | **95** |

ability of IBMX, given acutely, to protect against ACh-induced bronchoconstriction.

3.1.7 Effect of chronic treatment of rats with salbutamol on the ability of forskolin, given acutely, to protect against ACh-induced bronchoconstriction. **96**

3.1.8 Effect of chronic treatment of rats with salbutamol on the ability of levcromakalim, given acutely, to protect against ACh-induced bronchoconstriction. **97**

3.1.9 Effect of chronic treatment of rats with $PGE_2$ on the ability of $PGE_2$, given acutely, to protect against ACh-induced bronchoconstriction. **99**

3.1.10 Effect of chronic treatment of rats with $PGE_2$ on the ability of salbutamol, given acutely, to protect against ACh-induced bronchoconstriction. **100**

3.2.1 Binding of [$^{125}$I]ICYP to total β-adrenoceptors in lung membranes prepared from vehicle- and salbutamol-treated rats. **108**

3.2.2 Binding of [$^{125}$I]ICYP to $\beta_1$-adrenoceptors in lung membranes prepared from vehicle- and salbutamol-treated rats. **109**

3.2.3 Binding of [$^{125}$I]ICYP to $\beta_2$-adrenoceptors in lung membranes prepared from vehicle- and salbutamol-treated rats. **110**

3.2.4 Effects of chronic treatment of rats with salbutamol on the steady-state levels of $\beta_1$- and $\beta_2$-adrenoceptor mRNA. **111**

3.2.5 Effect of chronic treatment of rats with salbutamol on cholera toxin-induced cAMP accumulation in lung *ex vivo.* **112**

3.2.6 Lack of effect of chronic treatment of rats with salbutamol on $G_\beta$ expression in lung membranes. **113**

3.2.7 Effect of chronic treatment of rats with salbutamol on $G_{s\alpha}$ expression in lung membranes. **114**

3.2.8 Effect of treating rats chronically with salbutamol on the kinetics of salbutamol-induced cAMP accumulation in lung *ex vivo.* **115**

3.2.9 Effect of treating rats chronically with salbutamol on the concentration-response relationship of salbutamol-induced cAMP accumulation in lung *ex vivo*. **117**

3.2.10 Effect of propranolol on salbutamol-induced cAMP accumulation in salbutamol- and vehicle-treated rat lung *ex vivo*. **117**

3.2.11 Effect of treating rats chronically with salbutamol on $PGE_2$-induced cAMP accumulation in lung *ex vivo*. **118**

3.2.12 Effect of chronic treatment of rats with salbutamol on PDE isoenzyme activity in lung *ex vivo*. **119**

3.2.13 Effect of chronic treatment of rats with salbutamol on salbutamol-induced PKA activation *ex vivo*. **121**

3.2.14 Effect of chronic treatment of rats with salbutamol on cytosolic GRK activity in lung *ex vivo*. **122**

3.2.15 Effects of chronic treatment of rats with salbutamol on GRK2 protein expression. **122**

3.2.16 Lack of effect of chronic treatment of rats with salbutamol on β-arrestin expression in lung membranes. **123**

3.2.17 Effect of treating rats chronically with salbutamol on salbutamol-induced cAMP accumulation in trachea *ex vivo*. **125**

3.2.18 Lack of effect of chronic treatment of rats with salbutamol on $G_\beta$ expression in tracheal membranes. **125**

3.2.19 Effect of chronic treatment of rats with salbutamol on $G_{s\alpha}$ expression in tracheal membranes. **126**

3.2.20 Biochemical effects of chronic treatment of rats with salbutamol on the $\beta_2$-adrenoceptor signal transduction cascade *ex vivo*. **135**

3.3.1 Representative traces of lung resistance experiments. **139**

3.3.2 Effect of chronic treatment of rats with salmeterol on the ability of salmeterol, given acutely, to protect against ACh-induced bronchoconstriction. **140**

3.3.3 Time-response of salmeterol-mediated protection against ACh-induced bronchoconstriction. **141**

3.3.4 Effect of chronic treatment of rats with salmeterol on the ability of $PGE_2$, given acutely, to protect against ACh-induced bronchoconstriction. **142**

3.3.5 Effect of chronic treatment of rats with salmeterol on the ability of salbutamol, given acutely, to protect against ACh-induced bronchoconstriction. **143**

3.3.6 Binding of [$^{125}$I]ICYP to total β-adrenoceptors in lung membranes prepared from vehicle- and salmeterol-treated rats. **146**

3.3.7 Binding of [$^{125}$I]ICYP to $\beta_1$-adrenoceptors in lung membranes prepared from vehicle- and salmeterol-treated rats. **147**

3.3.8 Binding of [$^{125}$I]ICYP to $\beta_2$-adrenoceptors in lung membranes prepared from vehicle- and salmeterol-treated rats. **148**

3.3.9 Effect of chronic treatment of rats with salmeterol on the steady-state levels of $\beta_1$- and $\beta_2$-adrenoceptor mRNA. **149**

3.3.10 Effect of chronic treatment of rats with salmeterol on $\beta_2$-adrenoceptor expression in lung membranes. **150**

3.3.11 Lack of effect of chronic treatment of rats with salmeterol on $G_\beta$ expression in lung membranes. **151**

3.3.12 Effect of chronic treatment of rats with salmeterol on $G_{s\alpha}$ expression in lung membranes. **152**

3.3.13 Effect of chronic treatment of rats with salmeterol on salmeterol-induced cAMP accumulation in lung *ex vivo.* **153**

3.3.14 Effect of chronic treatment of rats with salmeterol on $PGE_2$-induced cAMP accumulation in lung *ex vivo.* **154**

3.3.15 Effect of chronic treatment of rats with salmeterol on cytosolic GRK activity in lung *ex vivo.* **155**

3.3.16 Effects of chronic treatment of rats with salmeterol on GRK2 protein expression. **155**

3.3.17 Metabolism of salmeterol in rats. **162**

## List of Tables


| Table | Title | Page |
|---|---|---|
| 1.1 | Regulation of adenylyl cyclase isoforms. | **35** |
| 1.2 | Distribution, subtype and function of β-adrenoceptors in pulmonary tissue. | **44** |
| 1.3 | Characteristics of the G protein-coupled receptor kinases. | **51** |
| 1.4 | Description of PDE isoenzyme families. | **63** |
| 1.5 | Tissue distribution and effects of β-adrenoceptor stimulation in humans. | **69** |
| 3.1 | Summary of the *in vivo* bronchoprotection investigation using rats treated chronically with vehicle and salbutamol. | **93** |
| 3.2 | Effect of chronic treatment of rats with salbutamol on the density of cell surface $\beta_1$- and $\beta_2$-adrenoceptors on rat lung membrane and on the affinity of [$^{125}$I] ICYP. | **107** |
| 3.3 | Summary of the *in vivo* bronchoprotection investigation using rats treated chronically with vehicle and salbutamol. | **138** |
| 3.4 | Effect of chronic treatment of rats with salmeterol on the density of cell surface $\beta_1$- and $\beta_2$-adrenoceptors on rat lung membranes and on the affinity of [$^{125}$I] ICYP. | **145** |

**List of Abbreviations.**

| | |
|---|---|
| [$^{125}$I]ICYP | [$^{125}$I]iodocyanopindolol |
| cAMP | adenosine 3',5'-cyclic monophosphate |
| AC | adenylyl cyclase |
| ATP | adenosine 5'-triphosphate |
| $NH_3Ac$ | ammonium acetate |
| $NH_3Cl$ | ammonium chloride |
| βAR | β-adrenoceptor |
| βARK | β-adrenoceptor kinase |
| βARB | β-adrenoceptor binding protein |
| BSA | bovine serum albumin |
| $CaCl_2$ | calcium chloride |
| PKA | cAMP-dependent protein kinase |
| CTP | cytidine 5'-triphosphate |
| CTx | cholera toxin |
| DMSO | dimethyl sulphoxide |
| $K_2HPO_4$ | dipotassium hydrogen phosphate |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethyleneglycol-bis(β-aminoethylether)-N, N, N', N'-tetraacetic acid |
| ERK | extracellular signal-regulated kinase |
| FCS | foetal calf serum |

| | |
|---|---|
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| GTP | guanosine 5'-triphosphate |
| GRK | G protein-coupled receptor kinase |
| IBMX | 3-isobutyl-1-methylxanthine |
| MgAc | magnesium acetate |
| $MgCl_2$ | magnesium chloride |
| MAPK | mitogen-activated protein kinase |
| MOPS | morpholinosulphonic acid |
| MBP | myelin basic protein |
| Org 9935 | 4,5-dihydro-6-(5,6-dimethoxybenzo[b]thiene-2-yl)-5-methyl-3(2H)pyridazinone (PDE3 inhibitor) |
| $PGE_2$ | prostaglandin $E_2$ |
| PKC | protein kinase C |
| PKG | cyclic GMP-dependent protein kinase |
| PTx | pertussis toxin |
| PMSF | phenylmethylsulphonylflouride |
| PI-3 | phosphatidylinositol 3-kinase |
| KCl | potassium chloride |
| $KH_2PO_4$ | potassium dihydrogen phosphate |
| PKC | protein kinase C |
| Rnasin | ribonuclease inhibitor |
| NaAc | sodium acetate |
| NaCl | sodium chloride |
| SDS | sodium dodecyl sulphate |

| | |
|---|---|
| Tris HCl | tris(hydroxymethyl)aminomethane hydrochloride |
| UTP | uridine 5'-triphosphate |

# Chapter 1

# Introduction

## *1.1 The molecular architecture of the β-adrenoceptor.*

### *1.1.1 The G protein-coupled receptor superfamily.*

The G protein-coupled receptor (GPCR) superfamily represents one of the largest and functionally diverse protein families, probably accounting for 1% of the genome's 100,000 genes (Clapham, 1996). Family members have been identified in organisms as evolutionarily distant as yeast and humans. The vast array of molecules recognised by GPCRs range from neurotransmitters, hormones, lipids and autocoids, to odorants, ions and photons. In addition to the expansive number of GPCRs, there is also heterogeneity within the G proteins themselves. G Proteins are comprised of 3 subunits that exist as αβγ heterotrimers when in their inactive state. The family of heterotrimeric G proteins includes more than 20 isoforms comprised from at least 23 $G_\alpha$ subunits, 5 $G_\beta$ subunits and 12 $G_\gamma$ subunits. The collection of effectors to which G proteins couple is also diverse and includes phospholipases A, C and D, adenylyl cyclases as well as calcium and potassium channels. Additionally, any single GPCR may interact with more than

one G protein and consequently activate more than one second messenger pathway.

*1.1.2 Structural homology among the GPCRs.*

GPCRs share a considerable degree of structural homology despite the large variety of ligands to which they bind. A comparison of the primary structures of GPCRs indicates that these proteins are patterned according to a common structural principle. The molecular architecture of these receptors is characterised by the presence of seven hydrophobic stretches of 20-25 amino acids, which are predicted to form an α helical conformation (Rees *et al.*, 1989). These transmembrane (TM) α helices (TM I-VII) are connected by alternating extracellular and intracellular loops of amino acids. Sequence homology between GPCRs is greatest within the TM regions and is highest between species homologues or related subtypes of receptor, and is lowest for unrelated GPCRs.

Molecular cloning studies have revealed three distinct mammalian β-adrenoceptors (βARs) (Frielle *et al.*, 1987, Dixon *et al.*, 1986, Chung *et al.*, 1987, Emorine *et al.*, 1989). Recently, a putative forth βAR has been proposed. This, so-called, "atypical cardiac" βAR has not yet been cloned, but there is sufficient evidence that it is pharmacologically distinct from the three known βARs (Kaumann, 1997, Kaumann and Molenaar, 1997). The three cloned βARs so far identified have distinctly different sequences. The human $\beta_1$AR has only 54% homology with the human $\beta_2$AR. The $\beta_3$AR is clearly different from either $\beta_1$-or $\beta_2$ARs - it shares about 50% amino acid sequence homology and conforms to the previously described "atypical" βAR found in adipose tissue (Emorine *et al.*, 1989). The most sequence homology between these receptors exists within the TM amino acids (TM II-VII). The primary structure of the human $\beta_2$AR is shown in figure 1.1.

Across the entire GPCR family few amino acid residues are fully conserved. Around twenty invariant amino acid residues are found, most of which reside in the hydrophobic receptor core. In particular, these invariant amino acid residues appear to occupy a region proximal to the

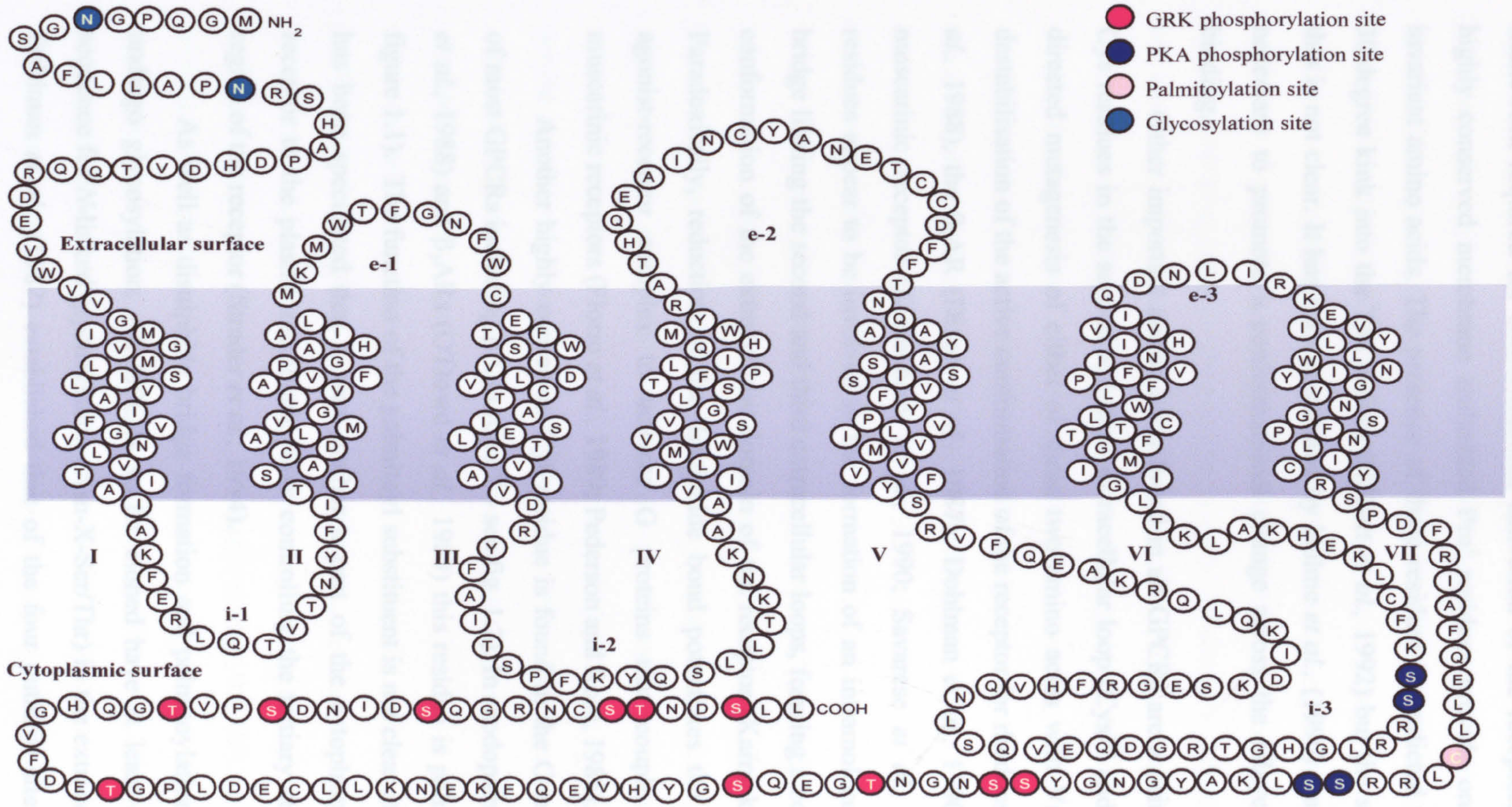



**Fig 1.1:** Primary structure of the $\beta_2$adrenoceptor. Circles with letters represent amino acids identified by the single letter amino acid code. Membrane-spanning domains are numbered with Roman numerals. Extracellular domains are designated by a prefix e-, and intracellular domains are designated by a prefix i-. The 11 potential sites for GRK-mediated phosphorylation on the C-tail of the $\beta_2$adrenoceptor are labelled, as are the potential phosphorylation sites of PKA. Also shown are the two extracellular sites of glycosylation and the putative intracellular site of palmitoylation. Adapted from O'Dowd *et al.*, (1989).

cytoplasmic membrane and are thought to provide a common structural framework required for conformational activation of the receptors. A series of highly conserved membrane embedded Pro[1] residues provide one example of invariant amino acids. The presence of these residues is predicted to introduce a 30 degree kink into the TM helices (Hilbert *et al.*, 1992) but the significance of this is not clear. It has been postulated by Hulme *et al.*, (1990) that they may be necessary to promote a conformational change across the bilayer after ligand binding.

Other important amino acids found in all GPCRs are a pair of conserved Cys residues in the second and third extracellular loop ($Cys^{106}$ and $Cys^{184}$). Site directed mutagenesis of either of these two amino acids with Val results in a destabilisation of the active conformation of the receptor for rhodopsin (Karnick *et al.*, 1988), the $\beta_2$AR (Dixon *et al.*, 1987a; Dohlman *et al.*, 1990) and the $M_1$ muscarinic receptor (Kurtenback *et al.*, 1990; Savarese *et al.*, 1992). These residues appear to be involved in the formation of an intramolecular disulphide bridge linking the second and third extracellular loops, forming a constraint in the conformation of the extracellular domain of the receptor (Karnick *et al.*, 1988). Paradoxically, reduction of the disulphide bond potentiates the ability of the agonist-receptor complex to activate G proteins that couple to βAR and muscarinic receptors (Florio *et al.*, 1989; Pederson and Ross, 1985).

Another highly conserved Cys residue is found in the C-terminal portion of most GPCRs including βARs ($Cys^{341}$ - see fig. 1.2). In rhodopsin (Ovchinnikov *et al.*, 1988) and $\beta_2$ARs (O'Dowd *et al.*, 1989) this residue is palmitoylated (see figure 1.1). The function of the palmitoyl substituent is not clear at present, but it has been speculated that it may anchor a part of the cytoplasmic tail of the receptor to the plasma membrane, thus controlling the tertiary structure of this region of the receptor (Strader *et al.*, 1994).

As well as disulphide bridge formation and palmitoylation, GPCRs also undergo glycosylation. All GPCRs so far cloned have at least one consensus sequence for *N*-linked glycosylation (Asn-X-Ser/Thr) in the extracellular domain. Dohlman *et al.*, (1987) established that of the four putative sites for *N*-linked

[1] See Appendix for list of abbreviations for amino acids.

glycosylation in the hamster $\beta_2$AR, only those sites in the N-terminal domain ($Asn^6$ and $Asn^{15}$) are utilised in the glycosylation of the native protein. The other consensus glycosylation sites are located at the C-terminus of the receptor. It is estimated that glycosylation accounts for 25% to 30% of the molecular mass of the native receptor. The functional significance of glycosylation is not clear (O'Dowd *et al.,* 1988). However, prevention of receptor glycosylation, either by inhibition of glycosylation enzymes in the cell or by site-directed mutagenesis of the potential glycosylation sites on the receptor, results in a decrease in the level of expression of the $\beta_2$AR, as well as changes in the appearance and migration of the receptor on SDS-polyacrylamide gels. Glycosylation does not affect ligand affinity for the receptor or coupling to G-proteins but may be important for the trafficking of the receptor through the cell during down-regulation, or for keeping the receptor correctly orientated in the lipid bilayer (see fig. 1.1).

*1.1.3 The ligand binding domain.*

Most modelling of GPCRs is based on the high resolution, electron cryo-microscopic structure of bacteriorhodopsin (Henderson *et al.,* 1990). In bacteriorhodopsin, binding of the retinal chromophore occurs in the central cavity enclosed by the seven TM domains where several amino acids contribute to the retinal binding site. Of particular importance is $Lys^{296}$ that becomes covalently bound to the retinal chromophore (Khorana, 1992). Exposure to light causes a series of conformational changes in rhodopsin that triggers its association with the G protein, transducin.

Several lines of evidence indicate that biogenic amine receptors, like the βAR, bind their ligands through a mechanism similar to that described for bacteriorhodopsin, although the formation of a covalent bond between receptor and ligand appears to be unique to rhodopsin among the GPCRs (Dixon *et al.,* 1987a). Initial studies to characterise ligand-binding sites of the biogenic amine receptors were carried out on ARs. Deletion studies on βARs were of limited success as many of the amino acid residues involved in ligand binding also appear to have important structural roles. Splicing regions of different receptors to form chimaeras proved useful in localising general regions of the receptor, but most

detailed analyses were provided by single amino acid substitutions, which were less likely to cause an overall disruption of the structure of the receptor protein.

In a series of deletion mutagenesis experiments on the βAR by Dixon *et al.*, (1987a), the hydrophilic loops connecting the seven TM hydrophobic domains were not found to be critical for ligand binding, indicating that the ligand binding pocket must reside in the TM domain. This is surprising, considering the hydrophilic nature of the small catecholamine ligands. Additional site-specific mutagenesis experiments have been performed by Strader *et al.*, (1988). In particular, replacement of acidic residues in the TM domain of the βAR with neutral amino-acids have implicated the side chain of $Asp^{113}$ in TM III as the counterion for the amine groups of βAR agonists and antagonists. These mutant receptors could still be fully activated by adrenaline, indicating that $Asp^{113}$ plays a role in ligand binding but not receptor activation. This Asp residue is conserved in all biogenic amine receptors, but is absent in other receptors. Another Asp residue, $Asp^{79}$ in TM II, is highly conserved too. Substitution of this Asp with Ala in the $\beta_2$AR results in a decrease in agonist affinity and efficacy but has no effect on antagonist interactions, consistent with a role for this residue in promoting receptor activation (Strader *et al.*, 1987).

Based on earlier pharmacological work, a second series of experiments indicated that the aromatic catechol moiety of βAR agonists binds to a pocket involving residues on TM V and TM VI. Two serine residues at positions 204 and 207 in TM V were demonstrated to be the most likely participants in hydrogen bonding between receptor and ligand. These residues are conserved in other receptors binding catecholamines (Strader *et al.*, 1989). Subsequent mutagenesis of the βAR has suggested a role for $Phe^{290}$ in TM VI in stabilising the interaction of the aromatic catechol-containing ring with the receptor (Dixon *et al.*, 1988). Based on the location of these residues in models of $\beta_2$AR structure, and on data from fluorescence-quenching experiments with the βAR antagonist carazolol, the ligand-binding pocket is estimated to be buried deep within the hydrophobic core of the protein about 11Å from the extracellular surface.

*1.1.4 The G protein-coupling domain.*

The biochemical and electrophysiological responses mediated by any given GPCR are primarily governed by the G protein(s) to which it interacts. It has become clear that there is a great deal of promiscuity among individual receptors allowing a single receptor to activate a number of second messenger pathways, although the predominant pathway varies between cell types. The most striking example of receptor promiscuity is that of the $\alpha_2$AR receptor which may couple to four different G proteins in the same cell (Wong *et al.*, 1991, 1992; Eason *et al.*, 1992; Federman *et al.*, 1992; Conklin *et al.*, 1993). Further heterogeneity may arise from a single G protein. For example, the βAR may stimulate AC and $Ca^{2+}$ channels through the interaction with $G_{s\alpha}$ (Birnbaumer *et al.*, 1990).

Through their expression in mammalian cells and Xenopus oocytes, βARs can couple to at least two distinct G proteins. The $\beta_1$- and $\beta_2$ARs are coupled primarily to the stimulatory G protein $G_s$ (see section 1.2.1), which activates adenylyl cyclase (AC) (Strosberg, 1993). The $\beta_3$AR is also coupled to $G_s$, albeit weakly (Liggett *et al.*, 1993) although in cardiac tissue it preferentially couples to a pertussis toxin-sensitive G protein that inhibits AC activity ($G_i$ - see section 1.2.1) (Gauthier *et al.*, 1996). The recently discovered atypical cardiac ($\beta_4$) AR has been shown to also couple to $G_s$, mediating cardiostimulation (Kaumann, 1997).

Most of the GPCR is buried in the cell membrane with only small regions of the receptor exposed on the cytoplasmic side. The cytoplasmic residues are mainly in the ends of the TM helices and their connecting loops, and it has become clear that many of the residues in these regions are involved in the interaction with G proteins. Of particular importance are those residues at the cytoplasmic ends of the TM helices and the portions of the cytoplasmic loops adjacent to the membrane surface. These include regions of the second intracellular loop (i2), the membrane-proximal regions of the C-terminal tail (i4) and, most importantly, the third intracellular loop (i3).

Initial studies, to define the regions of G protein coupling, were performed on βARs and demonstrated that removal of either end of the third intracellular

amino acid loop (i3) resulted in a receptor unable to couple to $G_s$ (Dixon *et al.*, 1987b). From a collection of chimaeric $\alpha_2$-$\beta_2$ARs, Lefkowitz *et al.*, (1989) found that an $\alpha_2$AR with sequences from the $\beta_2$AR third cytoplasmic loop and fifth and sixth TM regions was capable of coupling to $G_s$ to activate AC. More detailed analysis of i3, by limited deletions, showed that a small region of eight amino acids at the *N*-terminal portion of the loop (residues 222-229) was absolutely required for $\beta_2$AR-mediated activation of AC. In addition, deletion of 12 amino acids (residues 258-270) from the *C*-terminal portion of i3 impaired the receptor's ability to mediate stimulation of AC. It was demonstrated that the region extending from the *N*-terminus of TM V to the *C*-terminus of TM VI in the $\beta_2$AR was sufficient to cause coupling to $G_s$ in chimaeric $\alpha_2$-$\beta_2$ARs (Dixon *et al.*, 1987b; Kobilka *et al.*, 1988). Moreover, the *C*-terminal segment of the third cytoplasmic loop and the *N*-terminal portion of the cytoplasmic tail were critical in the formation of the $G_s$ binding site. The short extracellular loop between TM domains VI and VII (i.e. ~5 residues) suggests that these two regions lie in close proximity to each other on the cytoplasmic side of the membrane and may form a binding surface for $G_s$ (O'Dowd *et al.*, 1988).

To assess which receptor domains conferred specificity to $G_s$, mutants were constructed in which segments of 12-22 amino acids of the $\beta_2$AR were substituted with corresponding residues from the $\alpha_2$AR (Liggett *et al.*, 1991). Substitutions in the *N*-terminal portion (positions 216-237 - S1), *C*-terminal portion (positions 263-274 - S2) and the third intracellular loop/*N*-terminus of the cytoplasmic tail (positions 327-339 - S3) of the $\beta_2$AR were studied in various combinations. Substitution of region S2 into the $\beta_2$AR resulted in significant impairment in the ability of the receptor to couple to $G_s$. A smaller effect was observed upon substitution of region S3 into the $\beta_2$AR. Similar to the results shown by O'Dowd *et al.*, (1989), the S1 substitution failed to affect the ability of the $\beta_2$AR to couple to $G_s$. Combination of all three substitutions resulted in a receptor capable of coupling to $G_i$, in addition to a residual ability to couple to $G_s$.

The third intracellular loop (i3) is the most divergent domain among GPCRs. Secondary structure predictions indicate that regions of the *N*- and *C*-terminal portions of i3 may form amphipathic α-helices that are extensions of TM

V and TM VI. This led to the hypothesis that the amphipathic character of these α-helices is a critical determinant of the interaction of the $\beta_2$AR with $G_s$. Cheung *et al.,* (1991) demonstrated that peptides corresponding to the *N*- and *C*-terminal portions of i3 were able to activate the GTPase activity of $G_s$, but did not activate $G_i$ or $G_o$, unlike mastoparan, a bee venom peptide which activates G proteins and also forms amphipathic α-helices. This suggested that, in addition to helical properties, specific residues in these regions directly contribute to the receptor coupling selectivity to $G_s$ over other G proteins. It could, therefore, be predicted that the mechanism of receptor-mediated $G_s$ activation involves interaction with the amphipathic α-helices from the *N*- and *C*-terminal regions of i3 exposed during agonist-induced conformational changes in the receptor.

## *1.2 Coupling of the GPCRs.*

### *1.2.1. G protein structure and function*

#### *1.2.1.1 The α-subunit.*

As mentioned in section 1.1.1 more than 20 mammalian G protein α subunits ($G_\alpha$) have been identified, corresponding to 16 gene products divided into four major classes: $\alpha_{s(olf)}$, $\alpha_{i(o,\ t,\ g,\ z)}$, $\alpha_{q(11,\ 14,-16)}$ and $\alpha_{12(13)}$ (Simon *et al.,* 1991). G protein signalling involves a common mechanism of action. Binding of an agonist to its receptor causes a conformational change in the receptor, which allows it to interact with its heterotrimeric G protein resulting in the formation of a high affinity agonist-receptor-G protein complex. This interaction catalyses the exchange of GDP for GTP and, due to instability of the GTP-bound form of the heterotrimer, heterolytic dissociation occurs to form active $GTP_\alpha$ and βγ complexes. Acting either co-ordinately or independently these G protein subunits bind and activate an effector protein that, in turn, modulates the activities of downstream effector molecules. Concomitantly, dissociation of the G protein complex from the receptor also reduces the affinity of the agonist for its receptor. Intrinsic GTPase activity results in a slow breakdown of GTP in the α subunit

which causes the release of the G protein from its effector. The GDP bound form of the α subunit may then be recycled.

Classically, the $\beta_2$AR has been shown to couple to $G_s$ (Dixon 1987b), but it also can couple to $G_i$ (Xiao *et al.,* 1995). On purification, $G_{s\alpha}$ is found usually to be a mixture of two oligomers with differing α subunits ($M_r$ on SDS-PAGE gels 52 and 45 kDa respectively), and indistinguishable β and γ subunits. The α-subunit heterogeneity arises from alternative splicing of a single precursor mRNA, which results in polypeptides that contain (long form) or lack (short form) a sequence of 15 amino acid residues encoded by exon III of the gene for $G_{s\alpha}$. As many as four splice variants appear to be produced by this mechanism, and each short and long form may include or exclude a single serine residue at the splice junction (Kozasa *et al.,* 1988). When expressed in *E. coli* and purified, the proteins interact similarly with AC, the $G_{\beta\gamma}$ complex, and the $\beta_2$AR (Graziano *et al.,* 1989). The relative concentrations of the two forms of $G_{s\alpha}$ varies among cells and tissues but functional differences are not yet appreciated (Northup *et al.,* 1980; Sternwis *et al.,* 1981; Hildebrandt *et al.,* 1984). $G_{s\alpha}$ is so named due to the stimulatory action it has on AC; however it is also been reported to activate proteins including dihydropyridine-sensitive $Ca^{2+}$ channels in skeletal and cardiac muscle (Brown *et al.,* 1988), and high conductance $Ca^{2+}$-activated $K^+$ channels in smooth muscle (Li and Campbell, 1997).

$G_i$ was first visualised as a 41 kDa substrate for ADP-ribosylation by pertussis toxin and was later purified from rabbit liver by Bokoch *et al.,* (1983). The functional attributes related to $G_i$ were deduced by reconstitution of the rabbit liver protein into platelet and S49 cell membranes (Katada *et al.,* 1984a, b, c). The $G_i$ family is composed of at least three closely related α subunits that are encoded by different genes; they are designated $\alpha_{i1}$, $\alpha_{i2}$, and $\alpha_{i3}$. $G_{\alpha i1}$ has a mass of 41 kDa and is found in high concentrations in the brain. In contrast, $\alpha_{i2}$ is a 40 kDa protein that appears to be relatively ubiquitously distributed, whereas $\alpha_{i3}$ is a distinct 41 kDa protein whose precise tissue distribution is not fully known. The $G_{i\alpha}$ proteins are all involved in the regulation of signalling systems that can be disrupted by pertussis toxin in that they all act as substrates for ADP ribosylation. $G_{i\alpha}$ is known to mediate many functional responses. For example, Yatani *et al.,*

(1988) showed that the resolved α subunit of purified erythrocyte $G_i$ activates $K^+$ channels that are responsible for an outward rectifying current in atrial cardiomyocytes, this property is shared by $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$.

*1.2.1.2 The βγ subunits.*

Initial purification of $G_s$, $G_t$ and $G_i$ revealed 35 kDa polypeptides associated with the distinctive α subunits. Later analyses revealed that these polypeptides were the heterogeneous β subunit (Manning and Gilman 1983; Hildebrandt *et al.*, 1985). Contrary to the initial belief that the $G_{\beta\gamma}$ complex was little more than a carrier molecule for the α-subunit, it is now known that the βγ subunits can, themselves, play active roles in signal transduction through, for example, the regulation of $K^+$ channels, phospholipase Cβ, and certain isoforms of AC in animal cells (Clapham and Neer 1993; Sternweis, 1994).

Five isoforms of the β subunit (Watson *et al.*, 1994) and ten of the γ subunit (Ray *et al.*, 1995) have been identified thus far. The β isoforms share 50-90% identity in primary structure (Neer, 1995) but the sequences of the γ subunits are more diverse (30-80% identity) (Ray *et al.*, 1995). Whereas $G_\alpha$ subunits can be myristoylated or palmitoylated, the $G_\gamma$ of the $G_{\beta\gamma}$ dimer is farnesylated or geranylated (Ray *et al.*, 1995), thus, tethering the βγ to the plasma membrane. β subunits depend on γ to fold correctly - the two subunits cannot be dissociated from each other, except under denaturing conditions. Three-dimensional analyses of G-proteins reveal a structure in which the γ subunit is highly extended and embedded on one surface of the β subunit. The major, 300-residue *C*-terminal domain of the β subunit adopts the β-propeller fold, a motif that has been observed in a variety of other proteins (Murzin, 1992). The β-propeller is composed of seven repeats of ~43-residues, termed a WD repeat, which is formed by a series of seven, four-stranded antiparallel β-sheets, arranged like the blades of a propeller (Neer *et al.*, 1994). The γ subunit in the βγ dimer is composed of two helical segments joined by a loop. The *N*-terminal helix of γ engages the *N*-terminal helix of β as a coiled-coil. The second helix in γ overlays the fifth and sixth blades of β; the C-terminal loop of γ is buried, in part, in a hydrophobic pocket on the surface of β. Mutagenesis studies show that the coiled-coil contact

is required for stabilisation of the dimer (Lupas *et al.*, 1992; Garritsen *et al.*, 1993). Experiments conducted with chimaeric molecules have shown that segments corresponding to the fifth and sixth WD repeats in β (Garritsen and Simonds, 1994; Katz and Simon, 1995), and the second helical segment in γ (Spring and Neer, 1994), may encode the elements of selectivity of the two subunits.

There are two major contact sites between $G_\alpha$ and $G_\beta$ The first is the tethered prow of $G_\alpha$, the myristoylated or palmitoylated $G_\alpha$ N-terminus. The larger and more important site is the interface between the $G_\alpha$ switch regions and one of $G_\beta$'s negative faces. The apposition of the two subunits in this region shields this vital domain, protecting the $G_\alpha$ face until the cationic lever of the receptor "flips" the switch domain and triggers a conformational change in $G_\alpha$. After the replacement of GDP by GTP in the GTPase domain cleft, the $G_\alpha$ switch region springs shut. The freed $G_\alpha$ then is free to interact with other $G_\beta$ subunits. A schematic diagram of the crystallography of the G proteins subunits is shown in Figure 1.2.

*1.2.2 Adenylyl cyclase.*

Many GPCRs mediate their effects by interaction with the membrane-bound enzyme AC to increase or decrease cellular levels of cyclic AMP (cAMP). At least nine different forms of AC have now been discovered and there is increasing evidence that these isoforms are differentially regulated (Tang and Gilman, 1992; Iyengar and Birnbaumer, 1993). ACs are 150 kDa glycoproteins that convert ATP to cAMP, which acts as a cellular second messenger through activation of a variety of protein kinases.

All ACs so far identified are stimulated by $G_s\alpha$. The diterpene, forskolin, derived from the Indian plant *Coleus forskolhii*, is a potent activator of nearly all ACs. The analysis of nine types of recombinant mammalian ACs reveals that all ACs, except type IX, can be potently activated by forskolin (Premont *et al.*, 1996, Tang *et al.*, 1997) though to varying degrees. Examination of the amino acid sequences of the family members revealed 12 stretches of hydrophobic

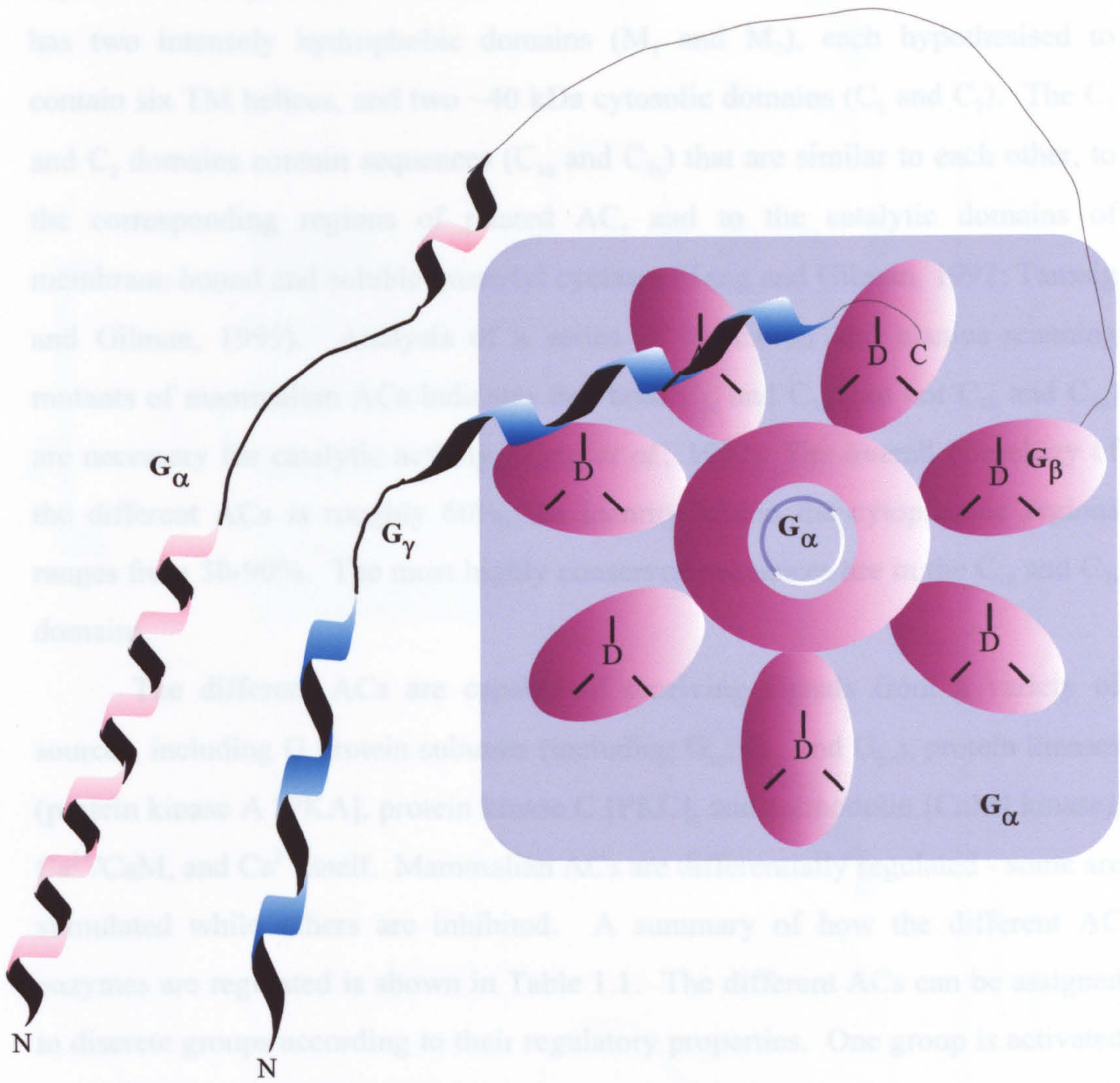



**Figure 1.2**: Crystallographic structure of a G protein. This schematic view shows the Gß propeller as seen from the cell membrane looking down the propeller shaft to $G_\alpha$. The outer part of the blades are least conserved. Previous work predicted correctly the ß N-terminal coiled-coil, the importance of the region now known as blade 5 in ß's interaction with γ, and the requirement of the $G_\alpha$ amino terminus for interaction with $G_{\beta\gamma}$. Conserved regions of 40 amino acid stretches, particularly a conserved aspartic acid (D), are needed for structural integrity. Adapted from Clapham, (1996).

residues in conserved positions. These are postulated to represent a TM span; they are arranged in two sets of six contiguous spans, separated by a large hydrophilic domain. This proposed topology of the AC enzymes is shown in Figure 1.3. The putative structure of AC contains a short variable N terminus (N), has two intensely hydrophobic domains ($M_1$ and $M_2$), each hypothesised to contain six TM helices, and two ~40 kDa cytosolic domains ($C_1$ and $C_2$). The $C_1$ and $C_2$ domains contain sequences ($C_{1a}$ and $C_{2a}$) that are similar to each other, to the corresponding regions of related AC, and to the catalytic domains of membrane-bound and soluble guanylyl cyclases (Tang and Gilman, 1992; Taussig and Gilman, 1995). Analysis of a series of truncation and alanine-scanning mutants of mammalian ACs indicates that both $C_{1a}$ and $C_{2a}$ (but not $C_{1b}$ and $C_{2b}$) are necessary for catalytic activity (Tang *et al.*, 1992). The overall homology of the different ACs is roughly 60%; the identity within the cytoplasmic regions ranges from 50-90%. The most highly conserved sequences are in the $C_{1a}$ and $C_{2a}$ domains.

The different ACs are capable of receiving signals from a variety of sources, including G protein subunits (including $G_{s\alpha}$, $G_{i\alpha}$ and $G_{\beta\gamma}$), protein kinases (protein kinase A [PKA], protein kinase C [PKC], and calmodulin [CaM] kinase), $Ca^{2+}$/CaM, and $Ca^{2+}$ itself. Mammalian ACs are differentially regulated - some are stimulated while others are inhibited. A summary of how the different AC enzymes are regulated is shown in Table 1.1. The different ACs can be assigned to discrete groups according to their regulatory properties. One group is activated by $Ca^{2+}$/CaM, a second is conditionally activated by $G_{\beta\gamma}$ subunits, and a third is activated by PKC. Similarly, different groups of ACs are inhibited by various signals. Most ACs seem susceptible to inhibition by $G_{i\alpha}$, whereas ore selective groups are inhibited by PKA, CaM kinase and $Ca^{2+}$.

As mentioned above, all mammalian ACs apart from IX are activated by forskolin. Forskolin binds to several intrinsic membrane proteins, including ACs, glucose transporters, voltage-gated $K^+$ channels, ligand-gated ion channels and P glycoproteins (Sunahara *et al.*, 1996). It was initially believed that this hydrophobic molecule bound in or near the membrane spans, but construction and expression of a soluble AC (Tang and Gilman, 1995; Dessauer and Gilman 1996)

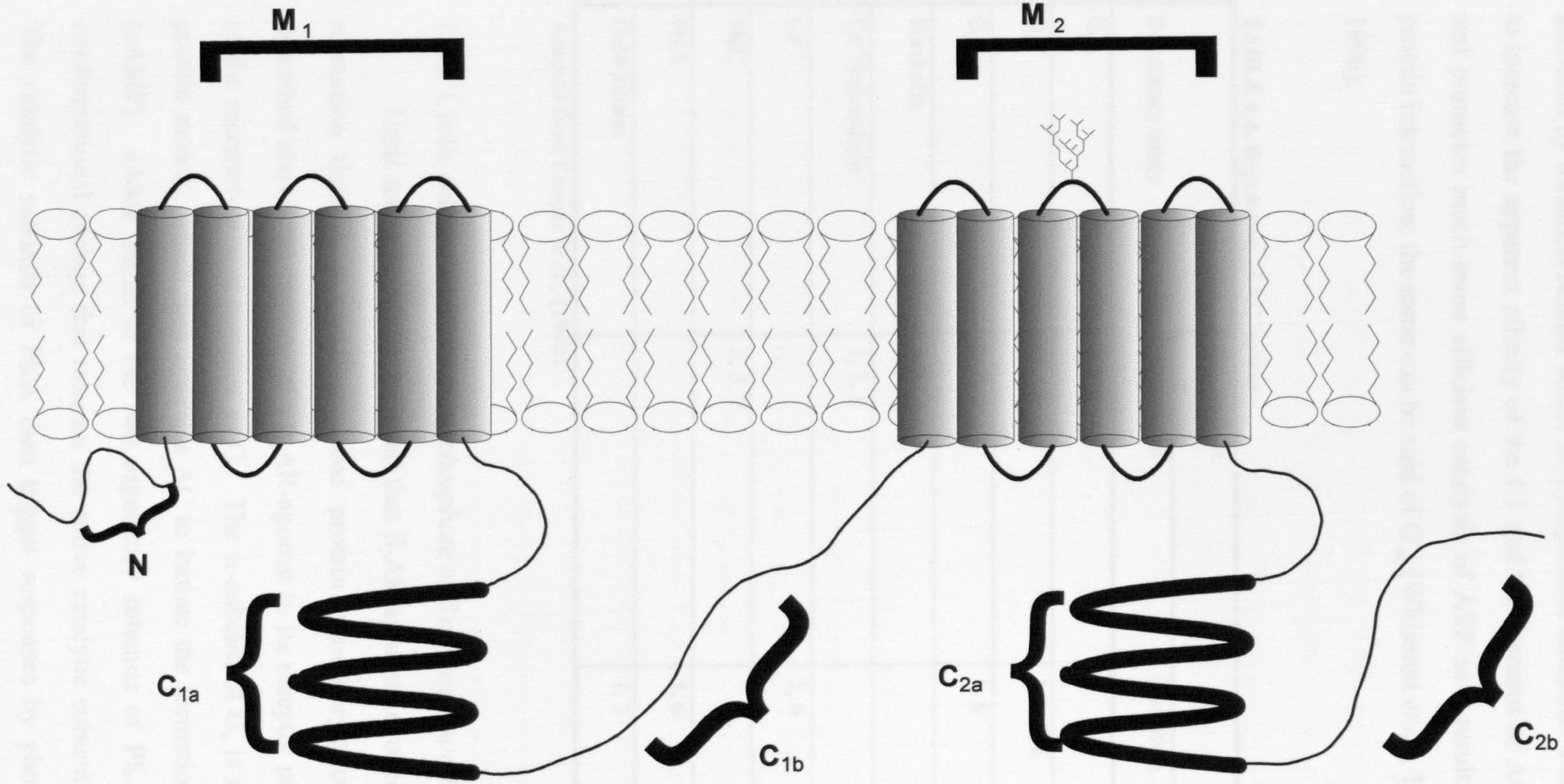



**Figure 1.3:** Topology of adenylyl cyclase. Cylindrical sections represent membrane-spanning regions, while bold lines indicate regions of high homology among all members of the AC family. Nomenclature is as follows: N, amino-terminal domain; $M_1$, first set of membrane-spanning regions; $C_{1a}$ and $C_{1b}$, the first large intracellular cytoplasmic domain; $M_2$, second set of transmembrane-spanning regions; and $C_{2a}$ and $C_{2b}$, second large intracellular domain (see section 1.2.2 for further details). Adapted from Sunahara *et al.,* (1996).

permitted unequivocal demonstration that the capacity of this enzyme to be activated by forskolin resides within its cytoplasmic domains. Forskolin is known to increase the apparent affinity of the C1 and C2 domains of AC for each other and promotes much more efficient catalysis of ATP as a result of this protein-protein interaction; the same can be said of $G_{s\alpha}$ (Whisnant *et al.*, 1996; Yan *et al.*, 1996).

**TABLE 1.1.** Regulation of AC isoforms.

| Regulatory entity | AC isoform | |
|---|---|---|
| | Stimulation | Inhibition |
| $G_{s\alpha}$ | 1-9 | |
| $G_{i\alpha}$ | | 1-3, 5, 6 |
| $G_{\beta\gamma}$ | 2, 4, 7 | 1, 8 |
| Forskolin | 1-9 | |
| $Ca^{2+}$/calmodulin | 1, 3. 8 | |
| $Ca^{2+}$ | | 5, 6 |
| PKC | 2, 5, 7 | |
| PKA | | 5, 6 |
| CaM kinase | | 1, 3 |

Adapted from Cooper *et al.*, (1998).

*1.2.3 Cyclic adenosine-3',5'- monophosphate and airway smooth muscle.*

Until recently, it was believed that $\beta_2$AR-agonists evoked smooth muscle relaxation through a cAMP-triggered protein phosphorylation cascade. As described above, the binding of a $\beta_2$AR-agonist to its receptor promotes coupling of the receptor-agonist complex to $G_s$. The $\alpha$-subunit of $G_s$ is released from the protein moiety, which then activates AC to initiate the formation of cyclic AMP (cAMP). cAMP binds to the two regulatory subunits of PKA and induces a conformational change that releases the active catalytic subunits of the enzyme. The catalytic subunits of PKA then trigger responses by phosphorylating key substrates, leading to smooth muscle relaxation (Torphy, 1994).

The precise way in which cAMP causes relaxation is not clear. Several actions of PKA, which can affect smooth muscle tone, have been identified - these include inhibition of inositol phosphate hydrolysis (Madison and Brown, 1988; Hall *et al.,* 1989), increase calcium uptake by internal stores (Mueller and Van Breeman, 1979), inactivation of myosin light chain kinase (Silver and Stull, 1982) and activation of cell membrane ion channels and transporters such as $K^+$ channels and the $Na^+/K^+$ ATPase (Kume *et al.,* 1989; Gunst and Stropp, 1988; Knox and Brown, 1991). The net effect of these processes is to decrease the intracellular calcium concentration and inhibit the interaction of the contractile proteins actin and myosin.

### *1.2.4 Non cAMP-mediated relaxation of airway smooth muscle:interaction with ion channels.*

More recently, results from several studies contest the established dogma that the cAMP/PKA cascade is the exclusive pathway for relaxation of smooth muscle. For example, studies with nitrates suggest that cyclic guanosine-3',5'-monophosphate (cGMP) may also be an important cellular second messenger (Nials *et al.,* 1993). There have also been studies showing that cAMP can activate cGMP-dependent protein kinase (PKG) in intact cell systems. Jiang *et al.,* (1992) reported that the increase in cAMP content accompanying treatment of bovine coronary arteries with isoprenaline resulted in a simultaneous activation of PKA and PKG. Both of these can relax smooth muscle - PKA by exerting an inhibitory effect on contractile protein function, and PKG by assisting in the reduction in cellular $Ca^{2+}$ (Sparrow *et al.,* 1984; De Lanerolle and Paul , 1991).

Work with porcine and ferret airway smooth muscle suggests that βAR-agonists can produce relaxation independently of cAMP via stimulation of $G_s$ that is coupled to large conductance, $Ca^{2+}$-activated potassium channels ($BKCa^{2+}$) (Huang *et al.,* 1993; Jones *et al.,* 1990; Miura *et al.,* 1992). Under conditions in which synthesis of cAMP does not occur and protein phosphorylation does not take place, patch-clamp techniques have shown that isoprenaline activates $BKCa^{2+}$. Application of $G_{s\alpha}$, together with a non-hydrolysable analogue of GTP

(GTPγS) to inside-out patches mimics the effect of isoprenaline (Kume *et al.*, 1992, 1994), whereas $G_{i\alpha}$ and $G_{o\alpha}$ were both inactive (Kume *et al.*, 1992). It is a reasonable supposition to assume that $\beta_2$AR-agonists can regulate $BKCa^{2+}$ by activating both cAMP-dependent and cAMP-independent pathways.

Agonists that open $BKCa^{2+}$ cause membrane hyperpolarisation, which, in turn, assists relaxation (Small *et al.*, 1988; Weston, 1989). Pharmacological evidence that $BKCa^{2+}$ have a role in airway smooth muscle relaxation was derived from studies in which the $BKCa^{2+}$ blocker charybdotoxin reduced, but did not abolish, the relaxation induced by $\beta_2$AR agonists (Huang *et al.*, 1993; Miura *et al.*, 1992). The fact that a component of the response is insensitive to charybdotoxin suggests that βAR-agonists also act through $BKCa^{2+}$-independent mechanisms. A summary of cAMP-dependent and independent regulation of ion channels is shown in Figure 1.4

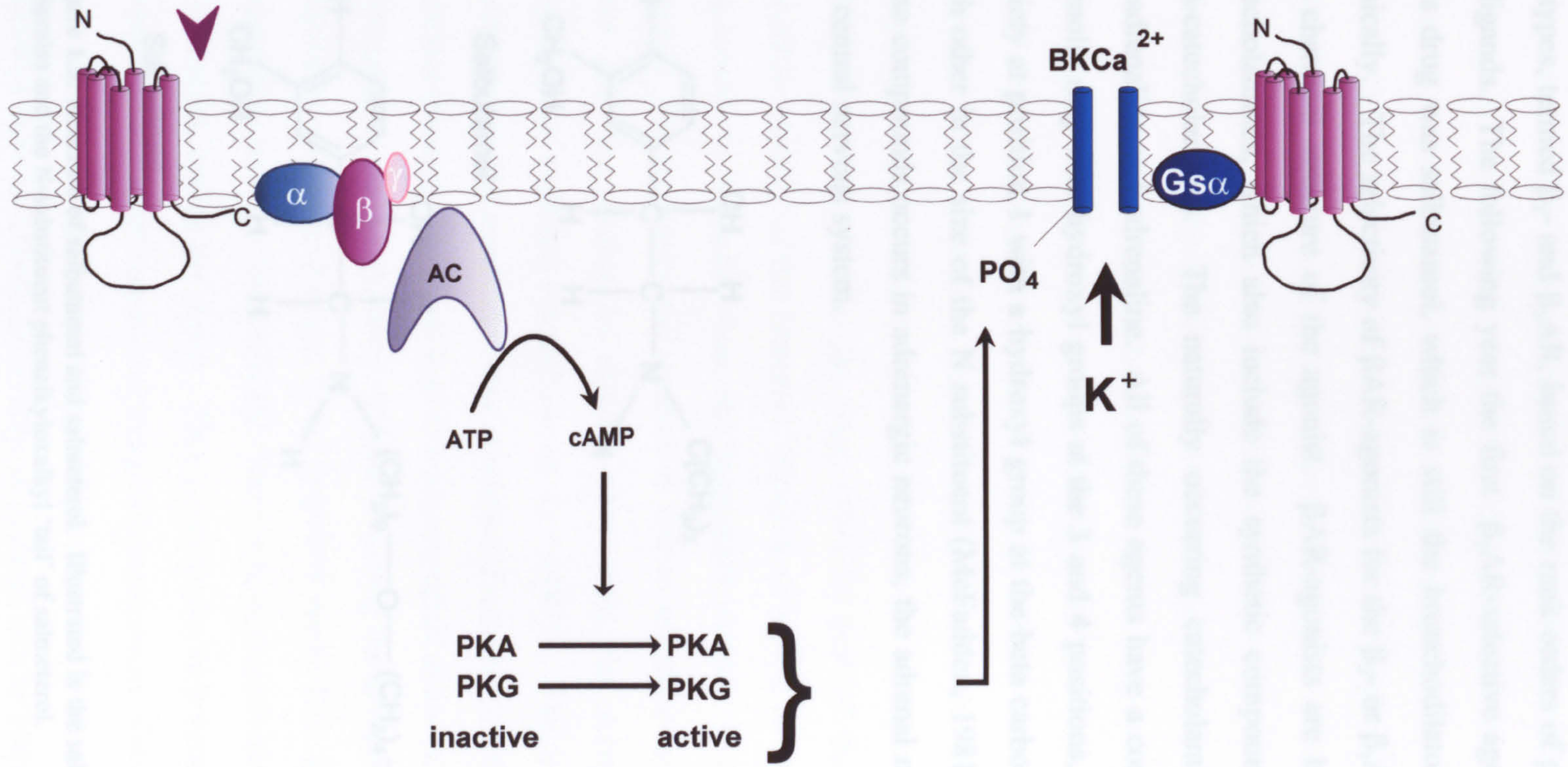
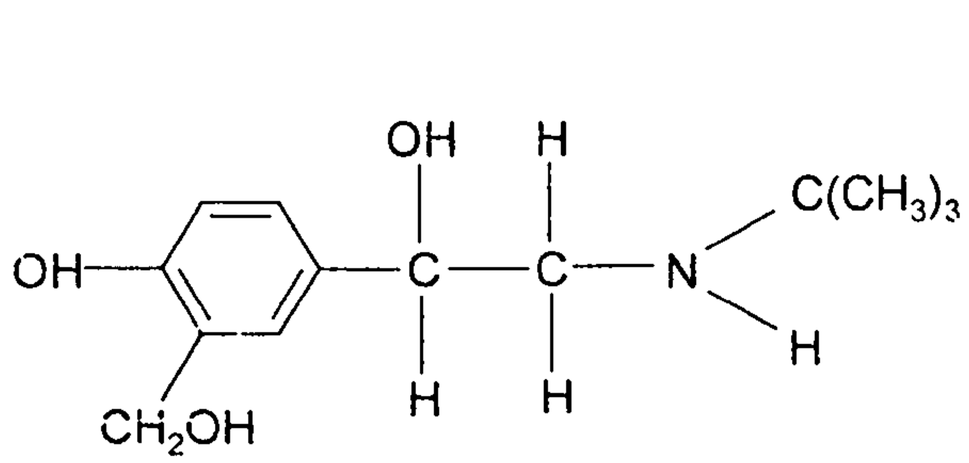



**Figure 1.4**: cAMP-dependent and independent regulation of high-conductance, calcium-activated potassium channels ($BKCa^{2+}$). The two potential protein phosphorylation cascades [PKA and PKG] by which ß-adrenoceptor agonists may regulate the activity of $BKCa^{2+}$ are shown. Activation of ß-adrenoceptors may also regulate $BKCa^{2+}$ activity directly using the stimulatory G protein subunit Gsα as a transducer. In this scheme, several distinct pathways converge to regulate the activity of a single effector ($BKCa^{2+}$). Modified from Torphy and Hall (1994).

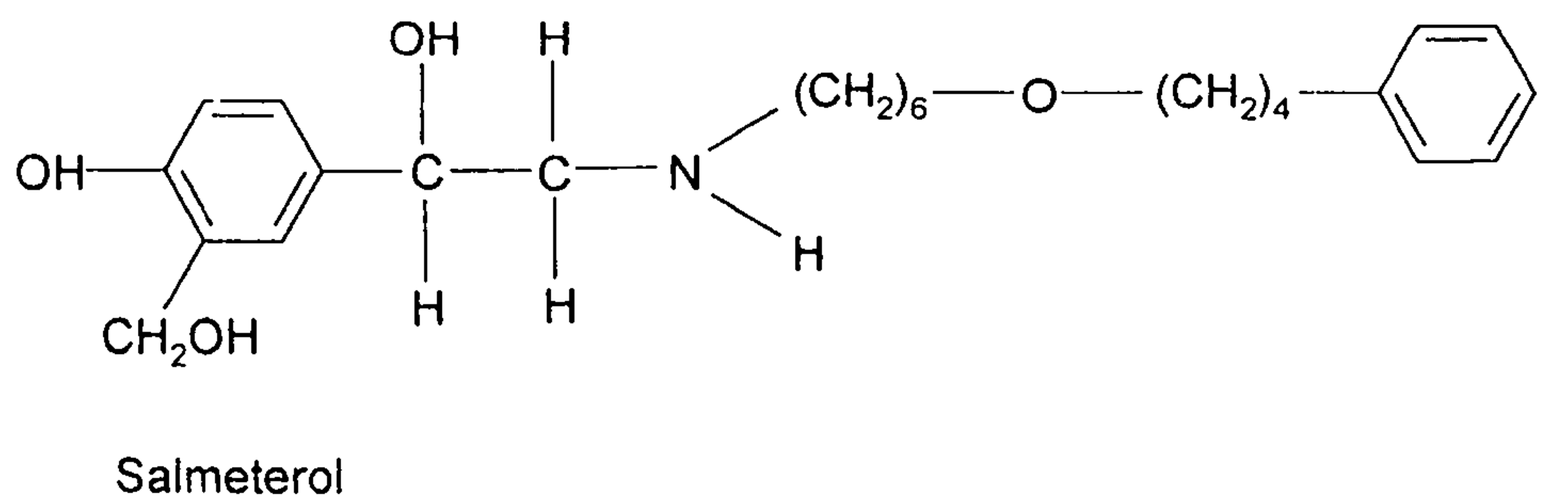
*1.2.5 $\beta_2$-adrenoceptor agonists.*

*1.2.5.1 Endogenous and exogenous agonists.*

Lands *et al*, (1967) proposed that β-ARs could be divided into two subtypes, termed $\beta_1$- and $\beta_2$AR, based on the rank orders of potency of a number of ligands. The following year the first $\beta_2$AR-selective agonist was described. This drug was salbutamol, which is still the bronchodilator most widely used clinically. The selectivity of βAR-agonists for the $\beta_2$- or $\beta_1$AR is determined by the chemical structure of the agonist. βAR-agonists are broadly divided into catecholamines, which also include the synthetic compound isoprenaline, and non-catecholamines. The naturally occurring catecholamines are dopamine, noradrenaline and adrenaline. All of these agents have a common structure of a phenolic ring with hydroxyl groups at the 3 and 4 positions, and an ethyl amino moiety at position 1 with a hydroxyl group at the beta carbon. They differ from each other in the size of the N substituent (McFadden, 1981). The synthesis of these compounds occurs in adrenergic neurons, the adrenal medulla, and parts of the central nervous system.




Salbutamol




Salmeterol

**Figure 1.5:** Structure of salbutamol and salmeterol. Illustrated is the saligenin active head of the molecules and the N-substituent phenalkyloxalkyl 'tail' of salmeterol.

*1.2.5.2 Salmeterol and salbutamol.*

Synthetic non-catecholamines have had the catechol nucleus replaced by either a resorcinol or saligenin ring. These drugs differ in their substitutions in the amine group and phenolic ring. The saligenins that include salbutamol and salmeterol have substitutions at the 3-hydroxy position. These modifications confer resistance to metabolism by catechol-*o*-methyltransferase resulting in a longer half-life, and reduce their efficacy for $\beta_1$ARs. Salmeterol differs from salbutamol in that it possesses a much longer substitution in the amino part of the molecule, namely a lipophilic N-substituent phenalkyloxalkyl 'tail'.

Salmeterol, in contrast to salbutamol, is a long-acting agonist at $\beta_2$ARs. This compound displays particularly tenacious and persistent agonist activity on airway smooth muscle *in vitro* and *in vivo*, in animals and humans (Ullman and Svedmyr, 1988). The most popular mechanism for the longevity of action of salmeterol on $\beta_2$ARs is the exosite theory. This theory requires the presence of an additional binding site for the drug, either as part of the receptor or in the immediate vicinity of the receptor. Binding of a drug to an exosite renders the drug unable to dissociate from the immediate vicinity of the active site of the receptor. The exosite-bound receptor is free to associate and dissociate with the active site, but unable to diffuse away from the receptor, even down a concentration gradient (Bradshaw *et al.*, 1987). The effect of an exosite mechanism would result in a long duration of action. If exosite binding was of sufficient strength, this could lead to a physiological action of the drug that cannot be reversed by simple washing, without affecting the competitive action of $\beta_2$AR antagonists, as the active site is not impeded upon. This theory was originally presented by Rocha e Silva (1969). There is now clear evidence for the existence of an exosite within the $\beta_2$AR. This putative site is proposed to be part of the receptor protein itself uninvolved in agonist recognition (Green *et al.*, 1996).

Both salbutamol and salmeterol produce relatively potent, concentration-dependent agonist activity on a range of isolated airway preparations. Salmeterol is more potent than salbutamol, but both compounds usually behave as partial agonists with respect to isoprenaline. The intrinsic activity of salbutamol and

salmeterol on airway smooth muscle relative to isoprenaline is 0.5 and 0.35 respectively. Salbutamol has a rapid onset of action (2-3 min) while salmeterol has been reported to have a substantially longer onset of action (approximately 30 min) (Nials *et al.*, 1994). This difference is due mostly to the different rates of diffusion of the compounds into tissues. One of the properties of salmeterol is the ability of the drug to reassert its action after washout. Moreover, hydrophilic βAR antagonists, such as sotalol, will block salmeterol-induced relaxation of guinea-pig trachea by pushing the active portion of the salmeterol molecule away from the active site of the receptor; however, removal of the antagonist allows salmeterol to reassert its activity without the addition of further drug. These results are consistent with the putative exosite theory of salmeterol binding.

Rhodes *et al.*, (1992) measured the partitioning of salmeterol into synthetic membranes. Salmeterol partitioned into the membranes extremely rapidly and virtually completely, resulting in an approximately 30,000:1 lipid-water ratio. These data suggest that there are two processes contributing to the duration of action of salmeterol. The first component results from the lipophilicity of the compound, with the membrane acting as a reservoir. The second, more major component, results from the compound being held in the vicinity of the active site of the $\beta_2$AR, consistent with exosite binding. Work has been presented by Green *et al.*, (1996) where hybrid $\beta_2$ARs were produced in which an amino acid sequence of the $\beta_2$AR was replaced by the corresponding section from the $\beta_1$AR by site-directed mutagenesis. The residues replaced were 149-158, believed to be associated with exosite binding (Green *et al.*, 1996). The affinity of salmeterol for the hybrid receptor was the same as for the wild type, however the modification resulted in a decreased persistence of agonist activity. When the corresponding $\beta_1$AR hybrid was constructed with key amino acids from the $\beta_2$AR, markedly enhanced persistence of agonist activity resulted. A summary of the $\beta_2$AR signal transduction cascade is depicted in figure 1.6.

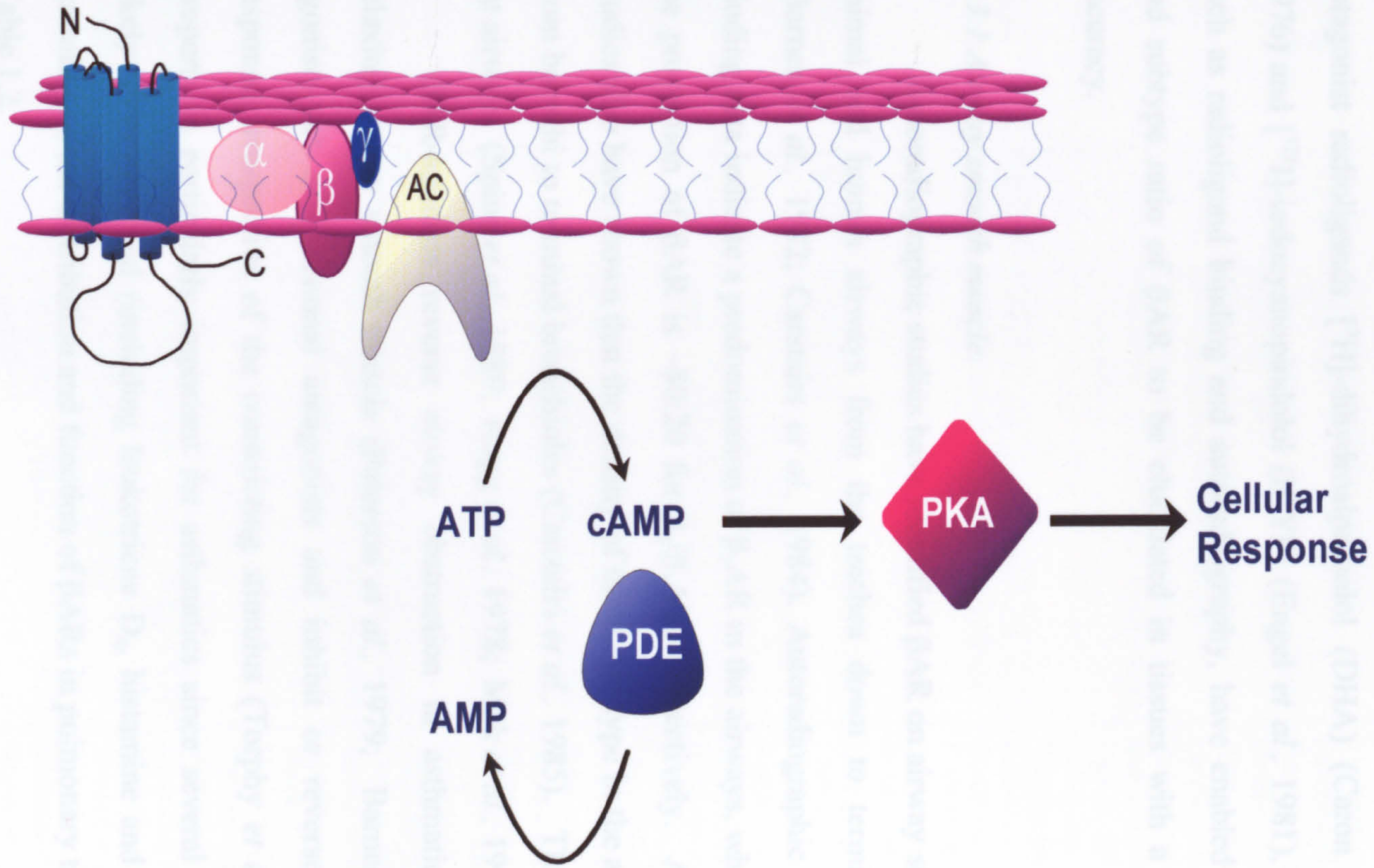



**Figure 1.6**: ß-Adrenoceptor signal transduction cascade. On stimulation by an agonist, a change in conformation of the ß-AR induces the α subunit of Gs to dissociate from the βγ subunits. The α subunit then stimulates adenylyl cyclase to catalyse the formation of cAMP, which activates PKA to phosphorylate a number of cellular proteins. cAMP is broken down to AMP by cyclic nucleotide phosphodiesterase (PDE) enzymes.

*1.3 Localisation and function of $\beta_2$-adrenoceptors.*

βARs are widely distributed throughout the lung and airways, and βAR-agonists have effects on a wide variety of pulmonarycells (Table 1.2). The lung alone is comprised of as many as 40 different cell types of which there are at least five major cell populations. The development of the highly selective βAR-antagonist radioligands [$^3$H]-dihydroalprenolol (DHA) (Caron and Lefkowitz, 1976) and [$^{125}$I]-iodocyanopindolol (ICYP) (Engel *et al.*, 1981), and techniques, such as radioligand binding and autoradiography, have enabled the localisation and subtype ratio of βAR to be elucidated in tissues with a high degree of accuracy.

*1.3.1 Airway smooth muscle.*

Autoradiographic studies have identified βAR on airway smooth muscle of animal and human airways from the trachea down to terminal bronchioles (Barnes *et al.*, 1982; Carstairs *et al.*, 1984). Autoradiographic and radioligand binding data indicate a predomination of $\beta_2$AR in the airways, whereas in the lung the proportion of βAR is ~80:20 for $\beta_2/\beta_1$ARs respectively. Autoradiographic studies also have shown that the density of the $\beta_2$-subtype in the airways increases from bronchi to terminal bronchioles (Carstairs *et al.*, 1985). This is also true for rat airways (Spina *et al.*, 1989; Rugg *et al.*, 1978; Mak *et al.*, 1994).

βAR-agonists reverse airway obstruction in asthmatics by primariiy relaxing airway smooth muscle (Paterson *et al.*, 1979; Barnes, 1986). βAR-agonists act as functional antagonists and inhibit or reverse the contractile response, irrespective of the constricting stimulus (Torphy *et al.*, 1983). This property is particularly important for asthmatics since several spasmogens are likely to be involved (including leukotriene $D_4$, histamine and bradykinin). A summary of the distribution and function of βARs in pulmonary tissue is shown in Table 1.2.

**Table 1.2:** Distribution, subtype and function of βARs in pulmonary cells and tissues.

| Location | βAR subtype | Function | References |
|---|---|---|---|
| Airway nerves | $\beta_1$AR<br>$\beta_2$AR | Modulation of neurotransmission in parasympathetic ganglia.<br>Modulation of cholinergic transmission at postganglionic cholinergic nerves.<br>Inhibition of excitatory NANC bronchoconstrictor responses | Skoogh & Svedmyr, 1989.<br>Rhoden *et al.*, 1988.<br>Aizawa *et al.*, 1991.<br>Bai *et al.*, 1988<br>Belvisi *et al.*, 1996.<br>Kamikawa & Shimo, 1990<br>Verleden *et al.*, 1993. |
| Airway epithelium | $\beta_2$AR | Unclear?<br>May promote release of airway relaxant factor.<br>Increases mucociliary clearance (by increasing beat frequency)<br>Increase in goblet cell secretion<br>Secretion of surfactant lipids from Clara cells | Emorine *et al.*, 1989.<br>Barnes *et al.*, 1982, 1986.<br>Goldie *et al.*, 1986.<br>Blair & Woods, 1969<br>Hesse *et al.*, 1981.<br>Hybinnette & Mercke, 1982.<br>Kanietzko & Kasparak, 1983<br>Van as, 1974.<br>Tokuyama *et al.*, 1990.<br>Widdicombe & Pack, 1982. |
| Blood vessels | $\beta_2$AR | Dilation and Increased blood flow.<br>Inhibition of microvascular leakage. | Barker *et al.*, 1988.<br>Boschetto *et al.*, 1989.<br>Erjefält & Persson, 1991.<br>Tokuyama *et al.*, 1991.<br>Sulakveldze & McDonald 1994 |
| Alveoli | $\beta_2$AR | Unknown | Carstairs *et al.*, 1985.<br>Barnes *et al.*, 1982.<br>Gatto *et al.*, 1987.<br>Spina *et al.*, 1989. |
| Mast Cells | $\beta_2$AR | Inhibits histamine release | Butchers *et al.*, 1980.<br>Peters *et al.*, 1982.<br>Church & Hiroi, 1987 |
| Eosinophils | $\beta_2$AR | Inhibition of oxidative burst, release of thromboxane/$LTC_4$, eosinophil chemotaxis | Yukawa *et al.*, 1990.<br>Dent *et al.*, 1994.<br>Munoz *et al.*, 1994.<br>Masayuma & Ishkana, 1985. |
| Macrophages | $\beta_2$AR | Unknown | Liggett, 1989<br>Hjemdahl *et al.*, 1990.<br>Fuller *et al.*, 1988 |
| Neutrophils | $\beta_2$AR | Inhibition of $Ca^{2+}$-dependent neutrophil activation of oxidative burst, and of mediator release. | Dulis & Wilson, 1980.<br>Galant & Allred, 1985.<br>Galant *et al.*, 1980.<br>Mack *et al.*, 1986.<br>Busse & Sosman, 1984.<br>Szefler *et al.*, 1987.<br>Williams *et al.*, 1976. |
| Mononuclear phagocytes/ lymphocytes | $\beta_2$AR | Diminishment of lymphokine secretion.<br>Reduction in T lymphocyte toxicity.<br>Decreased antibody production.<br>Reduced expression of IL-2 receptors.<br>Reduced mitogenesis. | Motulsky & Insel, 1982.<br>Williams *et al.*, 1976.<br>Bourne *et al.*, 1974.<br>Feldman, 1987.<br>Hellstrand *et al.*, 1985.<br>Kammer, 1988.<br>Khan *et al.*, 1985.<br>Sandes & Munson, 1984. |

### *1.3.2 $\beta_2$-adrenoceptor agonists and inflammation.*

It was the vasoconstrictor property of adrenaline that prompted the first successful trial of this drug in asthma in 1903 (Bullowa and Kaplan, 1903). The drug's bronchodilator action was established by work carried out in Prague in 1907 (Kahn, 1907). This action was soon considered important in asthma whereas the vasoconstrictor property remained important mainly for nasal therapies. In the 1960s and 1970s, Assem and Schild, and others, added "antiallergic" mast-cell inhibitory effects to the list of potentially important antiasthma actions and laid down that this property, in addition to bronchodilatation, resided in the $\beta_2$AR-agonist activity of these drugs (Assem and Schildt, 1969).

Short acting, selective $\beta_2$AR-agonists such as salbutamol and terbutaline were developed largely between 1965 and 1975. *In vitro*, these drugs exhibit potential anti-inflammatory activity through the potent inhibition of IgE-dependent mediator release from mast cells (Church and Hiroi, 1987), and by attenuating the increase in microvascular permeability induced by histamine, bradykinin and $LTB_4$ (Persson *et al.,* 1978). However, early studies showed that short-acting $\beta_2$AR-agonists failed to prevent the late asthmatic response (LAR) (Cockcroft and Murdock, 1987), implying that they did not prevent the characteristic inflammatory response *in vivo*.

There has been concern that $\beta_2$AR-agonists may have deleterious effects in asthma when taken regularly for prolonged periods. Anxiety arose following publication of case control studies from New Zealand and Canada which linked excess use of βAR-agonists with asthma mortality (Crane *et al.*, 1989; Spitzer *et al.*, 1992). Sears *et al.*, (1990) reported that regular use of fenoterol produced poorer asthma control than "as required" treatment, while a further study by van Schayck *et al.,* (1991), suggested that regular treatment of patients with inhaled salbutamol was associated with a more rapid decline in lung function than when the drug was used intermittently.

Eformoterol and salmeterol, long acting $\beta_2$AR-agonists introduced over the last decade, are capable of producing complete inhibition of IgE-dependent histamine, $LTC_4$ and $PGD_2$ release from human lung tissue (Butchers *et al.,* 1991; Johnson, 1994) but, whereas salbutamol is active for up to 4h, the effects of

salmeterol persist for >20h (Johnson, 1994). Long-acting $\beta_2$AR-agonists, in contrast to the short-acting compounds, are reported to prevent the accumulation of leukocytes in animal models of inflammation. Challenge studies in guinea-pigs with antigen and platelet activating factor (PAF) have shown that pre-treatment with salmeterol, but not salbutamol, inhibits the accumulation of eosinophils in the airways that occurs 24h post challenge (Whelan and Johnson, 1992; Sanjar *et al.*, 1991). Salmeterol and eformoterol also suppress $LTB_4$ synthesis by neutrophils (Johnson, 1990), the generation of $H_2O_2$ and thromboxane (TX) $B_2$ by eosinophils (Rabe *et al.*, 1990) and $TXB_2$ synthesis by human alveolar macrophages (Baker and Fuller, 1990), although these effects are only modest.

While there is evidence demonstrating that long-acting $\beta_2$AR-agonists may have anti-inflammatory properties *in vitro*, present information suggests that they probably have no significant anti-inflammatory effects in the bronchial mucosa in asthma. Roberts *et al.* (1992b) compared the effects of 6 weeks treatment of asthmatic patients with salmeterol (50μg twice daily) with those of placebo, analysing a wide range of indices of airway inflammation in both bronchial mucosal biopsies and bronchial alveolar lavage (BAL) fluid (Roberts *et al.*, 1992a,b, Gratziou *et al.*, 1992). In spite of improving symptoms and morning peak flow recordings, salmeterol had no effect on differential cell counts in the BAL fluid, albumin concentration or mediator levels (histamine, $PGD_2$ and tryptase), and failed to affect T cell numbers or activation status assessed by flow cytometry. In the biopsy specimens, there were no changes in numbers of mast cells, eosinophils or T cells, and no change in the thickness of the sub-basement collagen as assessed by electron microscopy. Similarly, a recent study found no change in BAL fluid cell profile, T cell activation status, or albumin and tryptase concentrations (Gardiner *et al.*, 1994) following 8 weeks treatment with salmeterol. Importantly, and bearing in mind the recent $\beta_2$AR controversy, there was no deterioration in these indices of inflammation in any of these studies, and no rebound increase in the $PC_{20}$ for methacholine 5 days after stopping treatment.

### *1.4 Regulation of $\beta_2$-adrenoceptor expression.*

#### *1.4.1 Receptor desensitisation and down-regulation: nomenclature.*

A common functional feature of cellular responses evoked by GPCR-agonists is that they are rapidly attenuated. This phenomenon, known as desensitisation, describes a readily reversible process whereby membrane receptors, usually after high concentrations of agonist, have impaired/reduced responses to subsequent stimulation. For GPCRs, this appears to be a multi-step or multifactorial response. Desensitisation may be a rapid event, occurring within seconds or minutes, involving phosphorylation of the receptor and/or G proteins that results in uncoupling of the receptor from its G protein and/or effector. The desensitisation process may also involve internalisation (or sequestration) of the receptor from the cell surface. This step is now viewed as a resensitisation process whereby the receptor is dephosphorylated by specific phosphatases (Pitcher *et al.*, 1995). In addition, longer exposure to agonist may result in down-regulation of receptors, a process characterised by a decrease in the number of cell surface receptors that can only be overcome by *de novo* protein synthesis.

Two major patterns of desensitisation and down-regulation have been characterised; receptor-specific or homologous desensitisation and receptor non-specific or heterologous desensitisation. Homologous desensitisation represents a situation whereby stimulation of a receptor by an agonist results in the desensitisation of responses evoked only by that agonist. Conversely, heterologous desensitisation occurs when treatment with an agonist results in an attenuation of responses mediated by agonists that through more than one receptor type. Over 20 years of work by numerous investigators has indicated that a variety of mechanisms contribute to the desensitisation of βARs. These mechanisms operate at the level of the receptor, G proteins, AC and beyond (e.g. phosphodiesterase (PDE) isoenzymes).

*1.4.2 The role of phosphorylation in receptor regulation.*

Phosphorylation of receptors is believed to be crucial in the desensitisation process, and βARs are known to be substrates for a number of cellular kinases. Evidence that desensitisation of the βAR involves phosphorylation of the receptor came from experiments where purified βAR from desensitised cells were functionally impaired when assessed in a reconstituted lipid system. This was the first evidence that the receptor was being covalently modified during the desensitisation process (Strulovici *et al.*, 1984). This modification was subsequently found to be due to phosphorylation, with stoichiometric phosphorylation of the receptor being found after exposure of cells to βAR agonists or other agents which raise intracellular cAMP levels (Benovic *et al.*, 1985). That increased cAMP was capable of phosphorylating receptors strongly suggested that PKA was involved. However, phosphorylation of βAR also was observed after agonist exposure of the cyc⁻ variant of the S49 mouse lymphoma cell. Because these cells lack functional $G_s$ and, therefore, do not exhibit increased cAMP contents upon agonist exposure, a unique kinase, which only requires agonist occupancy of the receptor in order to phosphorylate, was subsequently found (Benovic *et al.*, 1987). This kinase was termed β-adrenergic receptor kinase (βARK), and has now been cloned, the primary structure delineated, and subsequently has been found to be a member of a family of serine/threonine kinases, the GPCR kinases (GRKs), which phosphorylate a number of GPCRs (Benovic *et al.*, 1989).

*1.4.2.1 GPCR kinases.*

The GRKs are an important class of protein kinases involved in homologous desensitisation of GPCRs. The GRK family includes rhodopsin kinase (GRK1), the β-adrenergic receptor kinases (βARK1 and βARK2 or GRK2 and GRK3 respectively) and the GRK4 subfamily consisting of GRK4, GRK5 and GRK 6 (See Premont *et al.*, (1995) for review). Briefly, GRK1 phosphorylates rhodopsin in a light-dependent manner and mRNA for this kinase is found almost

exclusively in rod and cone retinal photoreceptor cells. βARK, initially described as an activity that phosphorylated $\beta_2$ARs, is now known to exist as two highly conserved isoforms (GRK2 and GRK3), which are known to phosphorylate a number of receptors. GRK4 is the most recent addition to the GRK family and phosphorylates a number of GPCRs, whereas GRK5 preferentially phosphorylates $\beta_2$AR (Premont *et al.*, 1994; Bullrich *et al.*, 1995). The six GRK proteins share 53-93% overall sequence homology. They also share a similar overall organisation, with the central kinase catalytic domain flanked by an approximately 185-amino acid N-terminal region thought to be important for activated receptor substrate recognition, and a C-terminal region responsible for enzyme location (Lefkowitz, 1993; Inglese *et al.*, 1993).

The family of GRKs possess the unique ability to specifically recognise and phosphorylate the agonist-occupied, or active, form of GPCRs. One important regulatory feature that distinguishes members of the GRK family is the mechanism by which these enzymes are targeted to their membrane-incorporated receptor substrates (Inglese *et al.*, 1993; Premont *et al.*, 1995). The βARKs, GRK2 and GRK3, are cytosolic proteins, but their substrate target, in this case the $\beta_2$AR, are integral membrane proteins. Therefore, phosphorylation of the receptor by these kinases requires their localisation at the plasma membrane. GRK2 and GRK3 are translocated to the membrane via their interaction with the βγ subunits of heterotrimeric G-proteins ($G_{\beta\gamma}$) (Pitcher *et al.*, 1992; Boekhoff *et al.*, 1994). The G-protein γ-subunit is geranylated, thus translocation of GRK2 and GRK3 indirectly involves isoprenylation mechanisms (Inglese *et al.*, 1993).

The interaction of GRK2 and GRK3 with $G_{\beta\gamma}$ involves a βγ binding domain encoded by the 125 amino acids C-terminal of the protein (Koch *et al.*, 1993), which bears striking homology to ~100 amino acid sequence designated as a pleckstrin homology (PH) domain (Touhara *et al.*, 1994). Effective membrane targeting of GRK2 and GRK3 requires the binding of both βγ subunits and phosphatidylinositol 4,5-bisphosphate ($PIP_2$) to the C-terminal PH domain of the kinases (Pitcher *et al.*, 1995). Membrane translocation of GRK2 and GRK3 can be impaired by polypeptides derived from its C-terminal domain that function to compete for G-protein βγ subunits (Koch *et al.*, 1993). The inhibition of GRK

BIBL LONDON UNIV

activity in the hearts of transgenic mice expressing the C-terminal βγ binding domain of GRK2 has firmly established a role for this domain in regulating the activity of GRK2 and GRK3 (Koch *et al.*, 1995). Liberation of the $G_{\beta\gamma}$ subunits from $G_\alpha$ occurs as a consequence of agonist stimulation and the subsequent coupling of the $\beta_2$AR to $G_s$ (Gilman, 1987; Birnbaumer *et al.*, 1990). Therefore, the translocation of GRK2 and GRK3 to the membrane might be considered a signal-dependent event, and the resulting receptor phosphorylation would be primarily agonist-dependent (Haga and Haga, 1992; Pitcher *et al.*, 1992). In contrast, membrane targeting of each of the other GRKs involves post-translational modifications that promote lipid association, such as farnesylation (GRK1), palmitoylation (GRK4, GRK6) or a polybasic carboxyl tail (GRK5), and would not necessarily be signal dependent (Inglese *et al.*, 1993; Casey, 1995). GRK5, the other GRK important in $\beta_2$AR phosphorylation, does not possess a PH domain, and the activity if this enzyme is not enhanced by $G_{\beta\gamma}$, though significant autophosphorylation is known to occur (Premont *et al.*, 1994).

GRKs generally phosphorylate GPCRs at several serine and threonine residues. However, for most GPCRs, little is known about the exact sites of GRK-mediated phosphorylation and the stoichiometry of phosphorylation differs depending on the receptor studied and the environment in which it is tested (Haga *et al.*, 1994; Premont *et al.*, 1995). The only GPCR in which the sites of GRK-mediated phosphorylation have been unambiguously identified is rhodopsin. GRK1 phosphorylates rhodopsin on specific residues located at the distal portion of the C-terminal cytoplasmic tail (C-tail) (Palczewski *et al.*, 1991 Ohguro *et al.*, 1995). Additionally, the phosphorylation sites for GRK2, GRK3 and GRK5 on rhodopsin have recently been identified from *in vitro* studies (Palczewski *et al.*, 1995). The serine residues phosphorylated by these GRKs are essentially identical, principally $Ser^{338}$ and $Ser^{343}$, GRK1 and GRK5 preferentially phosphorylate $Ser^{338}$, whereas GRK2 and GRK3 preferentially phosphorylate $Ser^{343}$. Mutational and proteolysis studies indicate that the sites of GRK2-mediated phosphorylation on the $\beta_2$AR lie potentially within the C-tail of this receptor too. Removal of the $\beta_2$AR C-tail by limited proteolysis (Dohlman *et al.*, 1987), truncation of the C-tail at position 365 (Bouvier *et al.*, 1988), and site-

directed mutations at serine and threonine residues within the C-tail, result in the loss of phosphoacceptor sites (Bouvier *et al.*, 1988; Hausdorff *et al.*, 1989, 1991). Protease digestion of $\beta_2$AR phosphorylated by GRK5 indicates that, like GRK2, GRK5 phosphorylates C-tail serine and threonine residues (Premont, 1994). All the sites of both GRK2- and GRK5-mediated phosphorylation were found to reside within a peptide comprising the C-terminal 40 amino acids of the $\beta_2$AR. There are seven potential phosphorylation sites within this stretch (four serine and three threonine residues), and GRK2 and GRK5 were shown to phosphorylate four and six sites respectively (Fredericks *et al.*, 1996). The complete sequence of putative phosphorylation sites for GRK2, GRK3 and GRK5 is shown in Figure 1.1. A summary of the characteristics of the GRKs is shown in Table 1.3.

**Table 1.3:** Characteristics of the GPCR kinases. Mechanisms of membrane targeting, major sites of expression, targets for phosphorylation and special features are shown.

| Family name | Common name | Membrane association | Tissue distribution[b] | Receptor substrates[a] | Special features |
|---|---|---|---|---|---|
| GRK1 | R kinase | Farnesylation | Retina>>Pineal | R, $\beta_1$AR, $\beta_2$AR | Auto-phospho-rylation |
| GRK2 | βARK1 | $G_{\beta\gamma}$, $PIP_2$ | Leukocytes>brain>> heart>lung>kidney | $\beta_2$AR, R. $\alpha_2$-$C_{10}$AR, $M_2$AChR, SPR, $A_1$-AR, $\alpha_2$-$C_2$AR, $M_3$AChR, $\beta_1$AR, δOR | PH domain |
| GRK3 | βARK2 | $G_{\beta\gamma}$, $PIP_2$ | brain>spleen>heart, lung, kidney | $\beta_2$AR, R, $M_2$AChR, SPR, $\alpha_2$-$C_2$AR, $\beta_1$AR, HTR, Odorant receptors, $M_3$AChR, | PH domain |
| GRK4 | IT-11 | Palmitoylation | Testis>>>brain, peripheral nerve | $\beta_2$AR | 4 splice variants |
| GRK5 | | Phospholipids | heart,placenta,lung> skeletal muscle>brain, lung,pancreas>kidney | $\beta_2$AR, R, $M_2$AChR, $\beta_1$AR, δOR | Auto-phospho-rylation |
| GRK6 | | Palmitoylation | brain, skeletal muscle> pancreas>heart,lung, kidney, placenta>liver | $\beta_2$AR, R, $M_2$AChR | Two genes |

Receptors that have been shown to be phosphorylated by the relevant kinase - R - Rhodopsin, $\beta_2/\beta_1$AR - $\beta_2$- or $\beta_1$AR receptor respectively, $\alpha_2$-$C_{10}$AR/$\alpha_2$-$C_2$AR - $\alpha_2$-adrenoceptors whose genes reside on human chromosomes 10 and 2 respectively, $M_2$AChR and $M_3$AChR - m2 and m3 muscarinic acetylcholine receptors, SPR - substance P receptor, $A_1$-AR - $A_1$-adenosine receptor, δOR - δ-opioid receptor, HTR - human thrombin receptor (assessed primarily by desensitisation).
[b]As determined by mRNA expression
Modified from tables presented by Freedman and Lefkowitz, (1996) and Chuang *et al.*, (1996).

*1.4.2.2 Second messenger kinases.*

In addition to phosphorylation by GRKs, many GPCRs are also phosphorylated by second messenger protein kinases - such as protein kinases A, G and C (PKA, PKG, PKC). This type of phosphorylation can provide a direct negative feedback, whereby the effector enzyme (e.g. PKA) shuts off its own stimulation. This pathway also allows a generalised non receptor-specific form of desensitisation, as activation of the relevant protein kinase by any connected biochemical pathway is sufficient to cause receptor phosphorylation. Unlike phosphorylation by GRKs, agonist occupancy of the substrate receptor does not markedly affect the phosphorylation kinetics effected by protein kinases (Benovic *et al.*, 1985).

Direct phosphorylation of the $\beta_2$AR has been shown in a reconstituted system (Benovic *et al.*, 1985). There are two consensus sites for PKA-mediated phosphorylation in the $\beta_2$AR sequence (Blake *et al.*, 1987; Bouvier *et al.*, 1989; Clark *et al.*, 1989), one in the third intracellular loop in a region that is essential for coupling to $G_s$, and the other at the N-terminal part of the C-terminus, which also appears to play a role in receptor-$G_s$ coupling (Strader *et al.*, 1987; O'Dowd *et al.*, 1988; Münch *et al.*, 1991). The site in the third intracellular loop is the preferred site (Bouvier *et al.*, 1989; Clark *et al.*, 1989). Because of the location in this coupling region, such phosphorylation *per se* is sufficient to impair the ability of the receptors to activate $G_s$ (Pitcher *et al.*, 1992). Phosphorylation of either of the sites discussed above would introduce a highly charged moiety into a region proposed to consist of amphipathic helices, and thus might directly impair coupling (Strader *et al.*, 1989). PKC can also phosphorylate the $\beta_2$AR, probably at the same sites that are utilised by PKA, again, with a clear preference for the site in the C-terminus of the third intracellular loop (Pitcher *et al.*, 1992; Bouvier *et al.*, 1987; Johnson *et al.*, 1990). The effects of PKC-mediated phosphorylation are, therefore, similar to those described for the PKA-mediated process (Bouvier *et al.*, 1991). PKA-mediated desensitisation is comparable to GRK-mediated desensitisation in terms of the extent of loss in receptor function (40-50% for PKA, 50-70% for GRK), but there are differences: PKA-mediated desensitisation

is considerably slower than the GRK-mediated process ($t_{½}$=15 min *vs.* 2 s respectively; Roth *et al.*, 1991), but is much more sensitive to agonist concentrations ($EC_{50}$ ≈ 10 nM *vs.* ≈ 300 nM) (Lohse *et al.*, 1990; Clark *et al.*, 1988 Hausdorff *et al.*, 1989) probably due to the amplification of signalling from receptor occupancy to PKA activation. Consequently, it has been speculated that PKA-mediated desensitisation of βAR may be a sensitive ubiquitous process occurring in response to low agonist concentrations, whereas GRK-mediated desensitisation might occur at sites where much higher concentrations of the relevant agonist acts (Lohse *et al.*, 1990). In support of this, GRK2 and GRK3 recently have been shown to be heavily concentrated at post synaptic locations, where a high concentration of neurotransmitter is expected (Attramadal *et al.*, 1992; Arriza *et al.*, 1992). Proposed PKA phosphorylation sites on the $\beta_2$AR are shown in Figure 1.1.

Two recent articles discuss the regulation of the βARKs (GRK2/3) by PKC. Chuang *et al.* (1995) reported that PKC phosphorylates and activates βARKs *in vivo* and *in vitro*, and Winstel *et al.*, (1996) showed that the activation is due to enhanced translocation of βARKs to the membrane. Thus, the increased activity after phosphorylation of βARKs by PKC was only seen when a membrane-bound substrate like rhodopsin was used, while the activity of βARKs towards a soluble peptide was even decreased after PKC-phosphorylation. In intact cells, PKC appears to induce a translocation of βARKs from the cytosol to the membrane. The PKC-phosphorylation site was mapped to the C-terminus of βARK, in a region critical for membrane binding (Winstel *et al.*, 1996).

### *1.4.3 Arrestins.*

Arrestins play an important role in the molecular mechanisms of desensitisation. Arrestin (a 48 kDa protein or S-antigen) was first implicated in signal quenching in rod outer segments (Wilden *et al.*, 1986). In reconstitution experiments the activation of cGMP PDE by photo-excited rhodopsin was only partially quenched by rhodopsin kinase. For full quenching the addition of arrestin was required. There are several members of the arrestin family. Arrestin (S-

antigen) from bovine retina binds to, and quenches, the signalling of phosphorylated rhodopsin (Wilden *et al.*, 1986). The observation that the ability of GRK2 to desensitize the $\beta_2$AR diminished during purification suggested the existence of a cofactor that was gradually being lost (Benovic *et al.*, 1987). This led to the identification of β-arrestin-1 and β-arrestin-2 (also called arrestin-3), which preferentially prevent activation of the $\beta_2$AR over rhodopsin (Attramadal *et al.*, 1992; Lohse *et al.*, 1990). Four subfamilies of arrestins have now been proposed that are based on sequence homology and tissue distribution: (i) visual arrestin (Shinohara *et al.*, 1987; Yamaki *et al.*, 1990), (ii) cone arrestin (X-arrestin or C-arrestin) (Murakami *et al.*, 1993), (iii) β-arrestins (Lohse *et al.*, 1990; Attramadal *et al.*, 1992) and the, as yet, uncharacterised D- and E-arrestins.

There are at least two polypeptide variants of β-arrestin-1 and β-arrestin-2 that are expressed as a consequence of alternative mRNA splicing (Stern-Marr *et al.*, 1993; Parruti *et al.*, 1993). Isoforms of β-arrestin-1 differ only by the insertion of eight amino acids between residues 333 and 334 (Parruti *et al.*, 1993), whereas variants of β-arrestin-2 are identical except for an 11 amino acid insert between amino acids 361 and 362 (Stern-Marr *et al.*, 1993). The existence of alternatively spliced variant β-arrestins increases the number of arrestin homologues that might contribute to the regulation of GPCRs outside the visual system and pineal gland to at least six.

Phosphorylation of the agonist-bound receptor by GRKs promotes the binding of the relevant arrestin to the receptor. It is this binding that appears to play a primary role in quenching signal transduction, apparently via directly disrupting the interaction between the receptor and G proteins (Benovic *et al.*, 1987; Lohse *et al.*, 1990, 1992). β-Arrestin inhibits signalling of GRK2-phosphorylated $\beta_2$AR by >75% (Lohse *et al.*, 1990). Together, β-arrestin and GRK2 strongly inhibit $\beta_2$AR GTPase activity and AC (Lohse *et al.*, 1992; Sohlemann *et al.*, 1995). The potency with which β-arrestin inhibits function of the $\beta_2$AR is increased 10-fold by GRK2-induced receptor phosphorylation but is unaffected by PKA-induced phosphorylation, which suggests that β-arrestin contributes to homologous but not heterologous desensitisation (Lohse *et al.*, 1992).

Desensitisation of the $\beta_2$AR in response to high concentrations of isoprenaline is amplified by overexpression of β-arrestin-1 (Pippig *et al.*, 1993). Overexpression of β-arrestins can also rescue the sequestration of $\beta_2$AR mutants defective in their ability to sequester without GRK involvement, although simultaneous overexpression of GRK enhances the sequestration observed (Ferguson *et al.*, 1996). Interestingly, receptor dephosphorylation by phosphatase 2A is blocked by arrestin, possibly indicating a role for this molecule in resensitisation (Palczewski *et al.*, 1989).

*1.4.4 Receptor sequestration.*

Agonist occupancy triggers phosphorylation of GPCRs by GRKs as discussed above. The phosphorylated receptor interacts with additional regulatory proteins, the arrestins, which block signal transduction. The uncoupled receptors can be subsequently removed from the plasma membrane in a process termed internalisation or sequestration, which involves endocytosis of the receptor (Premont *et al.*, 1995; Lohse *et al.*, 1996; Haga *et al.*, 1994). Agonist-mediated endocytosis has been observed for many GPCRs, including βARs (Von Zastrow and Kobilka, 1992). The predominant pathway for agonist-induced internalisation of GPCRs, including the $\beta_2$AR, is via clathrin-coated pits (Von Zastrow and Kobilka, 1992).

Clathrin-mediated endocytosis requires interaction of specific receptor domains with components of the endocytic machinery (Trowbridge *et al.*, 1993). In addition to their role in uncoupling GPCRs from G proteins, arrestins are involved in agonist-mediated receptor endocytosis (Ferguson *et al.*, 1996). Overexpression of β-arrestin-1/2 rescues agonist-mediated internalisation of an internalisation-defective mutant of the $\beta_2$AR, and overexpression of a dominant-negative β-arrestin mutant inhibits agonist-induced internalisation of the $\beta_2$AR. Arrestins may function as adaptor molecules that recruit cellular proteins to facilitate receptor endocytosis, or may directly mediate endocytosis themselves. Supporting an adaptor function of arrestins are the observations that β-arrestin1 and 2 bind to clathrin and are colocalised with clathrin and the $\beta_2$AR during early stages of endocytosis (Goodman *et al.*, 1997). Dynamin may be one protein that

interacts with arrestins to enhance endocytosis of some receptors (Zhang *et al.*, 1996). Dynamin is important for internalisation of the $\beta_2$AR, since over expression of a dynamin GTPase mutant inhibits agonist-induced endocytosis.

Receptor phosphorylation is a candidate for inducing agonist-dependent internalisation, since some GPCRs are rapidly phosphorylated in response to agonist binding and because serine/threonine-rich regions are known to be important for internalisation (Hausdorff *et al.*, 1991). However, internalisation of the $\beta_2$AR is unaffected by deletion of phosphorylation sites for GRKs and second messenger kinases (Hausdorff *et al.*, 1989; Strader *et al.*, 1987). Moreover, inhibitors of GRKs or PKA (Lohse *et al.*, 1990) and overexpression of a dominant-negative GRK mutant do not prevent receptor endocytosis (Kong *et al.*, 1994). Interest in this field was recently rekindled with the observation that overexpression of GRK2 restores agonist-induced internalisation of an internalisation-defective mutant $\beta_2$AR (Ferguson *et al.*, 1995). GRKs may facilitate internalisation by stabilising a receptor conformation or promoting the interaction of the receptor with a cellular element (possibly β-arrestin) that mediates receptor internalisation, even in the absence of GRK-induced receptor phosphorylation. Recently, Ruiz-Gómez and Mayor (1997) showed that GRK2 expression levels can modulate the extent of receptor sequestration at certain agonist concentrations, and also showed that GRK2 not only translocates to the plasma membrane, but colocalises with the $\beta_2$AR during receptor internalisation. These findings support the concept of GRK involvement in a correct conformation of the various domains of the $\beta_2$AR involved in sequestration. Also, the presence of GRK2 within internalisation vesicles may indicate that the endocytic system plays a role in the recycling of the kinase translocated to the plasma membrane upon receptor stimulation.

*1.4.5 Receptor cycling.*

After internalisation, a substantial number of receptors may rapidly reappear at the cell surface. Therefore, agonist-induced sequestration of GPCRs is thought to contribute to functional resensitisation. Resensitisation of cellular responses to agonists of the $\beta_2$AR is blocked by inhibitors of endocytosis or

receptor mutation (Barak *et al.*, 1994; Yu *et al.*, 1993). This implies that processing of the internalised receptor is necessary for resensitisation. This is likely to occur as a result of their dephosphorylation in endosomes (von Zastro and Kobilka, 1992) and occurs alongside the continual recycling of the endosome markers rab5 and transferrin receptors (von Zastro and Kobilka, 1992; Moore *et al.*, 1995).

Dephosphorylation may include dissociation of the ligand in acidified endosomes, followed by the dephosphorylation process mediated by GPCR phosphatase (GRP), dissociation of arrestins, and recycling of the receptor to the plasma membrane. In support of this are the observations that endosomes are enriched with GRP, a member of the PP-2A phosphatase family (Pitcher *et al.*, 1995), that significant dephosphorylation of the $\beta_2$AR only occurs at low pH (Krueger *et al.*, 1997) and that the $\beta_2$AR in endosomes is less phosphorylated than at the plasma membrane and does not resensitise if recycling is blocked (Pippig *et al.*, 1995; Sibley *et al.*, 1986). Sequestration appears to facilitate the localisation of the $\beta_2$AR to an acidified compartment, where a conformationally altered receptor associates with GRP and is then dephosphorylated (Krueger *et al.*, 1997). Receptor reappearance may be rapid, is temperature-dependent and does not appear to require *de novo* protein synthesis. A summary of the events occurring after agonist stimulation that eventually lead to either $\beta_2$AR recycling or degradation is given in Figure 1.7.

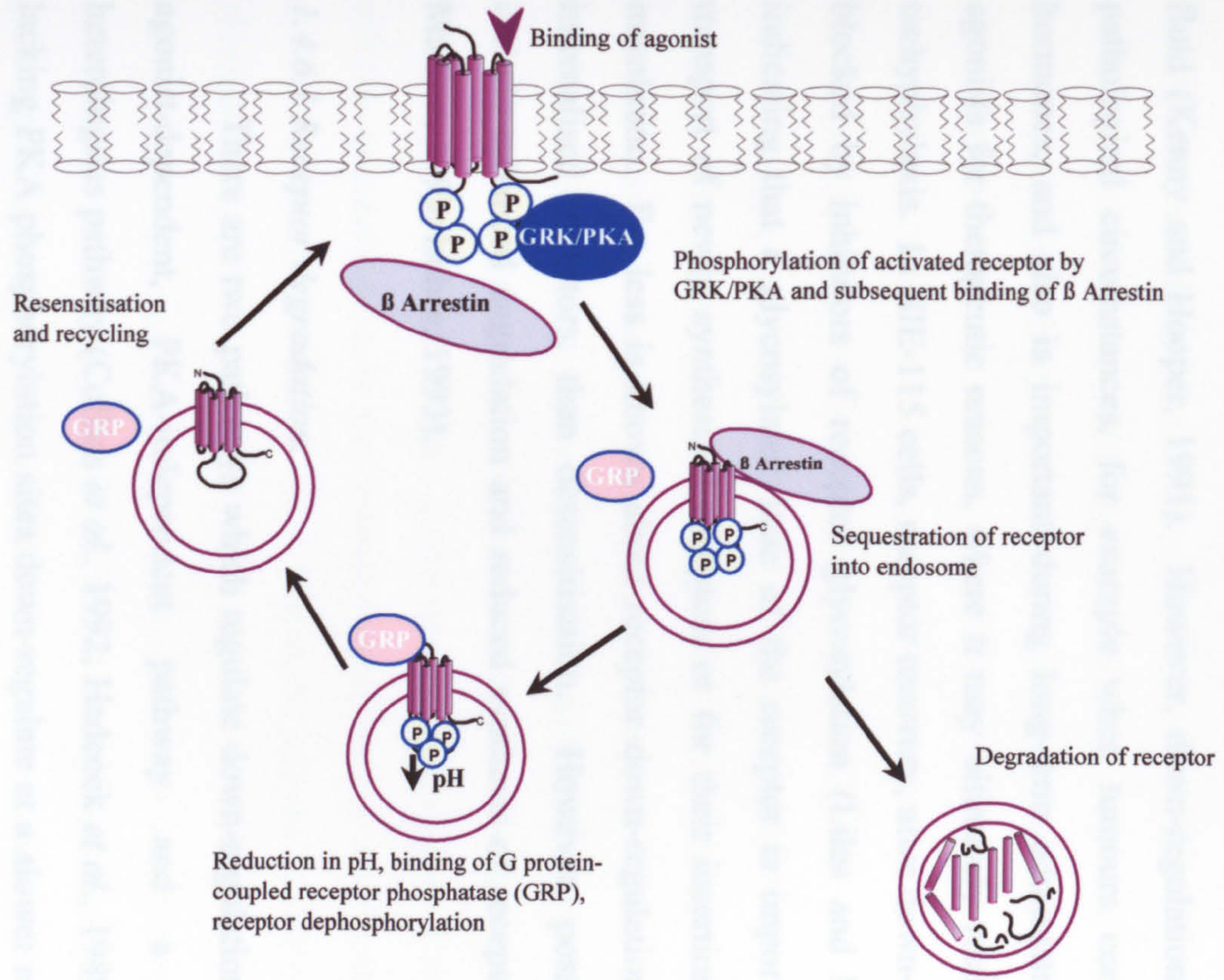



**Figure 1.7**: Endocytosis and cycling of ß2-adrenceptors after agonist stimulation. After phosphorylation of the receptor the adaptor protein ß-arrestin binds, and facilitates its internalisation into an endosome via the clathrin-coated vesicle pathway. Once the receptor has been successfully internalised it has two potential fates: degradation in a lysosome, or recycling back to the cell surface, which requires dephosphorylation of the receptor in an acidified endosome by G protein-coupled receptor phosphatases (GRP).

### *1.4.6 Down-regulation.*

As well as recycling, receptors may undergo down-regulation through degradative pathways. The process of down-regulation normally corresponds to a decrease in the number of cellular receptors and may occur by several mechanisms. Down-regulation is caused by long-term exposure to agonists for hours or days. Unlike internalisation, receptor reappearance following down-regulation requires protein synthesis, and is slow. From a physiological viewpoint, it is probably rare that a cell is continuously exposed to hormones and neurotransmitters, since they are usually rapidly removed from the extracellular fluid (Kenny and Hooper, 1991). However, down-regulation can occur under pathological circumstances, for example when tumours continuously secrete hormones, and also is important during long-term administration of receptor agonists for therapeutic reasons, where it may ultimately result in tolerance or tachyphylaxis. In NIE-115 cells, receptor recovery, after down-regulation, is also blocked by inhibitors of receptor glycosylation (Liles and Nathanson, 1986) indicating that a glycosylated state of the receptor is important in either the transport of newly synthesised receptors or for their insertion into the plasma membrane. Far less is known about receptor down-regulation, and the fate of internalised receptors, than desensitisation. However, possible mechanisms include enhanced degradation and reduced synthesis of receptors (Hadcock and Malbon, 1993; Lohse, 1993).

#### *1.4.6.1 Receptor degradation.*

There are two pathways which regulate down-regulation of the $\beta_2$AR: an agonist-dependent, PKA-independent pathway and a PKA-dependent, heterologous pathway (Collins *et al.*, 1992; Hadcock *et al.*, 1989). $\beta_2$AR mutants lacking PKA phosphorylation sites down-regulate at a slower rate than wild-type receptors, suggesting PKA-dependent phosphorylation enhances degradation. Conversely, GRK-dependent phosphorylation does not appear to enhance degradation, as mutant receptors lacking putative GRK phosphorylation sites down-regulate normally (Collins *et al.*, 1992; Hadcock and Malbon, 1993; Lohse, 1993). Long-term agonist exposure and subsequent G protein coupling or

uncoupling may result in a distinctive phosphorylation pattern or in a certain receptor conformation that exposes lysosomal targeting sequences. Little is known about lysosomal targeting of receptors for hormones and neurotransmitters. After a brief exposure to agonists, the $\beta_2$AR efficiently recycles and is rarely found in lysosomes (Von Zastrow and Kobilka, 1992). It is not known if long term exposure to agonists results in the targeting of these receptors to lysosomes. Continuous exposure may cause multiple rounds of endocytosis and recycling, some of which would be accompanied by mistargeting of a small proportion of receptors to lysosomes, that would eventually deplete the cell of receptors (see Figure 1.7). Mutations of tyrosine residues in the C-terminus of the $\beta_2$AR impairs down-regulation without affecting internalisation, implying that endocytosis and down-regulation are independent processes that rely on distinct signals (Valiquette *et al.*, 1990).

*1.4.6.2 Regulation of receptor synthesis.*

Another component of receptor down-regulation is reduced receptor synthesis. Regulation of receptor expression may occur through changes in gene expression of that receptor. In a cultured hamster cell line, down-regulation of $\beta_2$AR results in a rapid decline in the steady-state level of $\beta_2$AR mRNA (Hadcock *et al.*, 1989). These changes may occur through alterations in the stability of the mRNA and/or through changes in the rate of transcription of the gene. Receptor mRNA destabilisation is the prevailing mechanism and is responsible for the decline in receptor message after long-term agonist stimulation of the $\beta_2$AR (Hadcock *et al.*, 1989).

Short term exposure of cells or tissues to a $\beta_2$AR-agonist results in a transient increase in receptor mRNA levels through a direct increase in the rate of $\beta_2$AR gene transcription. This cAMP-mediated event relies on the binding of the transcription factor cAMP response element binding protein (CREB) to the cAMP response element (CRE), which is present in the promoter region of the $\beta_2$AR gene. Another CRE modulator is a CREB-related transcription factor that binds CREs in a dominant negative fashion, suggesting that under certain circumstances cAMP generation could also result in down-regulation of $\beta_2$AR gene transcription

(Collins *et al.,* 1991). Destabilisation of mRNA for GPCRs depends on cAMP generation and PKA activation. The involvement of PKA suggests phosphorylation or induction of a factor involved in selective degradation of receptor mRNA. A specific protein, termed βAR mRNA-binding protein (β-ARB), that selectively binds to $\beta_2$AR mRNA, is upregulated by $\beta_2$AR-agonists and may be involved in the reduced stability of mRNA induced by βAR-agonists, has recently been identified (Port *et al.,* 1992; Tholanikunnel *et al.,* 1995). βARB interacts with $\beta_2$AR mRNA, which then undergoes dramatic down-regulation. However, no such interaction is seen with mRNAs encoding the $\alpha_{1B}$-, $\beta_1$- or the $\beta_3$AR, which show little or no down-regulation.

### *1.4.7 Regulation of signalling downstream from the receptor.*

#### *1.4.7.1 Regulation at the level of G proteins.*

GPCRs may not be the only component of the G protein-coupled cascade to be regulated by agonist. Indeed, the expression and activity of several down-stream components of the pathway also may be altered. G proteins themselves provide one such example. Green *et al.,* (1990) observed that $G_{i\alpha1}$, $G_{i\alpha2}$ and $G_{i\alpha3}$ were down-regulated in a time- and concentration-dependent manner following chronic stimulation of adipocytes with both adenosine $A_1$ and EP prostaglandin receptor-agonists (Green *et al.,* 1990). The down-regulation at the protein level was not accompanied by any changes in the steady-state levels of the mRNA of the respective G protein subtype (Longabough *et al.,* 1989). $G_{s\alpha}$ levels were also reduced following stimulation of prostanoid IP receptors in NG108-15 cells (Mackenzie and Milligan, 1990) without changes in mRNA levels. Conversely, in the same cells, secretin and adenosine $A_2$ receptor stimulation (that also couple to adenylyl cyclase) did not affect $G_{s\alpha}$ levels. These apparently contradictory observations may be explained by the level of expression of the receptor stimulated. $G_{s\alpha}$ is down-regulated by stimulation with isoprenaline when $\beta_2$ARs are expressed at high (4 pmol/mg membrane protein) but not low (0.3 pmol/mg membrane protein) levels (Milligan *et al., 1995, Adie and Milligan, 1994).* The observations of Kim and Milligan, (1994) support the role of receptor availability in agonist-mediated G protein down-regulation. $G_{s\alpha}$ in NG108-15 neuroblastoma

× glioma cells transfected with $\beta_2$ARs is selectively down-regulated in response to isoprenaline or iloprost treatment. However, after the addition of an irreversible βAR antagonist, bromoacetyl alprenolol menthane, the potency and maximal ability of isoprenaline but not iloprost to induce $G_{s\alpha}$ down-regulation was diminished (Kim and Milligan, 1994).

In addition to an increased rate of G protein degradation, redistribution of G proteins from membranes to cytoplasm has also been observed following exposure of receptors to agonists. Kelly *et al.*, (1990) proposed that heterologous desensitisation observed following iloprost stimulation of neuroblastoma/glioma hybrid cells involved a functional loss of $G_{s\alpha}$ from the cell membrane. In S49 lymphoma cells, isoprenaline evoked a significant redistribution of $G_{s\alpha}$ (50%) to the cytoplasm (Ransnas *et al.*, 1989). The mechanisms involved in this redistribution are not known but the process may involve reversible palmitoylation, which is important in membrane association and has recently been shown to be altered upon G protein activation (Milligan *et al.*, 1995a). Haraguchi and Rodbell (1990) reported that exposure of rat adipocytes to isoprenaline caused redistribution of G proteins along with other plasma membrane elements (AC and 5'-nucleotidase) to a low-density membrane fraction that was generally rich in endosomes and golgi bodies (Kono *et al.*, 1977). The characteristics of this effect were indicative of an endocytic process, which was temperature-dependent, reversible and effectively abolished by agents that inhibited metabolism and lipolysis in adipocytes.

*1.4.7.2 Possible involvement of PDE up-regulation.*

Cyclic nucleotide PDEs (Beavo *et al.*, 1994) are a family of immunologically distinct isoenzymes whose function is to metabolise the second messenger purine nucleotides cAMP and cGMP, to their biologically inactive nucleotide 5'-monophosphates. Consequently, PDEs have a major role in regulating cellular cyclic nucleotide content. PDEs are presently categorised into 10 broad families that are distinguished by a number of criteria including substrate specificity, effect of cofactors ($Ca^{2+}$, calmodulin) and selective inhibition by standard drugs (see Table 1.4). The cAMP-specific, or PDE4, family has received

much attention recently as a potential effector of heterologous desensitisation of $G_s$-coupled receptors in asthma (Giembycz, 1996).

**Table 1.4:** Description of PDE isoenzyme families.

| **PDE family** | **Description** |
|---|---|
| PDE1 | Calmodulin-dependent |
| PDE2 | cGMP-stimulated |
| PDE3 | cGMP-inhibited |
| PDE4 | cAMP-specific |
| PDE5 | cGMP-specific |
| PDE6 | Photoreceptor isoenzyme |
| PDE7 | cAMP-specific |
| PDE8 | cAMP-specific* |
| PDE9 | cGMP-specific+ |
| PDE10 | Dual specificity (cAMP and cGMP)# |

N.B. For reference see review by Beavo *et al.*, (1994), apart from:-
* Soderling *et al.*, 1998, Hayashi *et al.*, 1998.
+ Fisher et al., 1998, Soderling *et al.*, 1998.
# Loughney *et al.*, 1999, Fujishige *et al.*, 1999, Soderling *et al.*, 1999.

Four genes (*PDE4A, PDE4B, PDE4C, PDE4D*) have been identified in the rat (Colicelli *et al.*, 1989; Davis *et al.*, 1989; Swinnen *et al.*, 1989a,b), mouse (Cherry and Davis, 1995) and in humans (Baecker *et al.*, 1994; Bolger *et al.*, 1993; Engels *et al.*, 1995; Livi *et al.*, 1990; McLaughlin *et al.*, 1993; Sullivan *et al.*, 1994; Obernolte *et al.*, 1993) that can encode multiple, immunologically-distinct PDE4 isoenzymes. There is also alternative splicing of mRNA of several groups to produce different protein forms. For example, the *PDE4D* gene can produce five products, *PDE4D1-5* (Conti *et al.*, 1995). Activation of the cAMP/PKA cascade can augment the activity of one or more PDE4 family subtypes by phosphorylation (Monaco *et al.*, 1994; Alvarez *et al.*, 1995; Sette *et al.*, 1994a,b) and/or gene induction which provide highly co-ordinated processes for short and long-term control of cAMP homeostasis. Recently, it has been suggested that induction of PDE4 could occur in certain diseases such as asthma when $\beta_2$AR-agonist therapy is taken chronically. Theoretically, induction of PDE

would lead to increased cAMP degradation and, thus, decreased responsiveness to stimulation of the signal transduction cascade (Torphy *et al.,* 1995; Giembycz, 1996). Although the functional consequences of this phenomenon are uncertain, a prediction based on *in vitro* experimentation, is a heterologous desensitisation of $G_s$-coupled receptors (Torphy *et al.,* 1995; Giembycz, 1996).

Seybold *et al.* (1998), investigated the effect of up-regulating PDE on $\beta_2$AR desensitisation in Jurkat T-cells and human blood T-lymphocytes. Concentration-response studies demonstrated that isoprenaline-induced cAMP accumulation was abolished when PDE4D1, 4D2, and 3B were up-regulated (Seybold *et al.,* 1998). The non-selective PDE3/4 inhibitor 3-isobutyl methylxanthene (IBMX), markedly restored the sensitivity of Jurkat T-cells to isoprenaline implying that this functional desensitisation was due to increase in PDE activity. Up-regulation of PDE4A and PDE4B in response to salbutamol and rolipram treatment in human monocytes also was shown to decrease cAMP elevation upon stimulation with $PGE_2$, showing an involvement of PDE up-regulation in heterologous desensitisation (Torphy *et al.,* 1995).

*1.4.7.3 Regulation of G protein signalling by RGS proteins.*

The slow rate of GTP hydrolysis from $G_\alpha$ subunits is regulated by interactions with GTPase-activating proteins (GAPs). GAPs were first recognised as regulators of protein synthesis factors and low molecular weight GTPases such as Ras. It is now understood that certain effectors in G protein-regulated pathways act as GAPs (Berstein *et al.,* 1992; Arshavsky *et al.,* 1992) and that there exists a family of GAPs for $G_\alpha$ proteins known as regulators of G protein signalling (RGS). There is a paucity of knowledge about the regulation of these proteins, but there are hints that they may be positioned at centres of signalling to intercept activated G proteins.

Currently 19 mammalian genes are known to encode proteins that contain the diagnostic RGS core domain. Typically, this 120-amino acid core is flanked on both sides by highly variable arms that constitute a 25 kDa protein (Tesmer *et al.,* 1997). The most obvious hypothesis for RGS action is that they act as inhibitors of GDP dissociation, blocking G protein activation or stimulators of

GTPase activity, and thereby facilitating deactivation. There is currently no evidence for the former action, but all of the mammalian RGS proteins examined biochemically act as GAPs (Chen *et al.,* 1996; Faurobert and Hurley, 1997; Berman *et al.,* 1996; Watson *et al.,* 1996; Hunt *et al.,* 1996). The most extensively studied RGS is RGS4, which has been expressed in bacteria and purified (Ross, 1995; Berman *et al.,* 1996; Nekrasova *et al.,* 1997).

Most RGS proteins tested to date appear to act as GAPs toward members of the $G_i$ subfamily, although several of these recognise $G_q$ family members (Hepler *et al.,* 1997). GAPs for $G_{s\alpha}$ and $G_{\alpha 12}$ have, thus far, not been detected, though inhibitory effects of RGS proteins on $G_s$-mediated signalling pathways have been observed. Chatterjee *et al.* (1997) reported the expression of a truncated form of RGS3 in baby hamster kidney cells impaired stimulation of cAMP accumulation by calcitonin gene-related peptide or pituitary AC-activating peptide. These peptides are presumably activating $G_s$-coupled receptors, so the question of mechanism is of considerable interest. No GAP activity between RGS3 and $G_{s\alpha}$ has been observed *in vitro* to show whether this RGS acts as an antagonist, blocking the binding of $G_{s\alpha}$ to AC. Currently, there is a dearth of information about the *in vivo* regulation of RGS proteins, and their purpose therein. The most obvious paradigm involves feedback desensitisation of activated, G protein-regulated pathways. Such desensitisation is well known in these signalling systems and regulation is exerted at many levels.

## *1.5. Adverse effects of $\beta_2$-AR agonists.*

### *1.5.1 Tolerance*

Although it is unusual for a drug (or class of drug) to be associated with either the deterioration of a condition it is supposed to alleviate or an increase the mortality from that disease, these are the two properties that have been attributed to $\beta_2$AR agonists. It is these two potentially severe adverse events that have resulted in controversy on how these drugs are to be used in the treatment of asthma. Increased mortality from asthma in England and Wales in the 1960's may

have resulted from the use of an extra-strength metered-dose inhaler preparation of isoprenaline. Indeed, there was no increase in asthma mortality in the USA during this time where the inhaler was not licensed (Stolley, 1972). More recent case control studies suggesting a link between asthma deaths and use of fenoterol in New Zealand (Crane *et al.*, 1989; Grainger *et al.*, 1991) have prompted concern that regular use of $\beta_2$AR agonists might increase asthma severity (Taylor *et al.*, 1993; Wahedna *et al.*, 1993). Because of the possible relationship between bronchodilator drug therapy and the rise in asthma deaths in several countries in the 1960's and 1970's, doubts have been cast on the safety of βAR agonists.

More recently, longer-acting $\beta_2$AR agonists have become available, and their use has become increasingly widespread in many countries. The issue of safety, particularly with these newer long-acting drugs, has been linked to the development of tolerance. Two studies performed by Lipworth and collaborators (Newnham *et al.*, 1994, 1995) with eformoterol, revealed a rightward shift in the dose-response curve to the bronchodilator activity of the drug in asthmatic patients after prior treatment with $\beta_2$AR-agonist compared with placebo. Bronchodilator subsensitivity was found to be greatest when measured 6 h after the final dose of formoterol. This was observed both in terms of $FEV_1$ (forced expiratory volume in 1 second) and $FEF_{25-75}$ (forced expiratory flow at 25, 50, and 75% of vital capacity), inferring that subsensitivity of $\beta_2$AR had occurred in both large and small airways. Interestingly, the finding of airway $\beta_2$AR subsensitivity was associated with significant down-regulation of lymphocyte $\beta_2$AR density. In this respect, there are now data using positron emission tomography to show that down-regulation of lung $\beta_2$AR closely follows that of lymphocyte $\beta_2$AR following exposure to $\beta_2$AR agonists, suggesting that lymphocyte $\beta_2$AR may be used as a surrogate for following changes in $\beta_2$AR density in the lungs (Quing *et al.*, 1994).

The effects of regular salmeterol therapy have also been studied by Grove *et al.*, (1995) who reported a rightwards-shift in the salbutamol bronchodilator dose-response curve after prior treatment with salmeterol compared with placebo. This reduction in bronchodilator response equated to a 2.5- and 4-fold greater dose of salbutamol being required to produce a given $FEV_1$ and a peak flow response respectively and was associated with a significant reduction in lymphocyte $\beta_2$AR

density. Taken together, the eformoterol and salmeterol studies described above indicate that prolonged receptor occupancy with long-acting $\beta_2$-AR agonists results in down-regulation and associated airway $\beta_2$AR subsensitivity.

*1.5.2 Other adverse effects.*

Skeletal muscle tremor is the most common adverse reaction to $\beta_2$AR agonists (Larsson and Svedmyr, 1977). Quantitative measurement of terbutaline-induced hand tremor in healthy volunteers has shown it to be the result of an increase in amplitude of movements within the frequency range of physiological tremor (Bengtsson and Fagerstrom, 1982). Tremor is mediated via $\beta_2$AR in skeletal muscle and could be therefore anticipated to be a response to all $\beta_2$AR agonists (Paterson *et al.*, 1983). Severity of muscle tremor may be used to limit the dose of $\beta_2$AR agonists given to asthmatics (Larsson and Svedmyr 1977).

Salbutamol, administered intravenously, may cause pronounced tachycardia in both asthmatic (Paterson *et al.*, 1971; Svedmyr and Thiringer, 1971) and healthy volunteers (Gundogu *et al.*, 1979). Tachycardia is only consistently observed when high doses of $\beta_2$AR agonists are used in the treatment of severe asthma attacks (Svedmyr, 1981). It might be expected that high doses of $\beta_2$AR agonists may induce potentially troublesome cardiac arrythmias, but generally this is not seen when $\beta_2$AR agonists are used as bronchodilators, even when administered intravenously for acute severe asthma. (Williams *et al.*, 1975, 1981). Nogrady *et al.*, (1977) observed that salbutamol produced a rise in plasma free fatty acids and hypokalaemia in patients with acute severe asthma. Although changes in plasma potassium have been claimed to exacerbate cardiac arrythmias (Haalboom *et al.*, 1985), it has yet to be demonstrated whether they significantly contribute to $\beta_2$AR agonist-induced cardiac side effects (Chapman *et al.*, 1985). In contrast, in digitalised patients, the marked hypokalaemia that maybe induced by intravenous salbutamol may precipitate serious ventricular arhythmias (Leitch *et al.*, 1976).

Sympathomimetic amines are known to decrease plasma potassium concentrations by a process mediated via $\beta_2$ARs (Corea *et al.*, 1981). This reduction in plasma potassium is due to an increase in the transport of potassium

ions into skeletal muscle (Vick *et al.*, 1972, Buur *et al.*, 1982). Intravenous $\beta_2$AR agonists can cause a significant reduction in plasma potassium in asthmatics (Nogrady *et al.*, 1977), but prior exposure to oral $\beta_2$AR agonists can blunt this effect via the phenomenon of tolerance (Nogrady *et al.*, 1977).

$\beta_2$AR agonists increase plasma glucose via a $\beta_2$AR-mediated increase in glycogenolysis (Rolf Smith *et al.*, 1984). Intravenous salbutamol produces a much greater increase in plasma glucose and in ketone bodies in diabetic patients compared to healthy subjects, so hyperglycaemic ketoacidosis may occur in diabetic asthmatics, depending on the level of tolerance which has developed to the metabolic side effects of $\beta_2$AR agonists (Gundogdu, 1979).

While $\beta_2$AR agonist-induced lipolysis is mainly mediated via $\beta_1$ARs (Paterson *et al.*,1983), $\beta_2$ARs appear to play a significant role in the degradation of stored triglycerides to fatty acids and glycerol (Rolf Smith *et al.*, 1984). Nogrady *et al.*, (1977) found that intravenous salbutamol significantly elevated plasma free fatty acid levels in patients with acute severe asthma. Harvey *et al.*, (1981) showed that intravenous salbutamol significantly raised plasma glucose, non-esterified fatty acid levels and glycerol levels. Again, this would be clinically relevant for diabetic asthmatics, who should have their plasma glucose, free fatty acids and ketone bodies monitored.

Bronchodilators, such as atropine, theophylline and $\beta_2$AR agonists, may decrease arterial oxygen tension ($PaO_2$) even though they simultaneously improve airway function (Larsson and Svedmyr,1977; Paterson *et al.*, 1979). $\beta_2$AR agonists can increase cardiac output and cause pulmonary vasodilatation. This may decrease $PaO_2$ by increasing blood flow through poorly ventilated areas of the lung, and increasing ventilation/perfusion mismatch (Tai and Read, 1967). Tai and Read (1967) warn that in severe asthmatic patients, where $PaO_2$ may be very low (Summer, 1985), the change in $PaO_2$ produced by $\beta_2$AR agonists may become clinically relevant. Thus, $PaO_2$ should be carefully monitored when $\beta_2$AR agonists are given for severe life-threatening asthma and supplemental oxygen should be available for use as required by patients. A summary of the adverse effects of $\beta_2$AR agonists is shown in Table 1.5.

**Table 1.5:** Tissue distribution and effects of βAR stimulation in humans.

| | $\beta_1$AR | $\beta_2$AR |
|---|---|---|
| Bronchial smooth muscle | No effect | Relaxation |
| Myocardial heart rate | Increase | Increase |
| Myocardial contractility | Increase | Increase |
| Vascular smooth muscle | Not present | Relaxation |
| Cyclic AMP (plasma) | Increase | Increase |
| Cell membrane $Na^+K^+$ATPase | No effect | Stimulation,↓plasma $K^+$ |
| Glucose (blood) | No effect | Increase |
| Skeletal muscle | No effect | Tremor/Weakness |
| Arterial oxygen tension | No effect | Decrease |

## *1.6 Use of animal models.*

Animal models of asthma and other pulmonary diseases have been developed in an attempt to study the underlying pathophysiology and to evaluate the potential of novel potential therapies. Whilst the use of cell lines is useful in assessing intracellular biochemical mechanisms involved in the pathology of asthma, there is a limit to what can be elucidated from a cell-based systems. Extrapolating from a cellular model to the human system is an ill-advised practise, so animal models have been developed in the hope that they mimic, to some extent, the human disease. Using animal models, important mechanistic questions, that are difficult or impossible to assess in man, can been addressed.

When developing animal models of pulmonary diseases, the experimentally-induced pathology may be characterised in terms of certain pathophysiological changes such as inflammation or smooth muscle contraction, or by indices of lung function. For the study of asthma, a variety of techniques, differing in levels of sophistication and the extent of physiological data they provide, have been developed to measure parameters of lung function in small laboratory animals. Some of the techniques have been developed from similar tests used clinically to investigate lung function and disease in humans. There are

a wide variety of animal species used in models of human airways disease including rats, guinea-pigs, rabbits, dogs, sheep, horses and non-human primates. All species have limitations in terms of the types of questions that can be addressed. For example, studies of cardiopulmonary physiology and function are more easily carried out in larger animals. On the other hand, studies looking at pathophysiological events in the airways are best approached in smaller species where cost is less prohibitive. Furthermore, in terms of the immunological factors that influence airway responses, some species are more fully characterised. Taken together, these observations point out that no single model allows investigation of all questions. Small animals, such as rats (Di Maria *et al.,* 1987) and guinea-pigs (Kallos and Kallos, 1984), are commonly used for the assessment of lung function in models of pulmonary diseases, such as asthma, and to evaluate the effects of drugs.

The advantage of using a rat model to assess long-term $\beta_2$AR agonist treatment resides in the fact that similar studies have been undertaken previously (Nishikawa *et al.,* 1993; Mak *et al.,* 1995) to assess down-regulation of βARs and mRNA levels in lung tissue *ex vivo*. Thus, it was reasoned that this model could be adapted to assess $\beta_2$AR desensitisation *in vivo* and the mechanisms that could account for this effect.

### *1.7. Scope of thesis.*

The factors that govern the expression and regulation of $\beta_2$ARs are fairly well known. The different facets of molecular machinery involved in receptor desensitisation are well characterised in isolated cell systems and tissues. However, there is a paucity of knowledge regarding the pathophysiological effects of chronic, high dose exposure to $\beta_2$AR agonists *in vivo*. Hence, the general aim of the research described in this thesis was to investigate the *in vivo* functional effects that occur following long term exposure of rats to two $\beta_2$AR agonists, salbutamol and salmeterol, and to investigate the molecular mechanism(s) involved in any desensitisation.

Case studies investigating increases in asthma deaths in several countries suggest a possible link with overuse of $\beta_2$AR agonist therapy and increased

mortality due to fatal asthma, so there is a direct clinical relevance in an investigation of the effects of chronic $\beta_2$AR agonist treatment. The initial aim of the study was to develop a viable model system using rats to study $\beta_2$AR desensitisation in response to chronic agonist treatment. Thereafter, detailed studies were performed to determine the nature of desensitisation (homologous or heterologous) and changes in the $\beta_2$AR signal transduction cascade that could account for this effect.

# Chapter 2

# Materials and Methods

## *2.1 Measurement of bronchoconstriction in vivo.*

### *2.1.i Animals.*

Male Sprague-Dawley rats (Charles River, U.K. Ltd., Margate, Kent) of 275-300g body weight were housed in a temperature-controlled (21°C) room with food and water freely available.

### *2.1.2 Animal preparation.*

Animals were sedated with 2% hypnorm (0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone) (0.3 ml/kg) and implanted subcutaneously with Alzet 7-day mini osmotic pumps (Alzet model 2001; Alzo Co., Palo Alto, CA). An incision (approx 1.5 cm) was made in the skin between the scapulae and a small pocket was formed by spreading apart the subcutaneous connective tissues with a haemostat. The pump was inserted into the pocket with the flow moderator pointing away from the head. The skin was then closed with sutures. The rate of infusion of fluid from the mini pump was 0.94-0.97 µl/h delivering 40 µg/kg/h of salbutamol or $PGE_2$, 10 µg/kg/h of salmeterol, or the relevant vehicle (0.9% w/v saline, 0.9% w/v saline/5% EtOH, 1 × PBS/0.1% glacial acetic acid respectively) for a period of 7 days. After the treatment period the animals were used to assess several indices of $\beta_2$AR desensitisation, either *in vivo* where lung function was measured or *ex vivo* using excised lung and tracheae, where various biochemical parameters were measured.

*2.1.3 Dissection and description of preparation.*

Rats treated for 7 days with $\beta_2$AR-agonists, $PGE_2$ or vehicle were anaesthetised with an intraperitoneal (i.p.) injection of urethane (12 ml/kg of a 25% solution w/v in saline) and placed on a heating pad (Homeothermic System, Harvard Apparatus Ltd., Kent), which maintained body temperature at 37°C. Urethane was used since it does not exert a depressant activity on smooth muscle contractility if used in moderate doses. The left jugular vein was cannulated for injection of drugs, and the left carotid artery was cannulated for monitoring blood pressure. The latter was achieved using an indwelling Portex cannula filled with heparin-saline (10 U/ml), linked to a pressure transducer (Druck blood pressure transducer, Druck Ltd., Groby, Leicestershire), which was connected to a recorder (Lectromed Multitrace 2, Ormed Ltd., Welwyn Garden City, Hertfordshire). The trachea was cannulated and the animal ventilated with a small constant volume respiration pump (Bioscience U.K., Sheerness, Kent), operating at 75 strokes per minute and a constant volume of 2 ml. Overflow pressure was measured by a modification of the Konzett and Rossler procedure (Konzett and Rossler, 1940) using a differential pressure transducer (Farnell Electronic Components Ltd., Leeds). Measurement of respiratory insufflation pressure is a crude and simple method for estimating airway resistance. That this method is appropriate for this purpose has been demonstrated by Martling *et al.*, (1984) who compared data obtained from overflow pressure measurements with estimates of pulmonary resistance and compliance, derived from measurements of air flow and transpulmonary pressure (see Figure 2.1 for diagram of *in vivo* air resistance setup).

*2.1.4 Measurement of ACh-induced bronchoconstriction.*

Bronchoconstriction was induced in anaesthetised rats by the administration of ACh. Three doses (100, 500 or 2000 μg/kg i.v.) of ACh were used in a dose-response investigation, given ten minutes apart, and randomised in each animal. This preliminary study was carried out to find a submaximal dose of ACh that could be used in subsequent studies.

2.1.5 *Measurement of bronchoprotection in rats treated chronically with salbutamol, salmeterol or $PGE_2$.*

Bronchoconstriction was evoked in $\beta_2$AR agonist-, $PGE_2$- and vehicle-treated rats by the injection of ACh (500 μg/kg i.v.). Once a constant degree of bronchoconstriction was achieved, salbutamol (100 μg/kg i.v.) was administered after a fifteen minute recovery period. Five minutes after salbutamol, ACh was administered again and any bronchoconstriction was noted. ACh was then administered at 15, 30, 45 and 60 minutes after salbutamol. This procedure was repeated using salmeterol (100μg/kg i.v.), $PGE_2$ (300 μg/kg i.v.), IBMX (300 μg/kg i.v.), forskolin (300 μg/kg i.v.) and levcromakalim (50 μg/kg i.v.). The degree of bronchoprotection in $\beta_2$AR agonist-, $PGE_2$ and vehicle-treated rats was then compared. Preliminary studies established that chronic treatment of rats with salbutamol or salmeterol did not affect resting mean arterial blood pressure or the magnitude of the depressor response evoked by any of the drugs used.

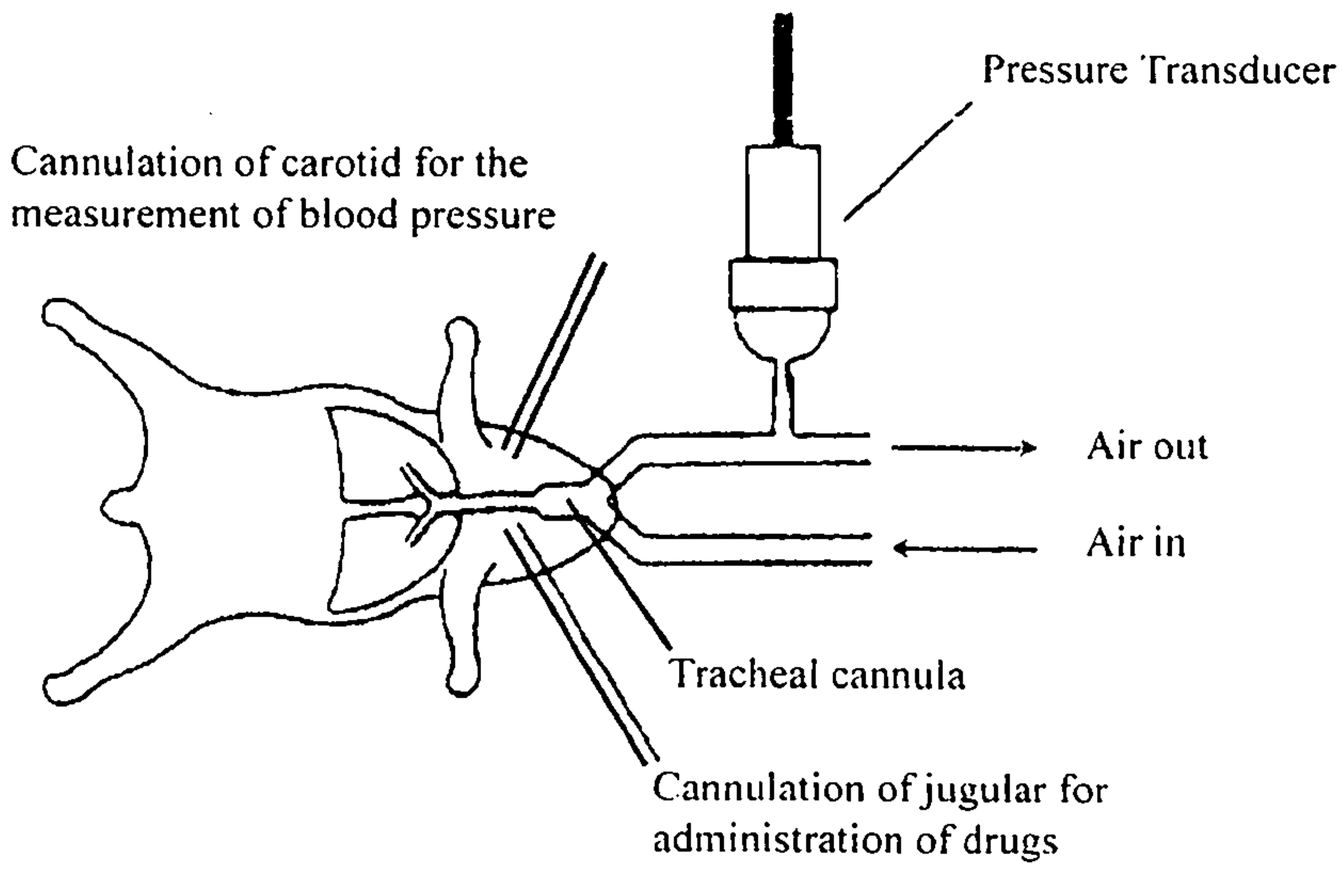



**Figure 2.1.** Diagram showing setup for *in vivo* lung airway resistance experiments. Anaesthetised animals had trachea, jugular vein and carotid artery cannulated. The tracheal and carotid cannulae were connected to pressure transducers to measure airway pressure changes and blood pressure respectively. Adapted from Raeburn *et al.*, (1992).

## *2.2 Radioligand binding studies.*

### *2.2.1 Preparation of lung membranes.*

Lung from rats treated for 7 days with salbutamol, salmeterol or vehicle was ground in liquid nitrogen, and suspended in 30 ml of 25 mM Tris HCl buffer (pH 7.4) containing 0.32 M sucrose at 4°C. The suspension was homogenised with a Polytron homogeniser (Kinematica, Basel, Switzerland) at setting 6 for 2 × 30s bursts, filtered through 2 layers of cheese cloth and centrifuged at 1000 g for 10 min at 4°C to remove debris. The supernatant was then centrifuged at 40,000 g for 20 min at 4°C and the resulting pellet washed twice in 5 ml of 25 mM Tris HCl (pH 7.4 at 4°C) and recentrifuged at the same speed. The purpose of the 2 washes was to remove any soluble interfering substances, such as exogenous drug still bound, endogenous neurotransmitters and guanine nucleotides, which may interfere with the radioligand binding assay. The final pellet was resuspended in Tris HCl (pH 7.4 at 37°C), aliquoted, snap frozen in liquid nitrogen and stored at -80°C.

### *2.2.2 Radioligand binding.*

Aliquots of lung membranes at a protein concentration of 10 μg per tube were incubated with [$^{125}$I]iodocyanopindolol (ICYP; specific activity: 200 Ci/mmol, Amersham International, UK) in the absence or presence of excess (-)-isoprenaline (200 μM) (to define non-specific binding), in 25 mM Tris-HCl buffer (pH 7.4) containing 154 mM NaCl and 1 mM ascorbic acid (to prevent oxidation of isoprenaline) in a final volume of 250 μl. Saturation curves were constructed using six concentrations of [$^{125}$I]ICYP from 3.125pM to 100pM.

The density of $\beta_1$ARs was analysed by ICYP saturation binding in the presence of 0.1 μM ICI 118551 (a $\beta_2$AR selective antagonist), a concentration at which greater than 99% of $\beta_2$ARs are occupied. Conversely, the density of $\beta_2$-adrenoceptors was analyzed by ICYP saturation binding in the presence of 0.1μM CGP 20712 A (a $\beta_1$AR antagonist) a concentration at which greater than 99% of $\beta_1$ARs are occupied (Lemoine & Kaumann, 1991; Kaumann & Lemoine 1987;

Hall *et al.*, 1990). Incubations were carried out in triplicate at 37°C for 120 min, which was found to be optimal for specific binding, and free and membrane-bound ligand was subsequently separated by rapid vacuum filtration over GF/C glass fibre filters pre-soaked for four hours with ice cold 25 mM Tris HCl buffer (pH 7.4) using a Brandel cell harvester. Filters were washed three times with 5 ml of ice-cold Tris buffer and placed in vials. The filters were counted in an Auto Gamma Counting System (model 5550, Packard Instrument Co., Downers Grove, IL, USA) at an efficiency of 80%. Specific binding was calculated by subtracting non-specific binding from total binding.

*2.2.3 Data analysis.*

The equilibrium dissociation constant ($K_d$) and maximal number of binding sites ($B_{max}$) for [$^{125}$I]ICYP, a non-selective βAR antagonist, were determined by Scatchard analysis (Scatchard, 1949) of saturation binding isotherms. Binding data were analysed using the computerised, iterative, non-linear regression program, Prism (Graphpad software, San Diego, Ca, USA).

*2.3 Northern blot analysis.*

*2.3.1 Probes for Northern analyses.*

Random primer labelling was carried out with an 851 bp *Sma*I/*Pvu*II fragment from the human $\beta_1$AR cDNA, a 439 bp *Sma*I fragment from human $\beta_2$AR cDNA and a 1272 bp *Pst*I fragment from rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA using [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol). Rat and human βAR cDNAs are approximately 90% homologous (Hamid *et al.*, 1991).

*2.3.2 Isolation of mRNA.*

Total RNA from rat lung was isolated by an adaptation of the method described by Chomczynski and Sacchi (1987). Frozen rat lung was pulverised under liquid nitrogen with a pestle and mortar and resuspended in 15ml of denaturing solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate - pH

7.0, 0.5% sarcosyl sulphate, 0.1 M 2-mercaptoethanol). The tissue was then homogenised using a Polytron homogeniser at setting 6 for 2 × 30s bursts. The following solutions were then added in sequence with vigorous shaking between each addition; 1 ml of 2M NaAc (pH 4.0), 10 ml phenol (water-saturated) and 2 ml chloroform:isoamylalcohol (49:1). The final solution was shaken for 15 s and cooled on ice for 15 min before centrifugation at 10,000 x g for 15 min. The upper aqueous phase was transferred to a new tube and 10 ml of propan-2-ol was added. RNA was allowed to precipitate overnight at -20°C before centrifugation at 12,000 x g for 20 min. The resulting pellet was resuspended in 5 ml of solution D and 5 ml of propan-2-ol before a second precipitation overnight at -20°C. The final RNA pellet was washed with 75% ethanol, dried under vacuum and resuspended in 0.5 ml of nuclease-free water. Poly $(A)^+$ RNA was then isolated from total cellular RNA using an mRNA isolation kit according to the manufacturer's instructions.

*2.3.3 Northern blotting.*

Total cellular RNA (20µg/lane) and denatured mRNA was subjected to size fractionation by gel electrophoresis on 1% agarose/formaldehyde gels containing 20 mM morpholinosulphonic acid (MOPS), 5 mM sodium acetate and 1 mM EDTA (pH 7.0). Blotting of mRNA to nylon $N^+$ membranes was achieved by capillary action using standard sodium citrate (SSC; pH 8.0) as a buffer. RNA was permanently fixed to the nylon membranes through ultraviolet irradiation using a UV Stratalinker-1800 (Stratagene, UK).

*2.3.4 Prehybridisations and hybridisations.*

Prehybridisations and hybridisations were carried out at 42°C with the probes labelled to approximately 1.5 x $10^6$ cpm/ml in a buffer containing 50% formamide, 2 x SSC, 50 mM Tris-HCl (pH 7.5), 5 x Denhardts solution, 0.1% sodium dodecyl sulphate (SDS), 5 mM EDTA and 250 µg/ml denatured salmon sperm DNA. Following overnight hybridisation, the blots were washed to remove non-specific binding of the cDNA probes. Washes were carried out for 20 min intervals in increasing temperature and reduced salt in the presence of 0.1% SDS to a final stringency of 0.1 x SSC at 65°C. The blots were then exposed to Kodak

X-OMAT film for suitable times. Autoradiographs were analysed by laser-scanning densitometry using the Protein and DNA Imageware systems (The Discovery Series, NY, USA). The values for optical density were corrected for variations in gel loading using GAPDH to give normalised band densities.

### *2.4 Cyclic AMP experiments.*

#### *2.4.1 Time-response studies.*

Excised lung from vehicle-, salmeterol- and and salbutamol-treated rats was chopped into pieces of ~5 $mm^3$ and incubated in Krebs Henseleit solution (118 mM NaCl, 5.9 mM KCl, 1.2 mM $MgSO_4.7H_2O$, 1.2 mM $NaH_2PO_4.2H_2O$, 2.5 mM $CaCl_2.6H_2O$, 10 mM glucose and 25 mM $NaHCO_3$) containing 10 μM indomethacin and bubbled with 95% $O_2$/5% $CO_2$ at 37°C. Tissue pieces were incubated for 30 min in the absence or presence of 0.1 mM IBMX, a non-selective phosphodiesterase inhibitor, at which point salbutamol was added to a final concentration of 10 μM. At the relevant time point (1, 2, 3, 5, 10, 15, 20, 30 min) tissue was removed and blotted on absorbent paper to remove excess buffer and then immersed in liquid nitrogen. Lung was kept at -80°C until cAMP mass was determined.

#### *2.4.2 Concentration-response studies.*

Excised lung pieces from vehicle- and $\beta_2$AR agonist-treated rats were treated as described in section 2.4.1, except that salbutamol (10 nM to 100 μM), salmeterol (1μM), $PGE_2$ (1 μM) and CTx (10 ng/ml-30 μg/ml) were used as stimuli. After 5 minutes (2 hours for CTx), tissue was blotted on absorbant paper and immersed in liquid nitrogen. In some studies, tissue was pre-incubated for 30 minutes with 1 μM propranolol prior to salbutamol (10 μM), to assess whether the response was mediated through βARs.

*2.4.3 Radioimmunoassay of cAMP.*

Frozen rat lung was homogenised in 2 ml of ice cold 1 M trichloroacetic acid (TCA) and centrifuged at 2500 g to precipitate particulate material. To 500 µl of the resulting supernatant, 50 µl of 25 mM EDTA was added followed by 500 µl of freon:tri-n-octylamine (1:1 v/v). The mixture was thoroughly vortex-mixed and centrifuged at 13,000 g for 2 minutes at room temperature. The top phase (360 µl) was removed and neutralised with 40 µl 120 mM sodium bicarbonate. The neutralised extracts were acetylated by the consecutive addition of triethylamine (20 µl) and acetic anhydride (10 µl), and cAMP mass was measured immediately by RIA. Briefly, to 200 µl of acetylated sample, were added 50 µl of adenosine-3',5'-monophospho-2-*O*-succinyl-3-[$^{125}$I] iodotyrosine methyl ester (approximately 2000-3000 dpm) in 0.2% BSA and 100 µl of anti-cAMP antibody in 0.2% BSA. After vortex-mixing, samples were incubated overnight at 4°C and free and antibody-bound cAMP was separated by charcoal precipitation with ice-cold potassium phosphate buffer (100 mM in 0.2% BSA - pH 7.4) and quantified by γ-counting. The detection limit and sensitivity ($IC_{50}$) of this assay are 10 and 145 fmol cAMP respectively.

*2.5 Preparation and assay of PKA.*

Since guinea-pig, human and mouse lung contains primarily the Type II isoenzyme of PKA (see Giembycz and Diamond, 1990a) we assumed this to be the case for rat lung. To prevent the inactivation of this enzyme resulting from drug treatment, each frozen tissue was homogenized (Polytron) for 1 × 15 s burst at setting 8 in 20 vol (w/v) of ice-cold phosphate buffer (5 mM $KH_2PO_4$, 5 mM $K_2HPO_4$, pH 6.8, 10 mM EDTA, 10 mM DTT and 500 µM IBMX) and supplemented with 20 mM NaCl and 140 mM KCl (Cherrington *et al.*, 1976). The homogenate was centrifuged at 1,000 × g for 15 min at 4°C to pellet cell debris. The resulting supernatant containing the enzyme was diluted 1:20 in phosphate buffer immediately before use.

PKA activity was estimated by measuring the phosphorylation of the peptide Kemptide as described by Giembycz and Diamond (1990b). Assays were performed in triplicate at 30°C and initiated by the addition of 25 µl of diluted

enzyme extract to 75 µl of a reaction cocktail containing 20 mM MOPS, 10 mM magnesium acetate, 2 mg/ml BSA, 100 µM IBMX, 100 µM ATP supplemented with approximately 100 cpm/pmol [$\gamma$-$^{32}$P]ATP, 100 µM Kemptide, and 20 µM cAMP. Reactions were terminated after 10 min by spotting 75 µl aliquots of the reaction mixture on to 2 × 2 cm$^2$ P81 phosphocellulose paper squares that were left for 30 s then were immersed in orthophosphoric acid (150 mM). The paper squares were then extensively washed with fresh orthophosphoric acid to displace any non-specifically bound ATP and inorganic phosphate, immersed in IMS (~5 min) and diethyl ether (~5 min) and allowed to dry. The bound radioactivity (representing phosphorylated substrate) was subsequently quantified by liquid scintillation counting in 4 ml ACS II (Amersham International, Bucks).

One unit of PKA activity is defined as that amount of PKA which catalysed the incorporation of 1 pmol of phosphate from ATP into the peptide substrate, Kemptide, in 1 min at 30°C. PKA activity is expressed as a ratio of the specific activity of PKA in the absence of exogenous cAMP divided by the specific activity of PKA in the presence of 20 µM exogenous cAMP, which maximally activates the enzyme.

### *2.6 PDE experiments.*

#### *2.6.1 Measurement of cAMP PDE activity.*

Vehicle- and salbutamol-treated rat lung were ground in liquid nitrogen and homogenised in ice-cold buffer (20 mM TEA - pH 8, 1 mM EDTA) supplemented with the proteinase inhibitors benzamidine (2 mM), leupeptin (50 µM), PMSF (100 µM), bacitracin (100 µM) and soybean trypsin inhibitor (20µg/ml) and used immediately as the enzyme source. Assays were performed in duplicate at 37°C and initiated by the addition of 30 µl lung membranes to 270 µl of a reaction cocktail containing (final concentration) 20 mM TEA (pH 8.0), 5 mM dithiothreitol (DTT), 5 mM bovine serum albumin (BSA), 5 mM magnesium acetate, 0.25 unit alkaline phosphatase, 1 mM EGTA, 1 µM cAMP (supplemented with *ca.*250 000 dpm of [8-$^3$H] cAMP and *ca.* 5 000 dpm [8-$^{14}$C] adenosine to

estimate recovery) and any drug(s) under evaluation or its vehicle. The reaction was terminated by the addition of 1 ml of a mixture of Dowex AG 1-x8 : methanol : water (1:2:1), vortex-mixed and placed in an ice bath until the end of the assay. Samples were then further vortex-mixed for 30 min before being centrifuged at 12000 g for 5 min at 4°C. The radioactivity in 700 µl aliquots of the resulting supernatants was determined by liquid scintillation counting in 2 ml ACS II scintillant (Amersham) at a counting efficiency of approximately 60%. PDE activity is expressed as the formation of adenosine 5' monophosphate from cAMP per minute per µg protein (i.e. the lysate derived per µg protein) at 37° C after correction for the recovery (routinely 65-85%) of [8-$^{14}$C] adenosine. In this study, PDE3 and PDE4 are defined as cAMP hydrolytic activity inhibited by Org 9935 (30 µM) and rolipram (50µM) respectively.

*2.7 Western blot analysis.*

*2.7.1 Extraction of total, cytoplasmic and membrane protein.*

Approximately 100 mg of rat lung were ground in liquid nitrogen and subsequently homogenised for 60 s in 2 ml of ice-cold 50 mM Tris-HCl (pH 7.4)/ 1 mM $MgCl_2$ containing 0.5 mM PMSF, 1 mM sodium orthovanadate, 10 µg/ml leupeptin, 100 µg/ml aprotinin, 5 mM NaF and 10 mM sodium pyrophosphate using a Polytron. The homogenates were centrifuged at 1000 x *g* at 4°C for 15 min and 500 µl of the supernatant containing total cellular protein was taken. The remainder of the supernatant was centrifuged at 100,000 x *g* at 4°C for 60 min. The resulting pellets were resuspended in the aforementioned buffer with proteinase inhibitors and the protein concentration was established.

*2.7.2 Western blot analysis.*

Cytosolic and membrane protein were mixed with one-fourth volume of 4 x Laemmli sample buffer, boiled for 5 min and loaded onto a 10% polyacrylamide Tris-HCl/SDS gel. Denatured proteins were then size-fractionated by gel

electrophoresis for 40 min at 40 mA. Following electrophoresis, proteins were transblotted onto hybond nitrocellulose paper in a buffer containing 200 mM glycine, 50 mM Tris, 20% [v/v] methanol, 0.03% [w/v] SDS at 300 mA for 3 h. The nitrocellulose membranes were blocked overnight at 4°C in washing buffer (10 mM Tris-base, 0.05% Tween 20) containing 5% skimmed milk. The membranes were then incubated at 25°C for 1 h with a rabbit polyclonal antibody specific to either $G_{s\alpha}$ (anti-$G_{s\alpha}$ and $G_{olf\alpha}$; New England Nuclear (NEN)/Dupont, NEI 805) (1 in 1000 dilution), $G_{\beta}$ (anti-$G_{\beta}$ (common β-subunit); NEN/Dupont, NEI 808) (1 in 1000 dilution), GRK2 (anti-GRK2, Santa Cruz, Ca USA) (1 in 500 dilution), $\beta_2$AR (anti-$\beta_2$AR, Santa Cruz, Ca USA) (1 in 500 dilution) or β-arrestin (anti-β-arrestin; Lohse *et al.*, 1990) (1 in 500 dilution). All antisera were raised to the C-terminal part of their respective peptides. The filters were then washed for 5 x 5 minutes at 25°C in washing buffer (without skimmed milk) before incubation for a further 1 h with a donkey, anti-rabbit horseradish peroxidase-conjugated antibody in washing buffer (diluted 1:4000). Finally the nitrocellulose filters were treated with ECL reagent according to the manufacturer's instructions and exposed to Kodak X-OMAT film for an appropriate time. Band density was quantified by laser scanning densitometry.

*2.8 Assessment of GRK activity.*

*2.8.1 Preparation of cytosolic and membrane fractions.*

The method used was based on that described by Benovic *et al.*, (1989). Fresh tissue was homogenised (Polytron) at low speed for 1 min in cell lysis buffer (10 mM Tris HCl - pH 7.4, 5 mM EDTA, 7.5 mM $MgCl_2$, 0.1 mM PMSF, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 10 μg/ml benzamidine) and centrifuged at 4°C at 800 x g for 5 min. The pellet, containing unbroken cells and tissue debris, was discarded and the supernatant was centrifuged at 48,000 x g for 20 min at 4°C to separate the plasma membrane and cytosolic fraction. The cytosol was then re-centrifuged at 150,000 x g for 60 min at 4°C and GRK activity in the supernatant was determined immediately according to the method of Mayor *et al.*, (1987). The membrane fraction was washed once in cell lysis buffer, and recentrifuged at

48,000 x g for 15 min at 4°C. The resultant pellet then was resuspended in cell lysis buffer, sonicated for 5 s prior to assay and protein determination.

*2.8.2 Preparation of rod outer segments.*

Previous studies have demonstrated that rhodopsin, contained in the rod outer segments (ROS) of bovine retina, can be phosphorylated by βARK in a light-dependent manner (Benovic *et al.,* 1986) and can be used to investigate βARK activity quantitatively (Mayor *et al.,* 1987). Rhodopsin was used in the present study to measure and compare βARK activity in saline- and salbutamol-treated rat lung. ROS were prepared from bovine retina by stepwise sucrose gradient sedimentation using the method described by Benovic *et al.,* (1987). Retinal rhodopsin kinase was degraded by treatment of the preparation with 5 M urea.

*2.8.3 Phosphorylation assay.*

Assays were performed in triplicate at 30°C and initiated by the addition of 30 µl (100 µg protein) of cytosol or membranes to 120 µl of a reaction mixture containing (final concentration) 20 mM Tris HCl - pH 7.4, 300 pmol urea-treated ROS, 50 µM [$\gamma$-$^{32}$P]ATP (2 to 5 cpm/fmol), 8 mM $MgCl_2$, 3 mM EDTA, 5 mM NaF, 12 mM NaCl, 70 µM PMSF, 7 µg/ml leupeptin, 3.5 µg/ml pepstatin and 7 µg/ml benzamidine in the absence and presence of light. After incubation for 45 min, the reaction was quenched by the addition of 900 µl of ice-cold buffer (10 mM Tris HCl - pH 7.4, 100mM NaCl, 10 mM NaF, 2 mM EDTA) and centrifuged at 57,000 x g for 15 min at 4°C. The resultant pellet was reconstituted in SDS sample buffer, electrophoresed on a 10% SDS polyacrylamide slab gel and stained with Coomasie blue. Bands corresponding to phosphorhodopsin (*Mr* 35 kDa) were excised from the gel and the associated radioactivity determined by liquid scintillation counting. One unit of activity is defined as that amount of GRK which catalysed the incorporation of 1 fmol phosphate from ATP into ROS in 1 min per mg protein at 30°C.

*2.9 Protein Estimations.*

Two assays were used to estimate protein levels. In radioligand binding experiments protein estimation was carried out according to the method of Lowry (1951). All other protein estimations were carried out using a BioRad kit according to the manufacturer's instructions. In all protein estimations, bovine serum albumin was used as standard.

*2.10 Reagents.*

Hypnorm™ was obtained from Janssen-Cilag, High Wycombe, Bucks, UK. Salmeterol was a gift from Glaxo-Wellcome, Stevenage, Herts, UK. Levcromakalim was a gift from Smithkline Beecham Pharmaceuticals, Betchworth, Surrey. [$^{125}$I]iodocyanopindolol ([$^{125}$I]ICYP; specific activity 2200 Ci/mmol) was obtained from New England Nuclear (Stevenage). Org 9935 (4,5-dihydro-6-(5,6-dimethoxybenzo[b]thiene-2-yl)- 5-methyl-3(2H)pyridazinone) was obtained from Organon Laboratories Ltd, Newhouse, Lanarkshire, UK. Rolipram was obtained from Schering AG, Berlin, Germany. CGP 20712A (11-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamine-3-[4-(1-methyl-4-trifluoromethyl-2-imidazoyl) phenoxy]-2-propanol methanesulphonate) was obtained from Ciba-Geigy, Basel, Switzerland. ICI 118551 (erythro (±) -1(7-methylindan-4-yloxyl-3-isopropylamino butan-2-ol) was obtained from Zeneca, Macclesfield, UK. The scintillation cocktail, Filtron X, was obtained from National Diagnostics, (Aylesbury, UK). Protein assay reagent and acrylamide:bisacrylamide (37.5:1; 40% solution) were obtained from Bio-Rad (Hemel Hempstead, UK.). mRNA isolation kits were obtained from Promega (Southampton). [$\gamma^{32}$P]ATP, [$\alpha^{32}$P]UTP and [$\alpha^{32}$P]dCTP (specific activities >3000 Ci/mmol), [$\gamma^{32}$P]ATP (specific activity 20-40 Ci/mmol ), adenosine 3,5'-cyclic phosphoric acid 2'-O-succinyl 3-[$^{125}$I]cAMP (specific activity 2000 Ci/mmol), nylon $N^+$ membranes, hybond nitrocellulose paper, ECL reagents, horse radish peroxidase-conjugated secondary antibody and ACS II scintillant were supplied by Amersham Life Sciences (Amersham, UK). Phenol was obtained from Rathburn Chemicals (Peebleshire). GF/C glass fibre filters, phosphocellulose, chloroform, ethanol, propan-2-ol,

formaldehyde, diethyl ether, Freon (1, 1, 2-trichlorotrifluoroethane), glacial acetic acid and Analar grade standard laboratory chemicals were obtained from BDH (Leicester). $G_{s\alpha}$- and $G_{\beta}$-specific antibodies were obtained from NEN/Dupont, Boston, MA, USA. $\beta_2$AR- and GRK2-specific antibodies were obtained from Santa Cruz Biotechnologies (Middlesex, UK). β-Arrestin-specific antibodies were a kind gift from Dr Martin Lohse, Institut für Pharmakologie und Toxikologie, Universität Würzburg, Germany; and Roche Diagnostics, Pharma Research, Penzberg, Germany. Donkey anti Rabbit antibodies were obtained from DAKO laboratories, Stevenage, Herts.

The following chemicals were obtained from Sigma (Poole, Dorset): (-)-isoprenaline, 2-mercaptoethanol, 50X Denhardts solution, acetylcholine (ACh), adenosine 3,5'-cyclic-monophospate (cAMP), adenosine 5'-triphosphate (ATP), agarose, aprotinin, ascorbic acid, benzamidine, bovine serum albumin (BSA), cAMP-specific antibody, cholera toxin (CTx), cytidine 5'-triphosphate (CTP), dimethyl sulphoxide (DMSO), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-bis(β-aminoethylether)-N, N, N', N'-tetraacetic acid (EGTA), foetal calf serum (FCS), formamide, forskolin, glycerol, guanidine thiocyanate, heparin sulphate, isoamyl alcohol (IAA), 3-isobutylmethylxanthine (IBMX), Kemptide, L-glutamine, leupeptin, magnesium acetate, morpholinosulphonic acid (MOPS), prostaglandin $E_2$ ($PGE_2$), phenylsulphonylflouride (PMSF), phosphoric acid, salbutamol, salmon sperm DNA, sodium acetate, sodium fluoride, sodium orthovanadate, sodium pyrophosphate, sorbitol, spermidine, trichloroacetic acid (TCA), trioctylamine, Triton X-100, Trizma™ base, Trizma™ hydrochloride, trypsin/EDTA, Tween-40, urethane.

*2.11 Statistical Analysis.*

Results are presented as mean values ± SEM. Difference for paired and unpaired data between values were tested for significance by a two tailed Students *t*-test as appropriate. A probability level of $p<0.05$ was considered significant. (An overview of the methods used in these studies is given in Figure 2.2).

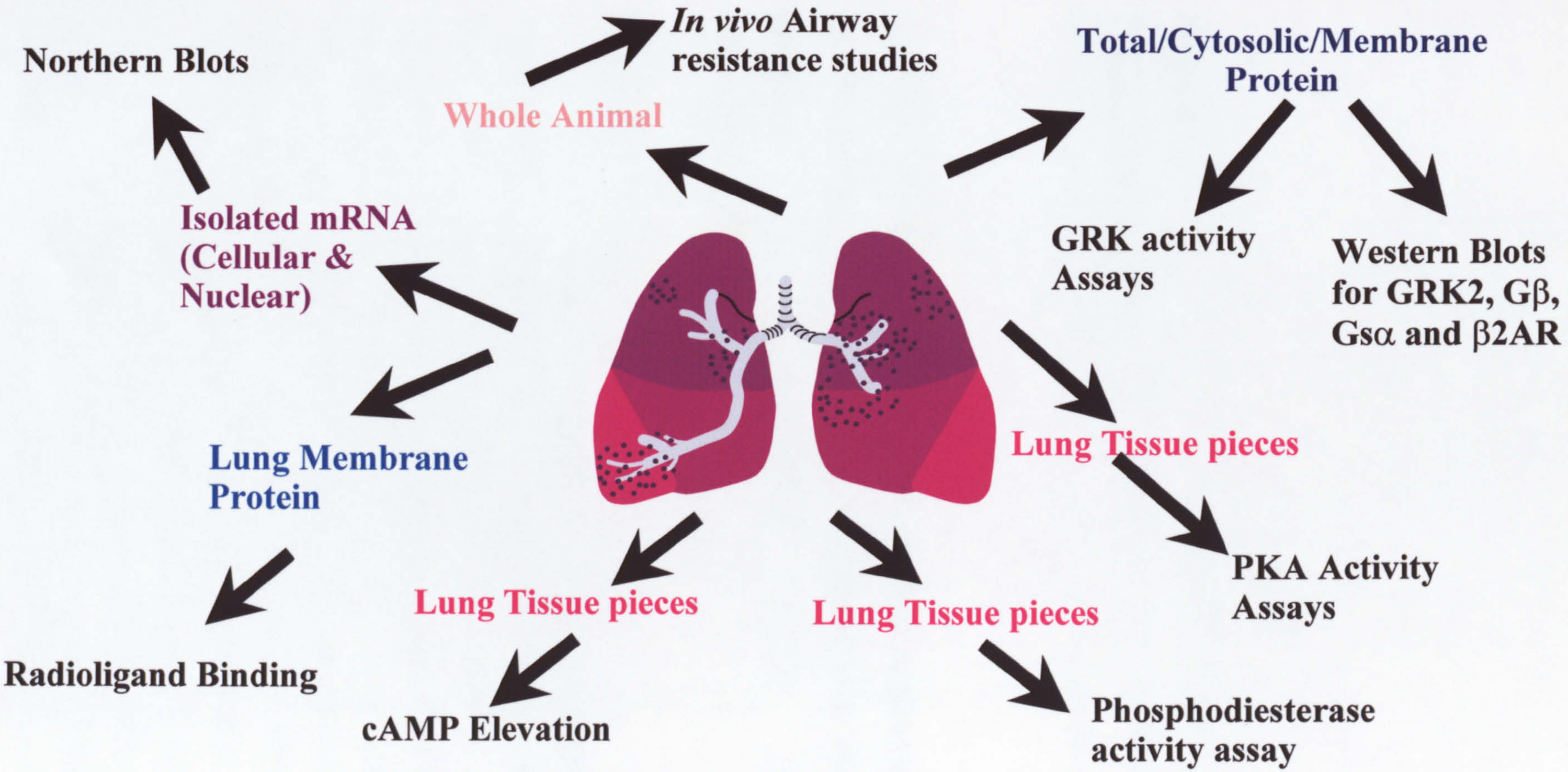



**Figure 2.2.** Summary of methodology.

# Chapter 3

## Results & Discussion

### 3.1 Effect of chronic treatment of rats with salbutamol and $PGE_2$ on the acute protective action of salbutamol, $PGE_2$, IBMX, forskolin and levcromakalim against ACh-induced bronchoconstriction *in vivo*.

*3.1.1 Introduction.*

Inhaled $\beta_2$AR agonists are regarded as the most effective bronchodilators for the therapy of asthma. However, since epidemics of asthma deaths in the UK in the 1960's (O'Donnell *et al.,* 1989) and New Zealand in the late 1970's (Grainger *et al.,* 1991), it has been suggested that overuse of $\beta_2$AR agonists may be associated with an increased risk of mortality. Indeed, many studies have shown tachyphylaxis to the protective effects of $\beta_2$AR against bronchoconstriction occurs in humans (Newnham *et al.,* 1994; Lipworth *et al.,* 1998), which would tend to reduce the clinical efficacy of these drugs (Cockcroft *et al.,* 1993).

It is well established that prolonged exposure of tissues to $\beta_2$AR agonists is associated with a rapid attenuation of responsiveness. This phenomenon, known as desensitisation, has been studied in detail, and is due to several mechanisms at different levels in the signal transduction pathway. There are two major patterns

of desensitisation, which have been characterised, homologous and heterologous desensitisation. Homologous, or agonist-specific, desensitisation involves phosphorylation of receptors by GRKs, binding of an additional factor termed β-arrestin, internalisation of the receptor into clathrin-coated pits and subsequently into vesicles. This process is fairly rapid and occurs within minutes of exposure to agonist. In contrast, heterologous, or non-agonist specific, desensitisation is postulated to be mediated by PKA and/or PKC, and also results in desensitisation of other G protein-coupled receptors. If exposure to agonist continues, down regulation of receptor number eventually occurs. This is observed as a loss of total receptor binding sites from the surface of the cell and is believed to occur via increased degradation of internalised receptors and reduced *de novo* synthesis of receptor protein (Chuang *et al.*, 1996; Böhm *et al.*, 1997).

$\beta_2$AR desensitisation has been studied extensively *in vitro* and the biochemical mechanisms responsible for this phenomenon are well defined. However, little is known of the biochemical processes of $\beta_2$AR desensitisation *in vivo*. To this end, a model of $\beta_2$AR desensitisation was developed which involved administering salbutamol (or salmeterol, see section 3.3) via the subcutaneous route to conscious rats at a constant rate for seven days. This model allowed an assessment of the ability of salbutamol and other drugs, given acutely, to protect the airways against ACh-induced bronchoconstriction *in vivo*. Moreover, changes in the $\beta_2$AR signal transduction cascade in lung and trachea that may account for any desensitisation could be be studied in detail *ex vivo* (section 3.2).

### *3.1.2 Dose-response relationship to ACh in anaesthetised rats treated chronically with saline and salbutamol.*

To ascertain the correct dose of ACh required to elicit a submaximal and reproducible bronchoconstriction, a dose-response study was performed measuring changes in tracheal pressure (Figure 3.1.1). ACh evoked a dose-dependent bronchoconstriction in both vehicle- and salbutamol-treated rats. The change in tracheal pressure obtained using ACh at 500 μg/kg i.v. was submaximal in both groups of animals, allowing increases or decreases in lung function to be

detected in response to pharmacological interventions and was used in all subsequent studies. There was no statistically significant difference in the magnitude of bronchoconstriction effected by ACh in vehicle- and salbutamol-treated rats.

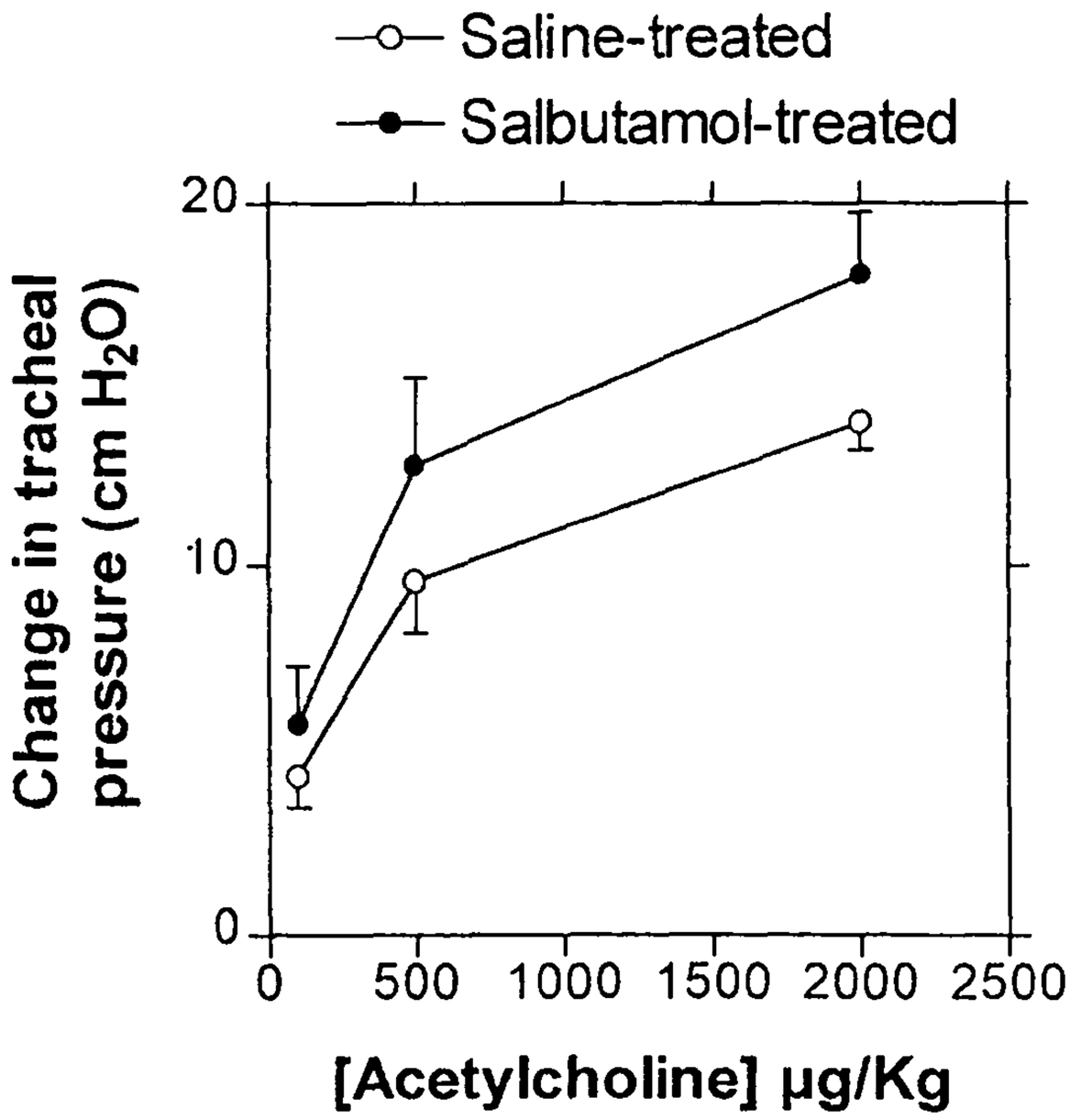



**Figure 3.1.1 Dose-dependence of ACh-induced bronchoconstriction in anaesthetised in rats treated chronically with saline and salbutamol.** Rats were anaesthetised with urethane and their carotid artery, jugular vein and trachea were cannulated as described in Materials and Methods. ACh (100, 500, or 2000 µg/kg i.v.) was then administered and changes in overflow pressure were measured using a modification of the technique introduced by Konsett and Rossler (1940). Data represent the mean ± SEM of four determinations in different rats.

### *3.1.3 Effect of repeated ACh challenge on the magnitude of bronchoconstriction in rats treated chronically with saline and salbutamol.*

The experimental protocol for the *in vivo* desensitisation study required repeated challenges with ACh to enable the bronchoprotective activity of a number of drugs to be assessed. To ensure that there was no change in the sensitivity of the preparation, with time, or in response to repeated drug treatment, ACh (500 µg/kg) was administered to rats at various intervals (0, 5, 10, 15, 30 and

45 minutes) and the bronchoconstriction noted. Figure 3.1.2 shows that there was no significant change in ACh-induced bronchoconstriction with time in vehicle- (open bars) and salbutamol-treated (closed bars) rats. Moreover, consistent with the data shown in figure 3.1.1 the magnitude of bronchoconstriction effected by ACh was the same in both groups of rats.

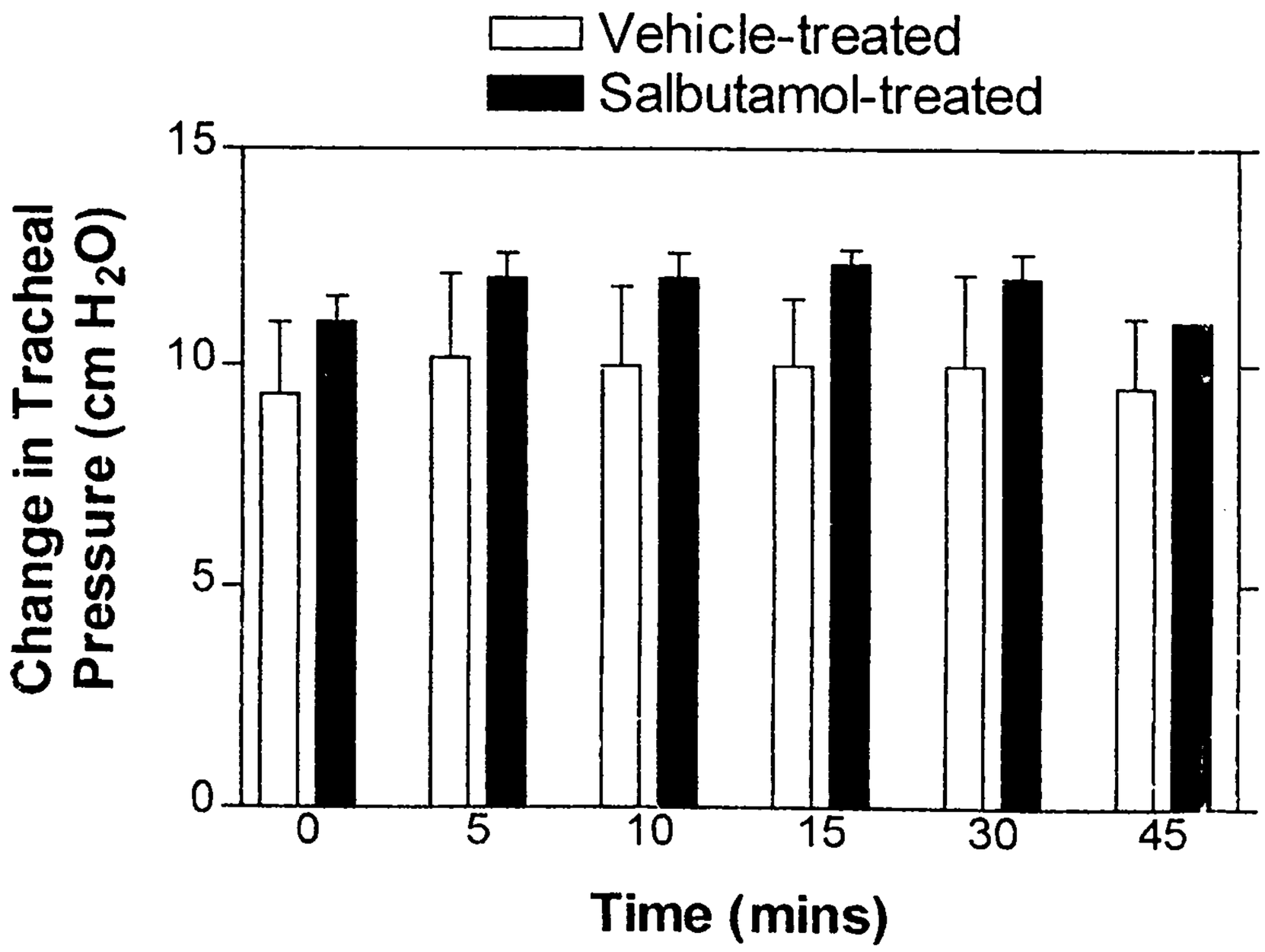



**Figure 3.1.2 Effect of repeated ACh challenge on the magnitude of bronchoconstriction in rats treated chronically with vehicle and salbutamol.** Rats were anaesthetised with urethane, and their carotid artery, jugular vein and trachea cannulated as described in Materials and Methods. ACh (500 μg/kg i.v.) was administered at 0, 5, 10, 15, 30 and 45 minutes, and changes in overflow pressure were measured using a modification of the technique introduced by Konsett and Rossler (1940). Data represent the mean ± SEM of four determinations in different rats.

*3.1.4 Effect of chronic treatment of rats with salbutamol on the ability of salbutamol, $PGE_2$, IBMX, forskolin, and levcromakalim, given acutely, to protect against ACh-induced bronchoconstriction.*

The ability of salbutamol (100μg/kg i.v.) to protect the airways against ACh-induced bronchoconstriction in vehicle- and salbutamol-treated rats was

examined. In salbutamol-treated rats, administration of salbutamol had no significant effect on ACh-induced bronchoconstriction whereas in vehicle-treated animals significant protection (~52%) was seen (see Figure 3.1.3 and Table 3.1 for summary of data, and Figure 3.1.4 for representative traces). In vehicle-treated rats, the protective effect of salbutamol persisted for 30 minutes before lung function began to recover.

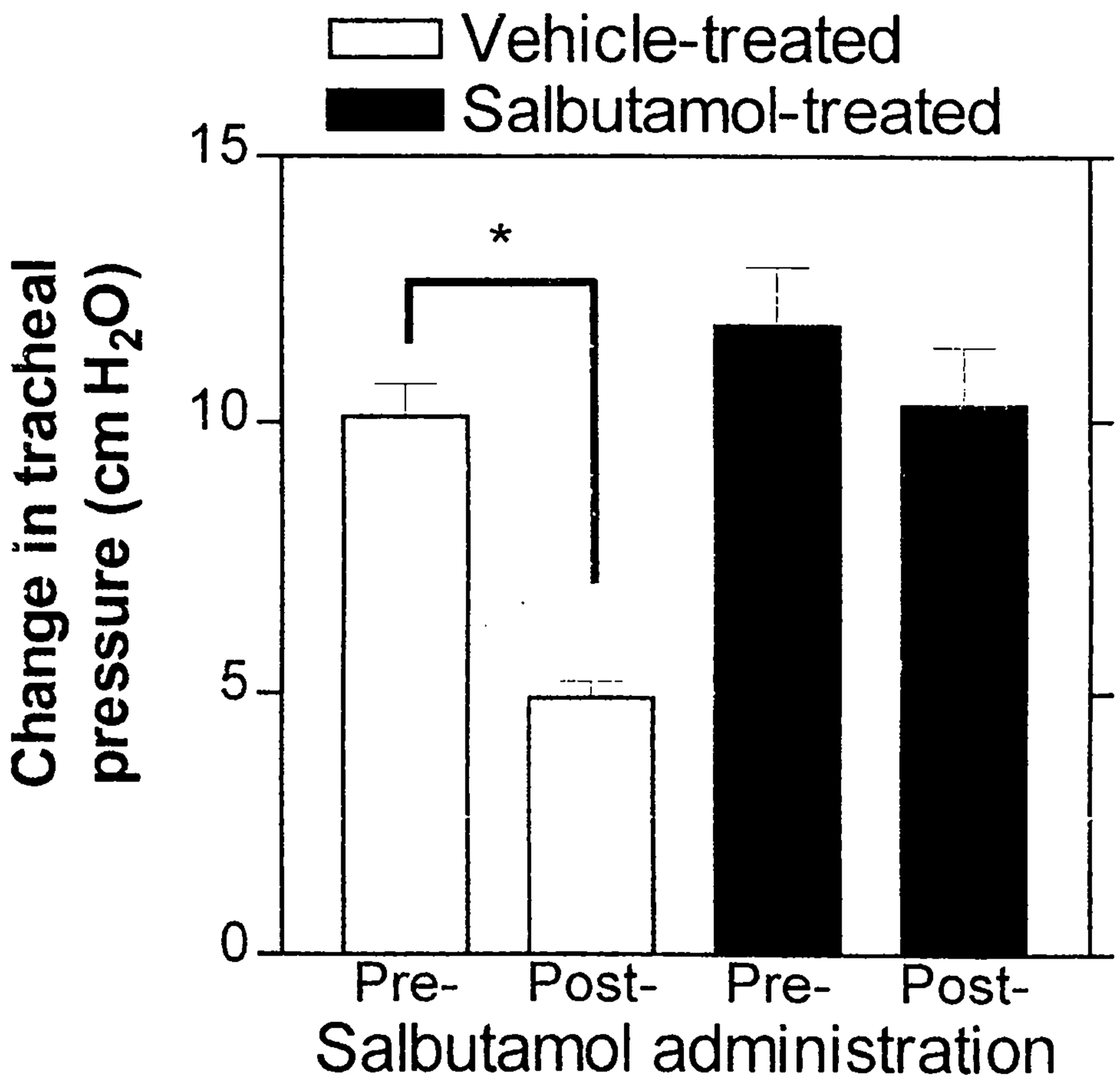



**Figure 3.1.3: Effect of chronic treatment of rats with salbutamol on the ability of salbutamol, given acutely, to protect against ACh-induced bronchoconstriction.** Rats were anaesthetised with urethane, and their carotid artery, jugular vein and trachea were cannulated as described in Materials and Methods. ACh (500 μg/kg i.v.) was administered and changes in overflow pressure were measured using a modification of the technique introduced by Konsett and Rossler (1940). When basal lung function was re-established, salbutamol (100 μg/kg i.v.) was given, and five minutes later ACh was administered again and any change in overflow pressure was noted. Data represent the mean ± SEM of four determinations from different rats, * $P<0.05$ significant protection - Student's paired t-test.

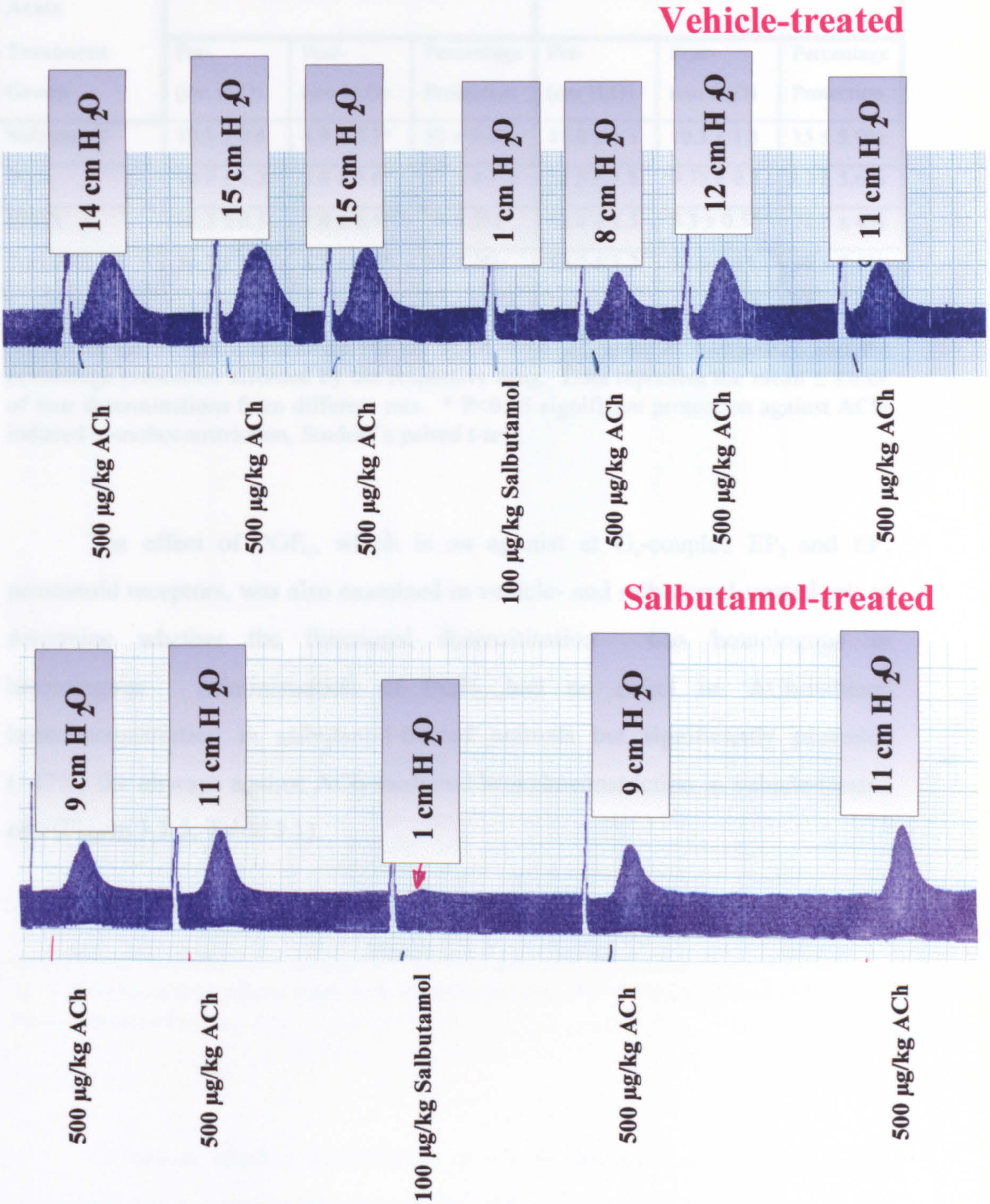



**Figure 3.1.3** Represenative trace of airway resistance experiments. Rats were anaesthetised with urethane, and their carotid artery, jugular vein and trachea were cannulated as described in Materials and methods. ACh (500 µg/kg i.v.) was administered and changes in overflow pressure were measured using a modification of the technique introduced by Konsett and Rossler (1940). When basal lung function was re-established, salbutamol (100 µg/kg i.v.) was given, and five munutes later ACh was administered again and any change in overflow pressure was noted.

**Table 3.1:** Summary of the *in vivo* bronchoprotection investigation using rats treated chronically with vehicle and salbutamol.

| **Acute Treatment Group** | **Vehicle-Treated** | | | **Salbutamol-treated** | | |
|---|---|---|---|---|---|---|
| | Pre- ($cm\ H_2O$) | Post- ($cm\ H_2O$) | Percentage Protection | Pre- ($cm\ H_2O$) | Post- ($cm\ H_2O$) | Percentage Protection |
| Salbutamol | 10.1 ± 0.6 | 4.9 ± 0.3* | 52 ± 3.4% | 11.8 ± 1.1 | 10.3 ± 1.1 | 15 ± 5.9% |
| $PGE_2$ | 10.9 ± 1.2 | 5.8 ± 0.8* | 47 ± 4.7% | 11.5 ± 1.8 | 9.75 ± 0.8 | 13 ± 5.6% |
| IBMX | 11.3 ± 0.5 | 7.0 ± 0.4* | 38 ± 3% | 12.4 ± 1.3 | 8.3 ± 0.5* | 32.3 ± 4% |
| Forskolin | 11.7 ± 1.0 | 8.3 ± 0.9* | 27 ± 2% | 13.7 ± 1.2 | 10.0 ± 1* | 30 ± 3.1% |
| Levcromakalim | 11.5 ± 1.3 | 7.5 ± 0.6* | 32 ± 3.6% | 10.8 ± 1.1 | 7.4 ± 1.0* | 35 ± 5.9% |

Shown is the bronchoconstriction (in $cm\ H_2O$) obtained upon ACh-exposure and the percentage protection afforded by the respective drug. Data represent the mean ± s.e.m. of four determinations from different rats. * $P<0.05$ significant protection against ACh-induced bronchoconstriction, Student's paired t-test.

The effect of $PGE_2$, which is an agonist at $G_s$-coupled $EP_2$ and $EP_4$ prostanoid receptors, was also examined in vehicle- and salbutamol-treated rats to determine whether the functional desensitisation was homologous or heterologous. Administration of $PGE_2$ had no effect on ACh-induced bronchoconstriction in salbutamol-treated animals but significantly protected (~47%) the airways against ACh-mediated bronchoconstriction in vehicle-treated rats (Figure 3.1.5; Table 3.1).

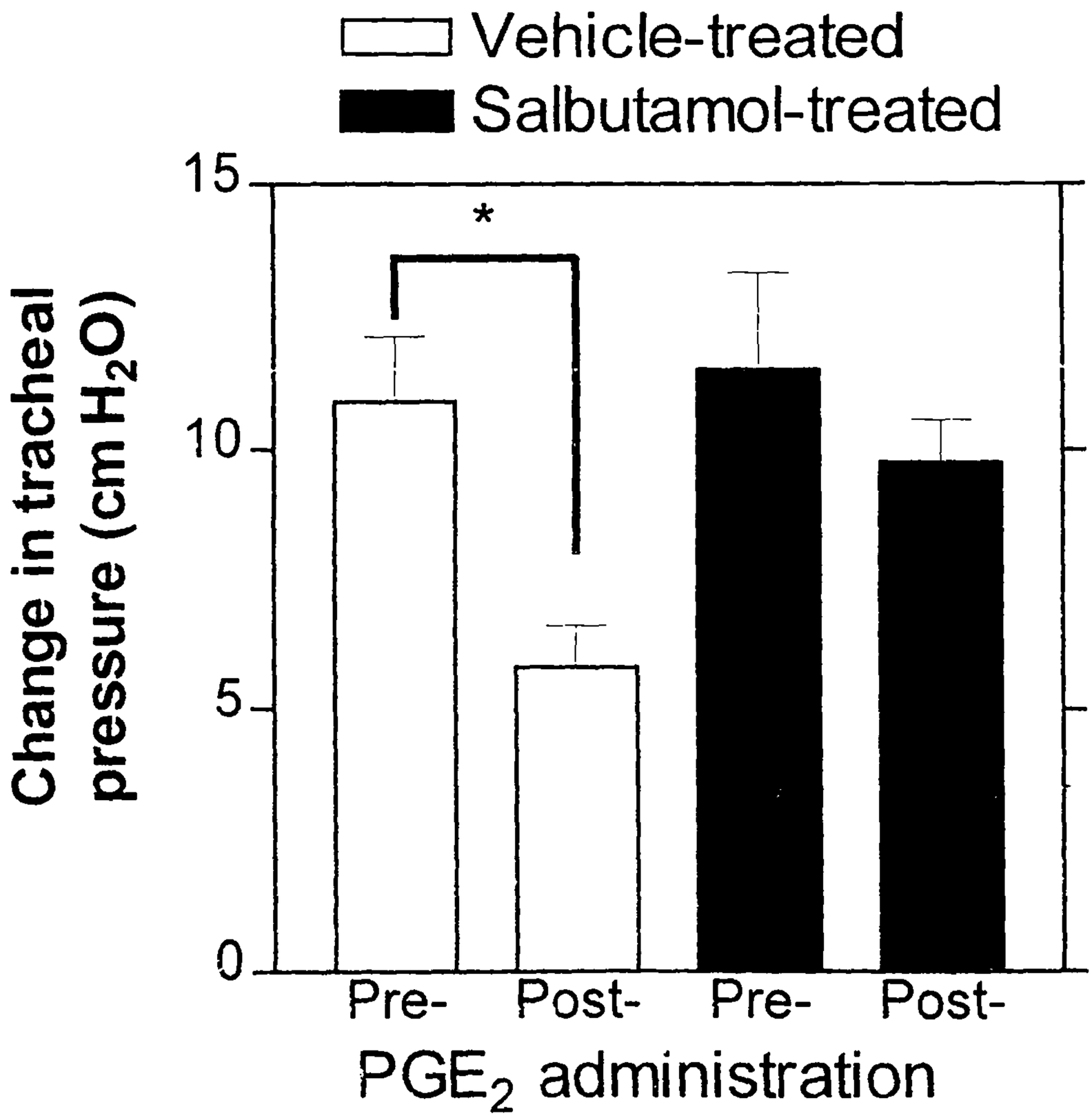



**Figure 3.1.5: Effect of chronic treatment of rats with salbutamol on the ability of $PGE_2$, given acutely, to protect against ACh-induced bronchoconstriction.** Rats were anaesthetised with urethane, and their carotid artery, jugular vein and trachea were cannulated as described in Materials and Methods. ACh (500 μg/kg i.v.) was administered and changes in overflow pressure were measured using a modification of the technique introduced by Konsett and Rossler (1940). When basal lung function was re-established, $PGE_2$ (300 μg/kg) was given, and five minutes later ACh was administered again and any change in overflow pressure was noted. Data represent the mean ± SEM of four determinations from different rats, * $P<0.05$ significant protection - Student's paired t-test.

To assess whether an inhibitor of cAMP degradation evoked a similar functional effect in rats treated chronically with salbutamol, the ability of the non-selective PDE inhibitor, IBMX, to protect against ACh-induced bronchoconstriction was investigated (Figure 3.1.6; Table 3.1). IBMX significantly protected the airways against ACh-induced bronchoconstricton in both salbutamol- (~32%) and vehicle-treated rats (~38%) by approximately the

same degree (Figure 3.1.6; Table 3.1) suggesting that the defect in signalling was at the level of cAMP formation.

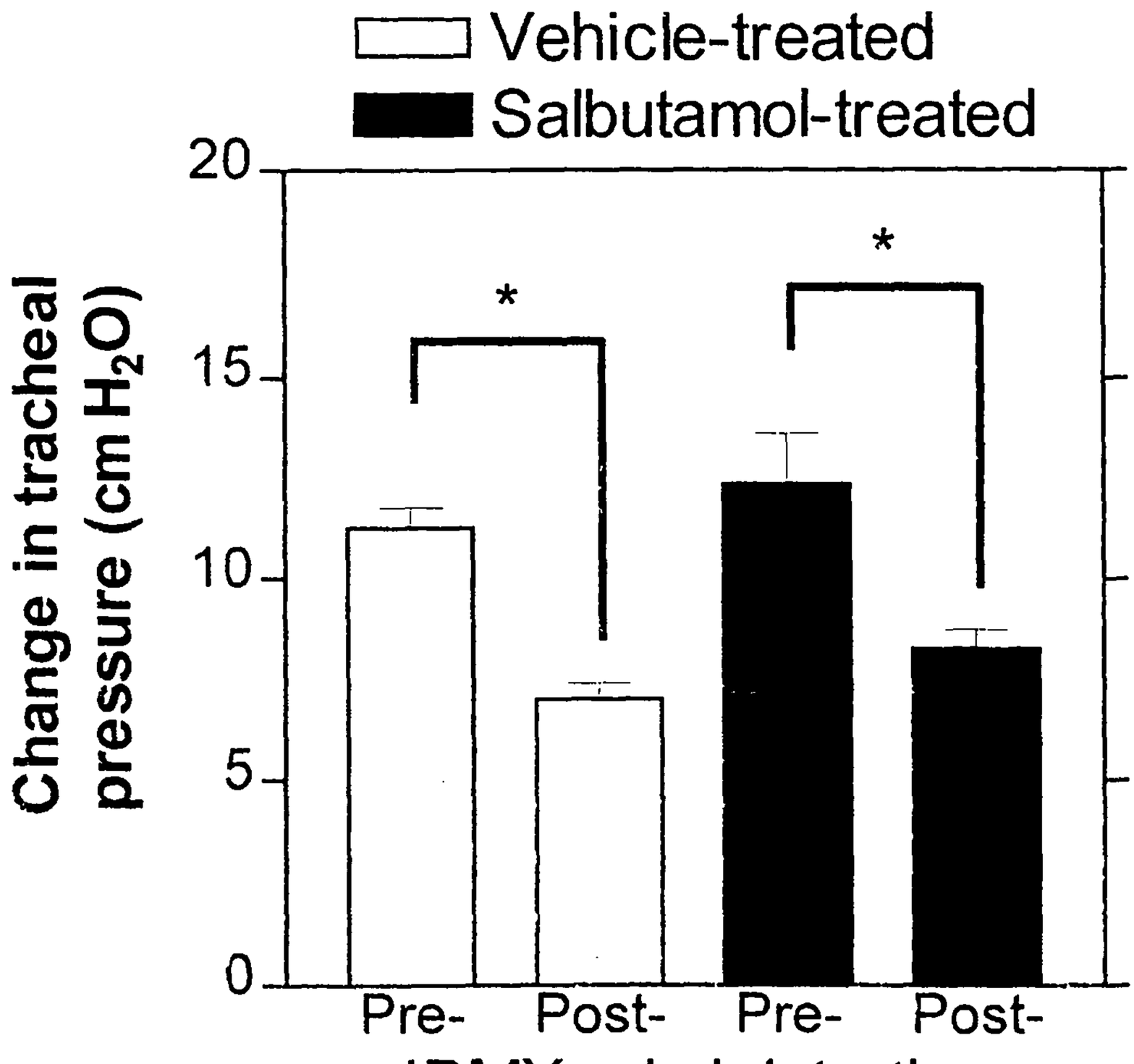



**Figure 3.1.6: Effect of chronic treatment of rats with salbutamol on the ability of IBMX, given acutely, to protect against ACh-induced bronchoconstriction.** Rats were anaesthetised with urethane, and their carotid artery, jugular vein and trachea were cannulated as described in Materials and Methods. ACh (500 μg/kg i.v.) was administered and changes in overflow pressure were measured using a modification of the technique introduced by Konsett and Rossler (1940). When basal lung function was re-established, IBMX (300 μg/kg) was administered, and five minutes later ACh was administered again and any change in overflow pressure was noted. Data represent the mean ± SEM of four determinations from different rats, *P<0.05 significant proteciton - Student's paired t-test.

The effect of chronic treatment of rats with salbutamol on the ability of forskolin, a direct AC activator, to protect against ACh-induced bronchoconstriction also was examined (Figure 3.1.7; Table 3.1). Forskolin significantly protected against ACh-induced bronchoconstriction in both salbutamol- (~30%) and vehicle-treated rats (~27%) (Figure 3.1.7; Table 3.1),

indicating that the mechanism(s) responsible for functional desensitisation was upstream of AC in the $\beta_2$-AR signal transduction cascade.

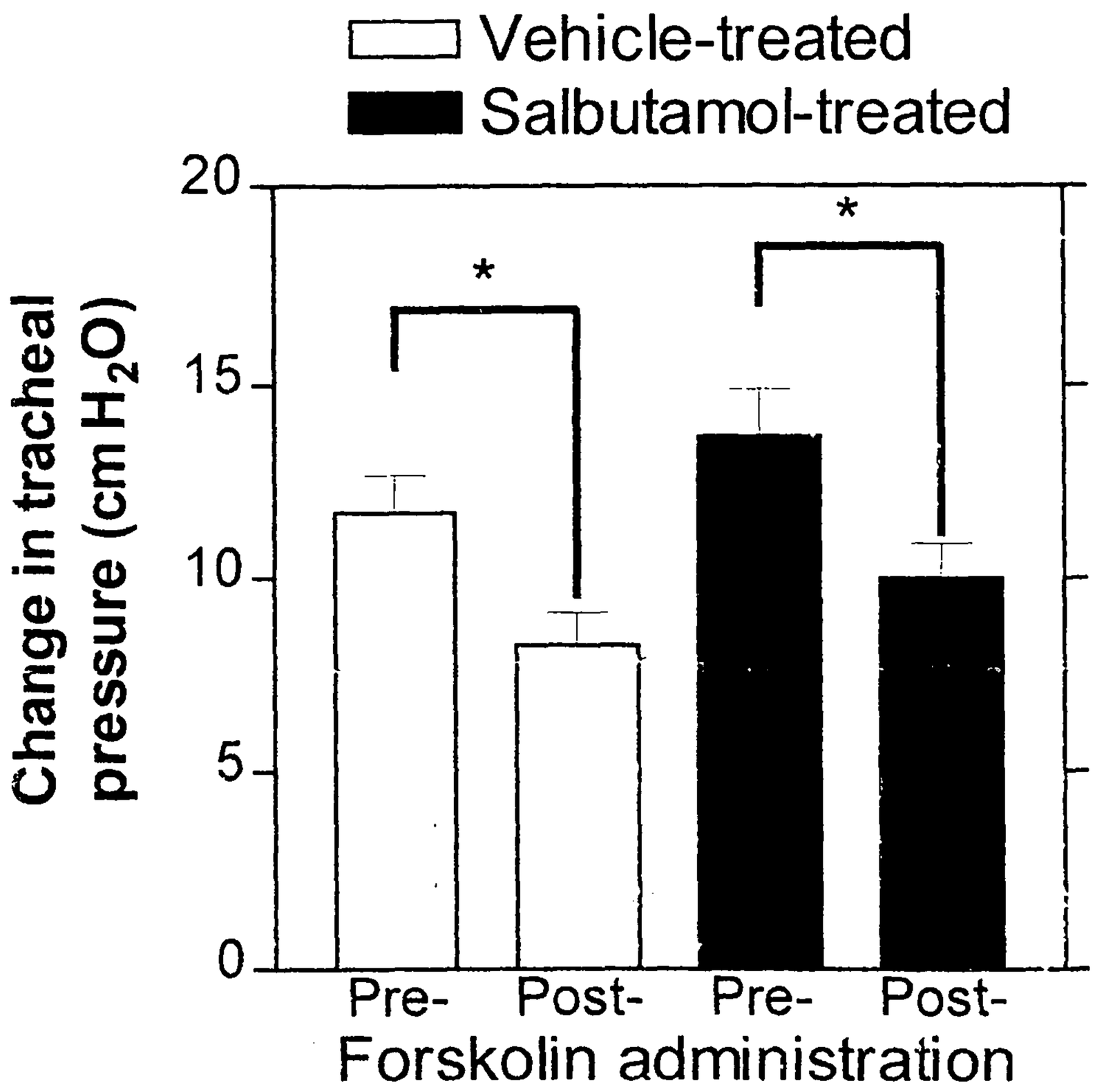



**Figure 3.1.7: Effect of chronic treatment of rats with salbutamol on the ability of forskolin, given acutely, to protect against ACh-induced bronchoconstriction.** Rats were anaesthetised with urethane, and their carotid artery, jugular vein and trachea were cannulated as described in Materials and Methods. ACh (500 μg/kg i.v.) was administered and changes in overflow pressure were measured using a modification of the technique introduced by Konsett and Rossler (1940). When basal lung function was re-established, forskolin (300 μg/kg) was given, and five minutes later ACh was administered again and any change in overflow pressure was noted. Data represent the mean ± SEM of four determinations from different rats, * $P<0.05$ significant protection - Student's paired t-test.

As a cAMP-independent control, the ability of the ATP-sensitive $K^+$-channel opener, levcromakalim, to protect against ACh-mediated bronchoconstriction was investigated (Figure 3.1.8; Table 3.1). Levcromakalim, which causes bronchodilatation by decreasing intracellular $Ca^{2+}$ via activating ATP-sensitive $K^+$ channels, evoked the same degree of protection against ACh-

induced bronchoconstriction in salbutamol- (~35%) and vehicle-treated rats (~32%).

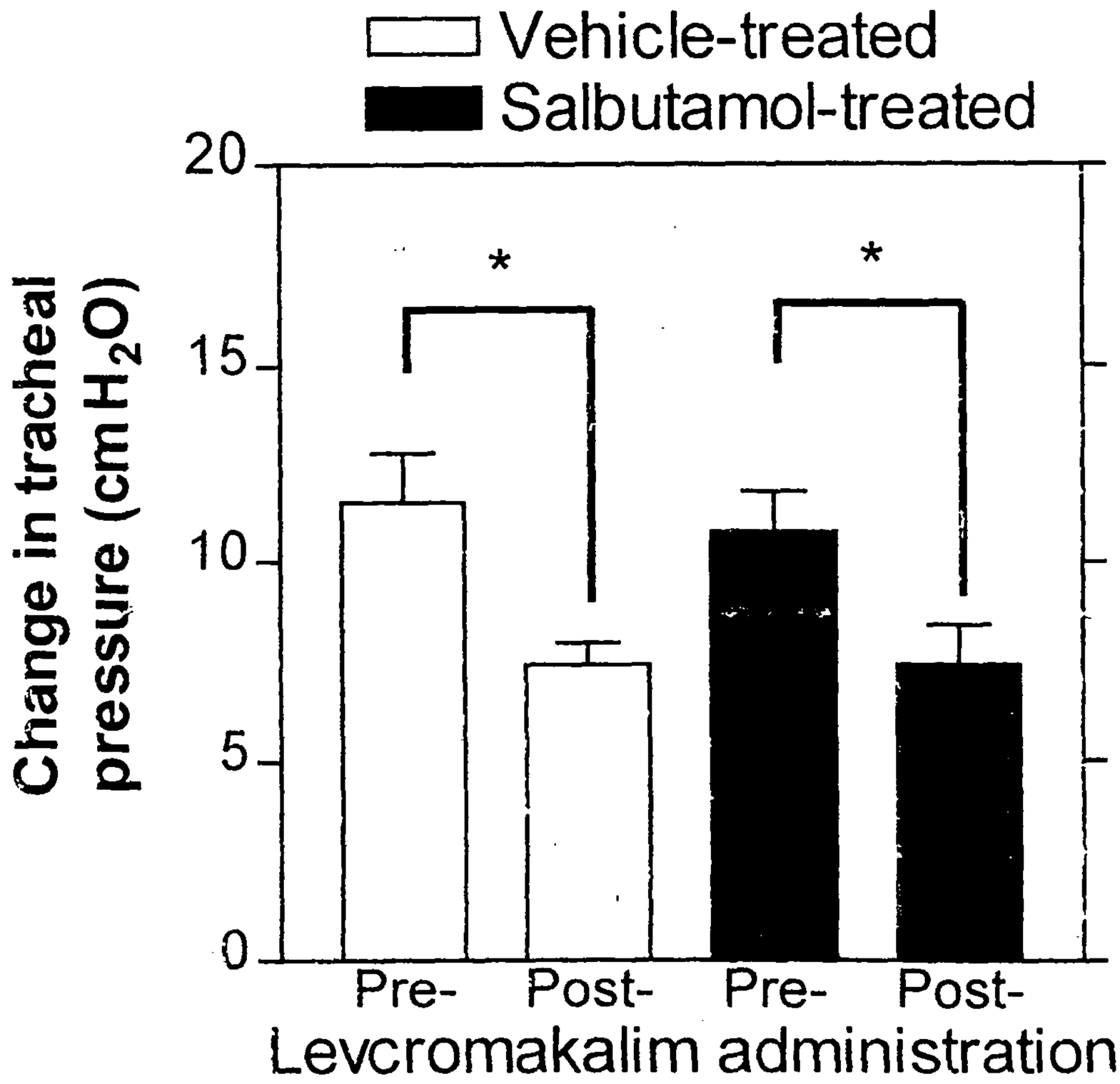



**Figure 3.1.8: Effect of chronic treatment of rats with salbutamol on the ability of levcromakalim, given acutely, to protect against ACh-induced bronchoconstriction.** Rats were anaesthetised with urethane, and their carotid artery, jugular vein and trachea were cannulated as described in Materials and Methods. ACh (500 μg/kg i.v.) was administered and changes in overflow pressure were measured using a modification of the technique introduced by Konsett and Rossler (1940) When basal lung function was re-established, levcromakalim (50 μg/kg) was given, and five minutes later ACh was administered again and any change in overflow pressure was noted. Data represent the mean ± SEM of four determinations from different rats, * $P<0.05$ significant protection - Student's paired t-test.

### *3.1.5 Effect of chronic treatment of rats with $PGE_2$ on the ability of $PGE_2$ and salbutamol, given acutely, to protect against ACh-induced bronchoconstriction.*

The effect of acutely-administered salbutamol and $PGE_2$ on ACh-induced bronchoconstriction was examined in rats treated chronically with $PGE_2$ (Figure 3.1.9). Administration of $PGE_2$ had no effect on ACh-induced bronchoconstriction in $PGE_2$-treated rats (9.2 ± 0.6 and 8.4 ± 0.5 cm $H_2O$ pre- and post-$PGE_2$ respectively, 9.5% mean protection). However, a significant protective effect (~48%) of $PGE_2$ against ACh-induced bronchoconstriction was seen in vehicle-treated rats (11.1 ± 0.6 and 5.8 ± 0.3 cm $H_2O$ pre- and post-$PGE_2$ respectively, 48.3% mean protection).

To assess whether heterologous functional desensitisaton could be demonstrated through a different $G_s$-coupled receptor, the bronchoprotective action of salbutamol against ACh-induced bronchoconstriction was investigated in $PGE_2$-treated rats (Figure 3.1.10). Administration of salbutamol had no effect on ACh-induced bronchoconstriction in $PGE_2$-treated rats (10.8 ± 0.5 and 9.2 ± 0.5 cm $H_2O$ pre- and post-$PGE_2$ respectively) under conditions where $PGE_2$ afforded significant protection (10.5 ± 0.7 and 6.3 ± 0.5 cm $H_2O$ pre- and post-$PGE_2$ respectively, 46.3% mean protection, $P<0.05$, n=4) (Figure 3.1.16).

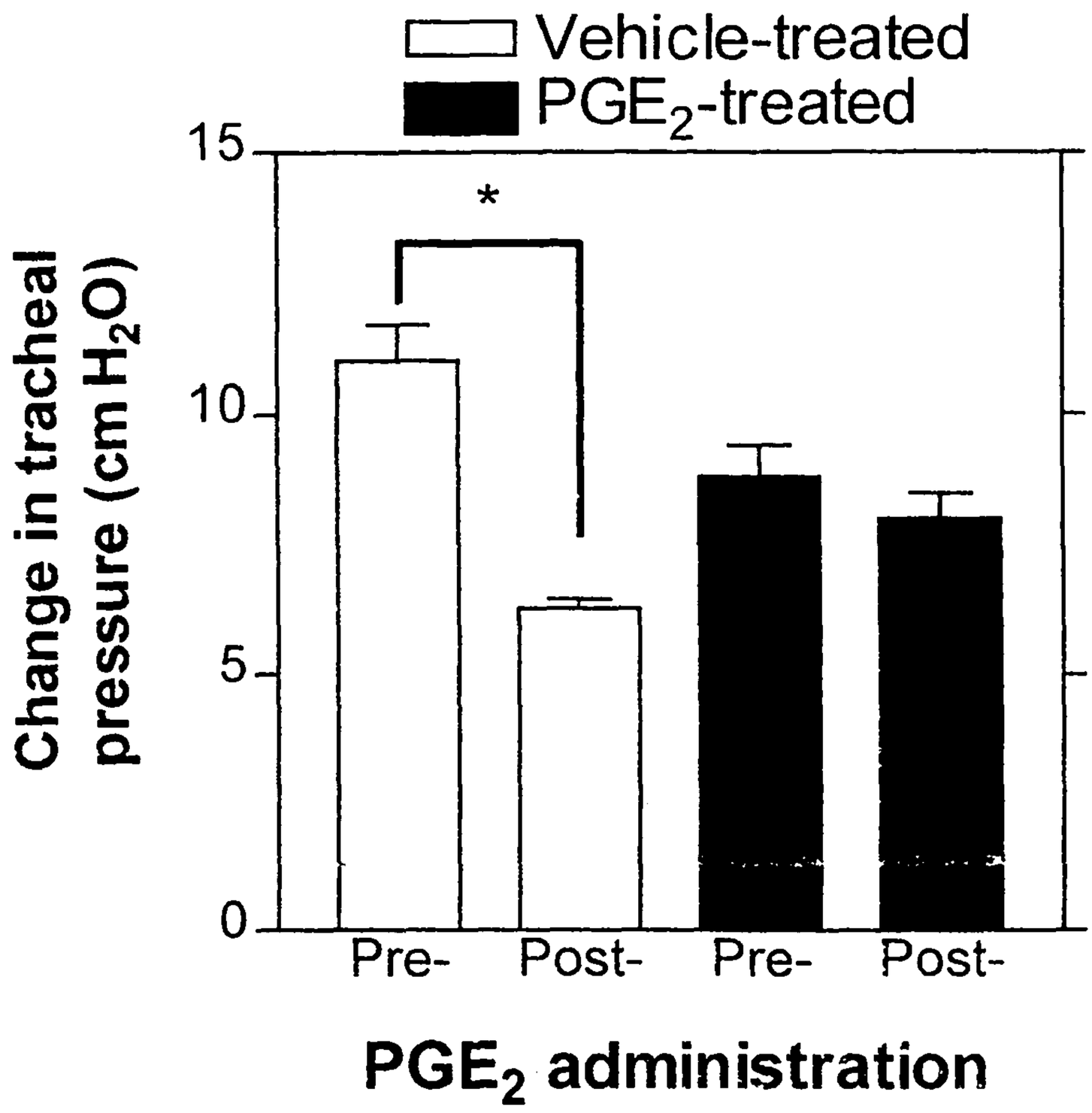



**Figure 3.1.9: Effect of chronic treatment of rats with $PGE_2$ on the ability of $PGE_2$, given acutely, to protect against ACh-induced bronchoconstriction.** Rats were anaesthetised with urethane, and their carotid artery, jugular vein and trachea were cannulated as described in Materials and Methods. ACh (500 μg/kg i.v.) was administered and changes in overflow pressure were measured using a modification of the technique introduced by Konsett and Rossler (1940). When basal lung function was re-established, $PGE_2$ (300 μg/kg) was given, and five minutes later ACh was administered again and any change in overflow pressure was noted. Data represent the mean ± SEM of four determinations from different rats, * $P<0.05$ significant bronchoprotection - Student's paired t-test.

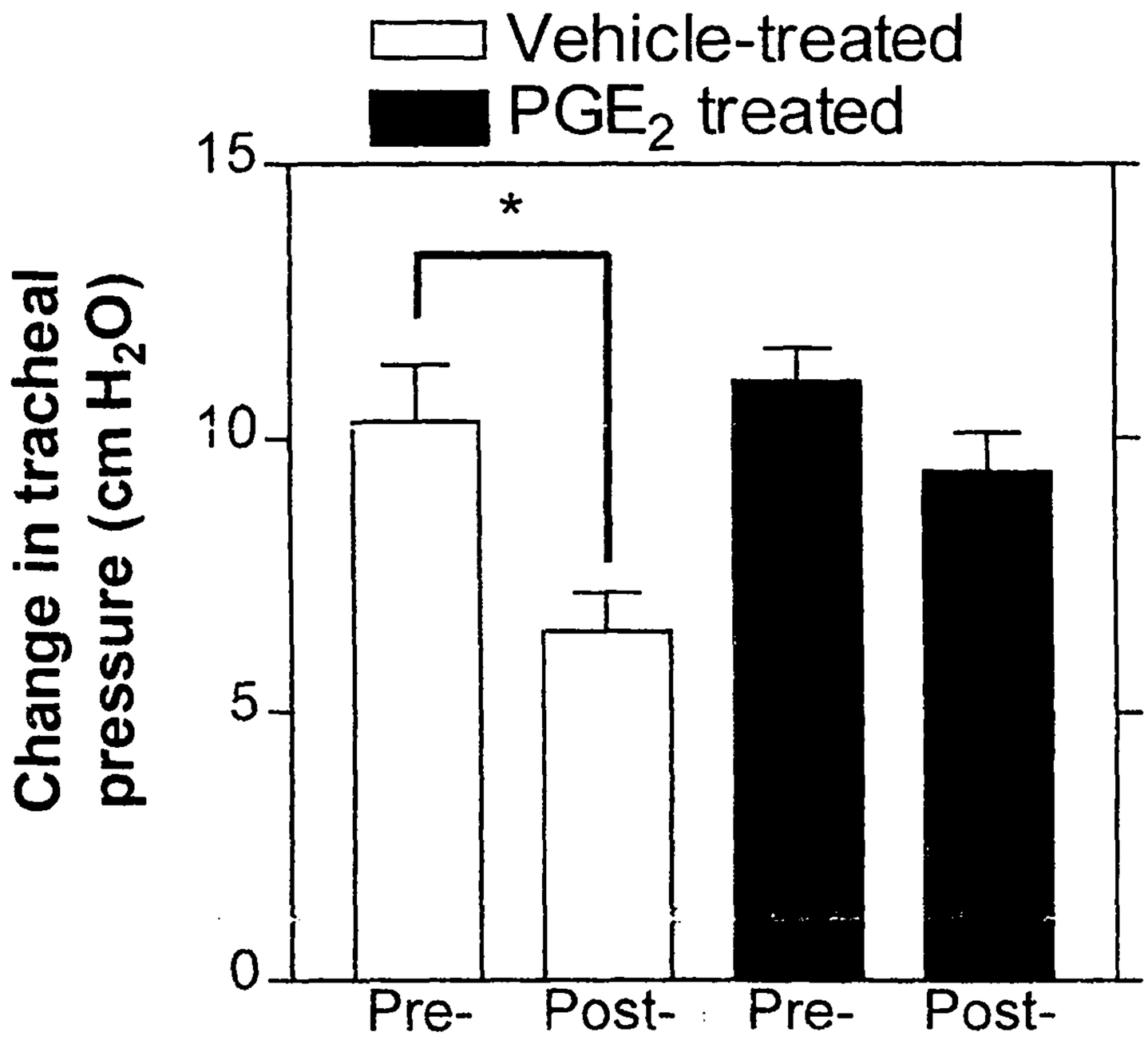



**Figure 3.1.10: Effect of chronic treatment of rats with $PGE_2$ on the ability of salbutamol, given acutely, to protect against ACh-induced bronchoconstriction.** Rats were anaesthetised with urethane, and their carotid artery, jugular vein and trachea were cannulated as described in Materials and Methods. ACh (500 μg/kg i.v.) was administered and changes in overflow pressure were measured using a modification of the technique introduced by Konsett and Rossler (1940). When basal lung function was re-established, salbutamol (100 μg/kg) was given, and five minutes later ACh was administered again and any change in overflow pressure was noted. Data represent the mean ± SEM of four determinations from different rats, * $P<0.05$ significant bronchoprotectoin - Student's paired t-test.

### *3.1.6 Discussion.*

The primary objective of this study was to assess if a constant infusion of salbutamol into rats would promote pulmonary $\beta_2$AR desensitisation at a functional level. Initially, a dose-response study was performed with ACh to establish a submaximal concentration of ACh with which to perform the

investigation. The dose-response study demonstrated that exogenous ACh at a 500μg/kg i.v. elicited highly reproducible and sub-maximal bronchoconstrictor responses that were not significantly different between vehicle- and salbutamol treated animals. Given that chronic treatment of rats with salbutamol resulted in profound pulmonary $\beta_2$AR desensitisation (see section 3.2.2), these data suggest that neither sympathetic tone nor endogenous catecholamines regulate baseline airway calibre, which is consistent with the sparse innervation of airway smooth muscle with catecholeamine-containing varicosites in this species (Bradley *et al.*, 1970, El-Bermani, 1978). The submaximal dose of ACh was also used to assess whether repeated challenges would alter the magnitude of the bronchoconstriction which could impact upon the interpretation of the protection studies. However, there was no change in response to ACh with repeated challenge, and no significant difference in response between vehicle- and salbutamol-treated rats.

Having established the conditions and validated the methods for assessing ACh-induced bronchoconstriction *in vivo*, the effect of chronic treatment of rats with salbutamol on the ability of salbutamol, given acutely, to protect against ACh-induced bronchoconstriction was investigated. In vehicle-treated rats, salbutamol was effective at protecting against ACh-induced bronchoconstriction - a mean of 52% protection of the initial response - whereas in rats treated chronically with salbutamol, the ability of salbutamol to protect was lost indicating functional desensitisation. *in vivo* desensitisation of β-adrenoceptors has been demonstrated in tracheae studied *ex vivo* taken from rats treated with isoprenaline (Avner and Noland, 1978; Taylor, 1987). In another study, Nerme *et al.*, (1978) observed a 50% reduction in the maximal relaxing effect of isoprenaline in lung parenchymal strips taken from guinea pigs treated for 7 days with 60 μg/kg/h isoprenaline.

The importance of the observations made in the present study may have relevance to the clinical phenomenon of tolerance. Tolerance or desensitisation of human airway smooth muscle to $\beta_2$AR agonists *in vitro* has been demonstrated, although the concentration of agonist necessary is high and the degree of desensitisation is variable (Guillot *et al.*, 1984). *In vivo*, Lipworth and his colleagues (Newnham *et al.*, 1994, 1995) have reported that four weeks treatment

of asthmatic subjects with inhaled eformoterol produces tachyphylaxis to the bronchodilator effects of these drugs. Tolerance to inhaled $\beta_2$AR agonists after regular treatment of human subjects has also been observed with short-acting $\beta_2$AR agonists against various challenges that cause bronchoconstriction (O'Connor *et al.*, 1992, Cockcroft *et al.*, 1993, Gibson *et al.*, 1978). This effect has been shown to render the airways twice as sensitive to allergen and exercise, and to increase the late phase asthmatic response and associated inflammation (Barnes, 1995) and may be relevant to the reduced asthma control seen with regular use of inhaled $\beta_2$AR agonists (Sears *et al.*, 1990, Taylor *et al.*, 1993). The cause(s) of tolerance is unclear. It has been reported that airways resected from asthmatic patients fail to relax normally to isoprenaline, supporting a possible defect in βAR function (Goldie *et al.*, 1986, Cerrina *et al.*, 1986, Bai *et al.*, 1992), although it is unknown whether this is the result of treatment or a consequence of the disease itself. Nevertheless, down-regulation of βAR number in lung and airways smooth muscle has been reported in animals given isoprenaline and noradrenaline chronically by infusion (Nishikawa *et al.*, 1993, 1994; Neeme *et al.*, 1990) and is associated with a reduced functional responsiveness towards βAR agonists *ex vivo* (Nishikawa *et al.*, 1994; Neeme *et al.*, 1990). Thus, it is possible that the loss of protection against various bronchoconstrictor challenges in humans is due, at least in part, to pulmonary $\beta_2$AR desensitisation.

The question of which of the two major patterns of desensitisation, homologous (agonist-specific) or heterologous (non-agonist specific) (Chuang *et al.*, 1996), had occurred in salbutamol-treated rats then was investigated by assessing the ability of $PGE_2$, an agonist that acts through $G_s$-coupled receptors of the $EP_{2/4}$ subtype, to protect against ACh-induced bronchoconstriction. After 7 days constant infusion of salbutamol, the ability of $PGE_2$ to protect against ACh-induced bronchoconstriction dropped from 46.9% protection to 12.8% demonstrating that a functional heterologous desensitisation had been induced. These results are consistent with a study by Travis *et al.*, (1997) showed that a reduction in the tissue concentration of nitric oxide-containing factors allowed tachyphylaxis to pituitary adenylyl cyclase-activating polypeptide- (PACAP)-induced vasodilatation to occur *in vivo*, and that this process led to the

heterologous desensitisation of βAR-mediated responses in anaesthetised Sprague-Dawley rats. In another investigation, Zeiders *et al.*, (1999) observed heterologous desensitisation of the ability of isoprenaline and glucagon to activate cardiac AC after 12 hours treatment of rats with isoprenaline. Collectively, therefore, these results support the supposition that heterologous desensitisation can occur *in vivo*.

To assess whether the PDE isoenzyme system was still functioning in rats given a chronic salbutamol infusion, the ability of the non-selective PDE inhibitor, IBMX, to protect against ACh-induced bronchoconstriction was studied. IBMX afforded equal protection against ACh-mediated bronchoconstriction in both vehicle- and salbutamol-treated animals, suggesting that PDE expression and activity are not altered and that desensitisation is the result of compromised cAMP formation (see section 3.2).

This conclusion is in contrast to the findings of Torphy *et al.*, (1995) and Seybold *et al.*, (1998) who found that up-regulation of PDE4 in U937 and T-cells respectively was responsible for the heterologous desensitisation evoked in cells treated with salbutamol. The reasons for this discrepancy are unexplored but could involve the time of agonist exposure, concentration of agonist at the $\beta_2$AR, difference in cell types or a combination of all three. The level of receptor expression in the tissue of interest will also affect desensitisation (Milligan, 1993). The ability of forskolin to protect against ACh-mediated bronchoconstriction was evaluated to determine whether chronic treatment of rats with salbutamol had affected AC-catalyses cAMP formation. Forskolin, like IBMX, significantly protected against ACh-induced bronchoconstriction in both vehicle- and salbutamol-treated rats, suggesting that the molecular site(s) of desensitisation is upstream of AC and that the expression of $G_s$ or coupling efficiency to AC could be impaired (see section 3.2).

βAR-agonists can also relax smooth muscle by directly activating ATP-sensitive $K^+$-channels ($K_{ATP}$) (Jones *et al.*, 1993). To ensure that desensitisation to salbutamol selectively affected the cAMP/PKA cascade the ability of the $K_{ATP}$ channel opener levcromakalim to protect against ACh-mediated bronchoconstriction was investigated. In both the vehicle- and salbutamol-treated

groups levcromakalim effected an equivalent anti-spasmogenic influence, indicating that the desensitisation is selective for drugs that act through $G_s$-coupled receptors.

To assess whether the functional *in vivo* desensitisation observed after chronic treatment with salbutamol could be mimicked using a different $G_s$-coupled receptor system, identical experiments were performed with rats treated chronically with $PGE_2$. After 7 days of chronic treatment, the bronchoprotective action of $PGE_2$ and salbutamol against ACh-mediated bronchoconstriction were significantly blunted. These data show that heterologous desensitisation can be demonstrated *in vivo* using a different $G_s$-coupled receptor pathway. The major mechanism responsible for this heterologous cross-desensitisation seemingly lies between the receptor moiety and AC. However, there are at least two other points in the signal transduction cascades of both receptor systems that are shared - PKA, PDE isoenzymes - and either of these could be involved in heterologous desensitisation mediated by both receptor systems to a limited degree.

In summary, the rat model used in this study proved a useful tool for studying *in vivo* functional desensitisation in response to chronic salbutamol and $PGE_2$ treatment. The data presented above demonstrate that chronic exposure of rats to salbutamol is associated with a significant loss in salbutamol- and $PGE_2$-induced bronchoprotection, and that the primary defect is upstream of AC. In addition, chronic treatment of rats with $PGE_2$ also results in a heterologous functional desensitisation to the bronchoprotective actions of $PGE_2$ and salbutamol implying a common mechanism of cross-desensitisation.

## 3.2 An investigation of the biochemical effects associated with pulmonary $\beta_2$-adrenoceptor desensitisation in rats treated chronically with salbutamol

### *3.2.1 Introduction.*

In asthma, β-AR agonists mediate their beneficial effects primarily by activating $\beta_2$ARs on airway smooth muscle, which mediate relaxation. The intracellular mechanisms responsible for this effect are now reasonably well established. When an agonist binds to the $\beta_2$AR a change in conformation occurs which promotes coupling to a stimulatory G protein ($G_s$). The α-subunit of $G_s$ is released from the αβγ heterotrimer and subsequently activates AC, which catalyses the formation of cAMP from ATP. cAMP acts as a second messenger by activating PKA, which then phosphorylates hydroxy amino acid residues on a number of cellular proteins (Hausdorff *et al.,* 1990). Termination of cAMP signalling is mediated by cyclic nucleotide PDEs, which are a family of enzymes that metabolise cAMP and cGMP to their functionally inactive nucleotide 5'-monophosphates. PDEs comprise the only degradation pathway for cAMP and cGMP in the cell, and play a critical role in the regulation of intracellular cyclic nucleotide levels (Beavo, 1995). PDE isoenzymes are currently classified into ten broad families based on their substrate specificity, sensitivity to selective inhibitors and cofactors, immunological characteristics and primary structure (Beavo *et al.,* 1994; Hayashi *et al.,* 1998; Soderling *et al.,* 1998a,b; Fisher *et al.,* 1998).

A common functional feature of cellular responses effected by GPCR agonists is that they are subject to rapid desensitisation. Desensitisation has been described in a number of hormone and neurotransmitter systems, and has been extensively studied for $\beta_2$AR/adenylyl cyclase cascade. Several mechanisms at different levels in the signal transduction pathway have been identified that regulate the responsiveness of the cell to βAR agonists. In particular, $\beta_2$AR signalling is regulated by the intracellular kinases GRKs, PKA and/or PKC. $\beta_2$AR

phosphorylation by PKA, produces heterologous desensitisation, whereas GRK2 promotes rapid uncoupling of the $\beta_2$AR from $G_s$. However, the extent to which either of these desensitisation mechanisms is used appears to be highly cell-specific (Shih *et al.*, 1994). If agonist stimulation continues, down regulation of receptors occurs (as described in Introduction), observed by a loss of total receptor binding sites from the surface of the cell. This process is postulated to occur via increased degradation of internalised receptors and reduced *de novo* synthesis of receptors (Chuang *et al.*, 1996, Böhm *et al.*, 1997).

Another mechanism, possibly involved in desensitisation of receptors, is an up-regulation of PDE enzymes in response to repeated agonist exposure. It has been recently suggested that induction of PDE4 could occur in diseases such as asthma in response to chronic treatment with $\beta_2$AR agonists (reviewed in Giembycz, 1996). The functional consequences of this biochemical effect are uncertain, but data based on *in vitro* experiments would suggest heterologous desensitisation of $G_s$ coupled receptors. Up-regulation of cAMP-hydrolysing PDE isoenzymes in response to chronic treatment of asthmatic subjects with $\beta_2$AR agonists could prove deleterious by reducing the therapeutic effects of the agonist treatment and the anti-inflammatory actions of endogenous activators of adenylyl cyclase (Torphy *et al.*, 1995; Giembycz, 1996; Seybold *et al.*, 1998).

Milligan proposed a different mechanism of signal attenuation in 1993 - down-regulation of G protein abundance (for review see Milligan, 1993), in which exposure of cells to agonists of receptors linked to G proteins can result in down-regulation of cellular levels or redistribution of G proteins from membranes to the cytosol. Agonist-induced reduction of G protein levels has been observed for members of each of the $G_s$, $G_i$ and $G_q$ families of G proteins, and is dependent upon the level of receptor expression and generally restricted to the G protein(s) with which the receptor interacts.

Having demonstrated a functional attenuation of the ability of both salbutamol and $PGE_2$ to protect against ACh-mediated bronchoconstriction in rats treated for 7 days with salbutamol, experiments were performed to assess aspects of the $\beta_2$AR signal transduction cascade affected by this treatment that could account for the desensitisation.

### *3.2.2 β-Adrenoceptor density and mRNA expression.*

One possible explanation for the loss in protection against ACh-induced bronchoconstriction is down-regulation of $\beta_2$AR number. Indeed, chronic treatment of rats with salbutamol was associated with a significant reduction (30%) in the density of $\beta_2$-, but not $\beta_1$-, ARs in lung parenchyma (Figures 3.2.1, 3.2.2, 3.2.3), without a significant change in the affinity of [$^{125}$I]ICYP (Table 3.2.2). This resulted in a reduction of the $\beta_2$:$\beta_1$ subtype ratio from 2:1 to 1.45:1.

*In vitro* experiments were also performed on lung parenchyma to assess whether chronic treatment of rats with salbutamol affected the steady-state level of βAR mRNAs. After 7 days of chronic treatment with salbutamol, the steady-state mRNA level, expressed as ratio to the housekeeping gene GAPDH, of $\beta_1$- and $\beta_2$-ARs was unchanged when compared to vehicle-treated animals (Figure 3.2.4).

**Table 3.2. Effect of chronic treatment of rats with salbutamol on the density of cell surface β-ARs on rat lung membranes and on the affinity of [$^{125}$I]ICYP.**
Rats were implanted sub-cutaneously with osmotic mini-pumps delivering saline or salbutamol (40 μg/kg/hr). After seven days, lung membranes were prepared and total, $\beta_1$- and $\beta_2$-AR density and the affinity of [$^{125}$I]ICYP were determined as described in Materials and Methods. Data represent the mean ± s.e.m of eight independent observations.

| | Total βARs | | $\beta_1$ARs* | | $\beta_2$ARs** | |
|---|---|---|---|---|---|---|
| | Kd (pM) | Bmax (fmol/mg protein) | Kd (pM) | Bmax (fmol/mg protein) | Kd (pM) | Bmax (fmol/mg protein) |
| Vehicle | 7.1 ± 1.1 | 176 ± 11 | 22 ± 3.2 | 63 ± 8 | 5.1 ± 0.9 | 125 ± 6.5 |
| Salbutamol | 10 ± 3.1 | 118 ± 19† | 18 ± 3.2 | 60 ± 12 | 8.3 ± 1.9 | 87 ± 12† |

†, $P < 0.05$, significant reduction in Bmax compared to vehicle-treated rats, Student's paired t-test.
*$\beta_1$-Subtype defined in the presence of the selective $\beta_2$-AR antagonist, ICI 118551 (0.1 μM).
**$\beta_2$-Subtype defined in the presence of the selective $\beta_1$-AR antagonist, CGP20712A (0.1 μM).

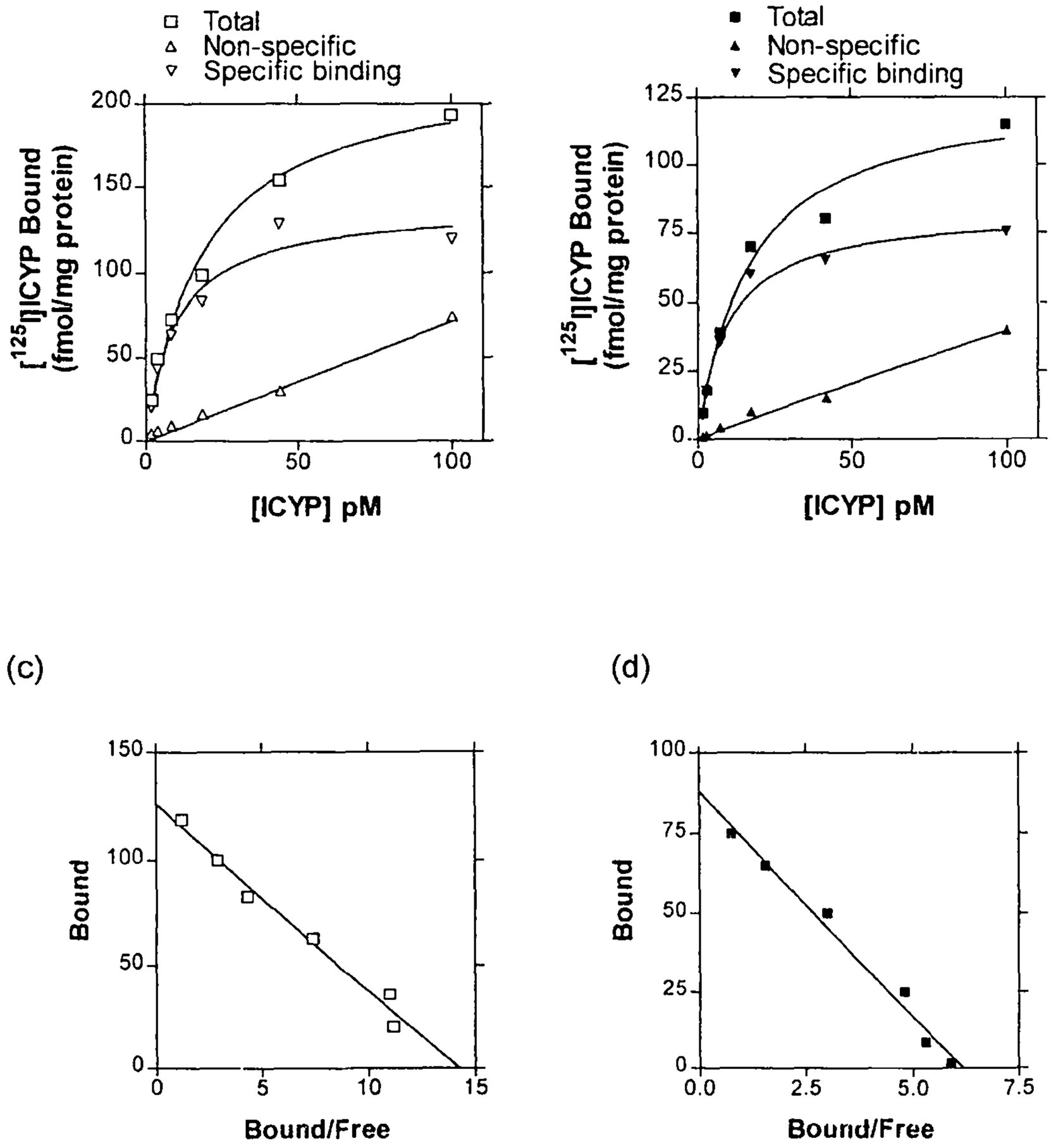



**Figure 3.2.1: Binding of [$^{125}$I]ICYP to total β-adrenoceptors in lung membranes prepared from vehicle- and salbutamol-treated rats.** Panels (a) and (b) show representative saturation isotherms for [$^{125}$I]ICYP binding to vehicle- (open symbols) and salbutamol-treated (closed symbols) lung respectively. Experiments were performed at 37°C in 25 mM Tris buffer containing 154 mM NaCl and 1 mM ascorbic acid (pH 7.4) at a protein concentration of 10 μg/tube. Non-specific binding was defined with 200 μM isoprenaline. Panels (c) and (d) show Scatchard transformations of the data in (a) and (b) respectively. Results are representative of eight determinations from different animals. See section 2.2 for further details.

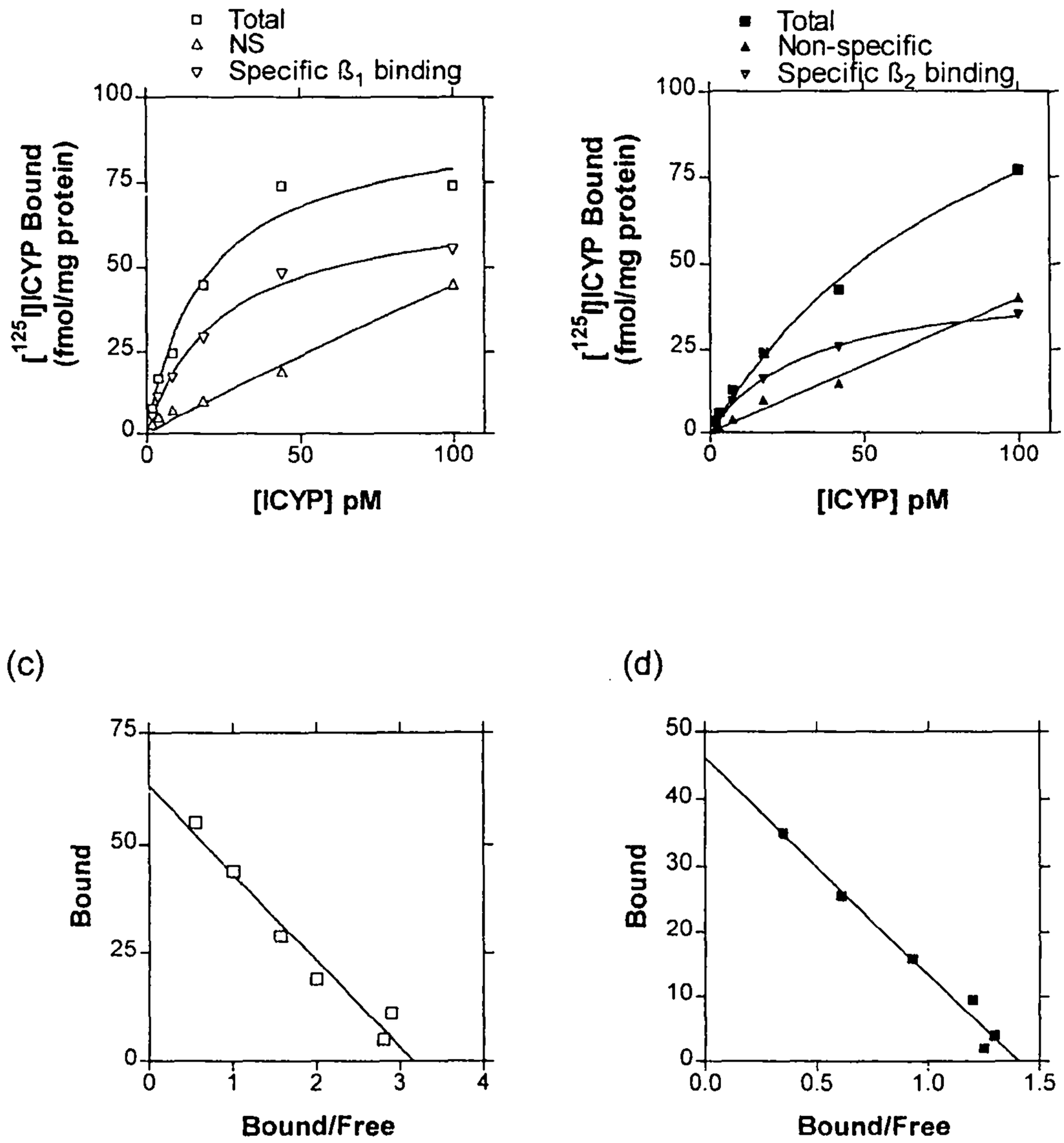



**Figure 3.2.2: Binding of [$^{125}$I]ICYP to $\beta_1$-adrenoceptors in lung membranes prepared from vehicle- and salbutamol-treated rats.** Panels (a) and (b) show representative saturation isotherms for [$^{125}$I]ICYP binding to vehicle- (open symbols) and salbutamol-treated (closed symbols) lung respectively. Experiments were performed as described in the legend to Figure 3.2.1 but the density of $\beta_1$-ARs was determined in the presence of 0.1 μM ICI 118551. Panels (c) and (d) show Scatchard transformations of the data in (a) and (b) respectively. Results are representative of eight determinations from different animals. See section 2.2 for further details.

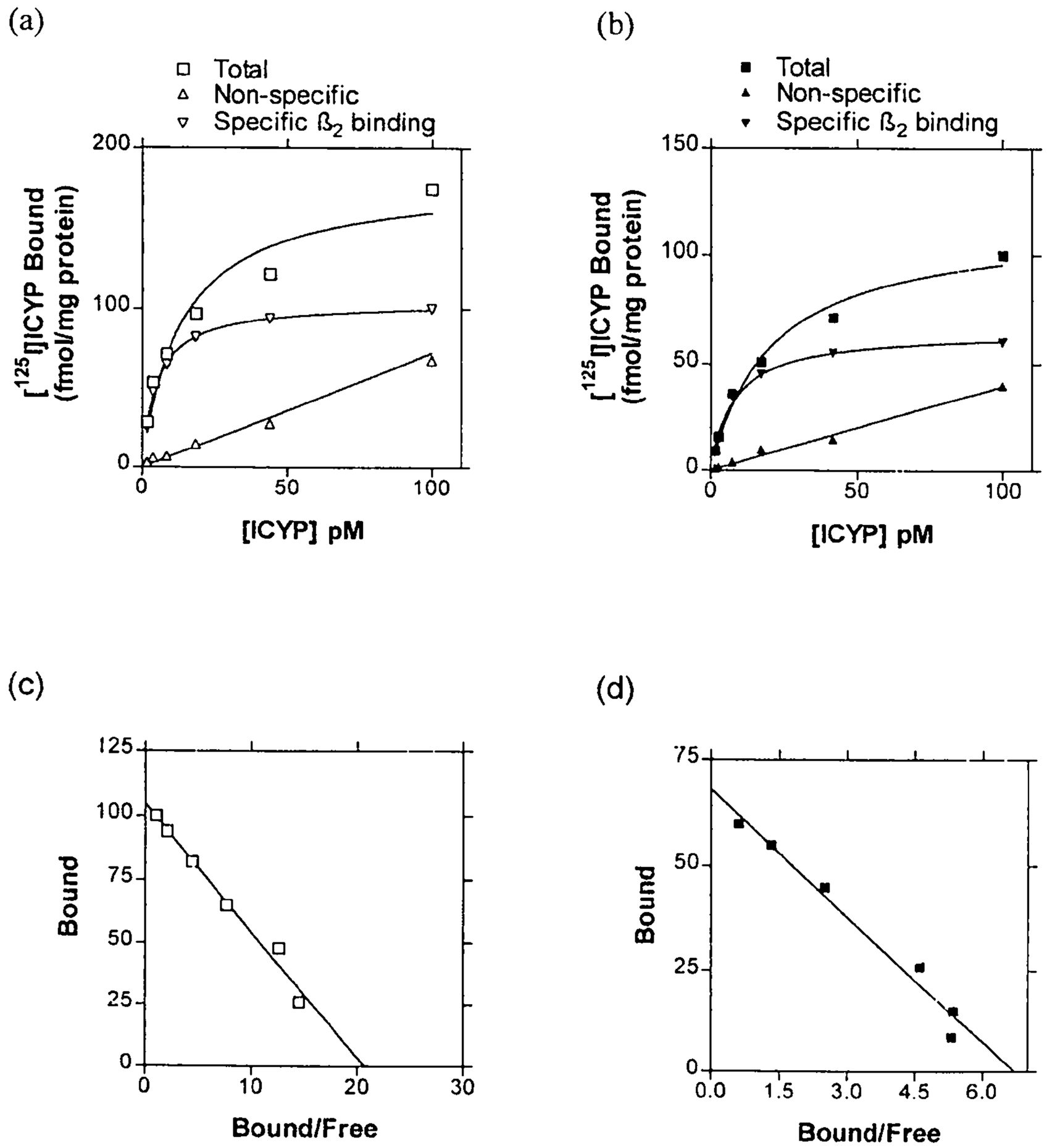



**Figure 3.2.3: Binding of [$^{125}$I]ICYP to $\beta_2$-adrenoceptors in lung membranes prepared from vehicle- and salbutamol-treated rats.** Panels (a) and (b) show representative saturation isotherms for [$^{125}$I]ICYP binding to vehicle- (open symbols) and salbutamol-treated (closed symbols) lung respectively. Experiments were performed as described in the legend to Figure 3.2.1 but the density of $\beta_2$-ARs was determined in the presence of 0.1 μM CGP 20712 A. Panels (c) and (d) show Scatchard transformations of the data in (a) and (b) respectively. Results are representative of eight determinations from different animals. See section 2.2 for further details.

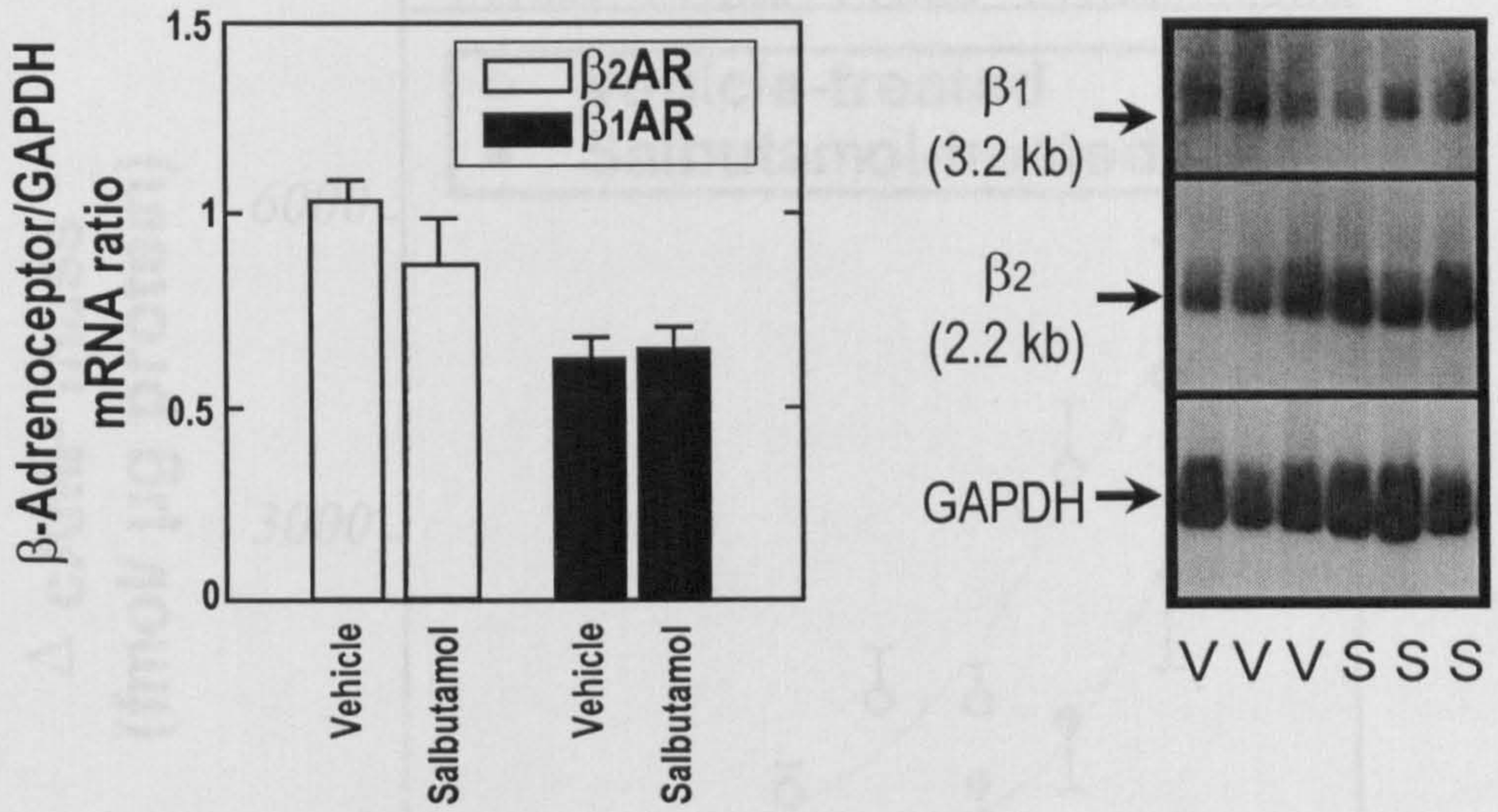



**Figure 3.2.4. Effects of chronic treatment of rats with salbutamol on the steady-state levels of $\beta_1$- and $\beta_2$-adrenoceptor mRNAs.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering salbutamol (40 μg/kg/h) or vehicle (0.9% saline w/v). After seven days, lung was excised and $\beta_1$- and $\beta_2$-AR mRNA was extracted and levels estimated by Northern analysis. Panel (a) shows the mean results of six independent determinations taken from different animals expressed relative to the "house-keeping" gene GAPDH. In panel (b) a representative autoradiogram is shown where S and V refer to salbutamol- and vehicle-treated animals respectively.

### *3.2.3 Effect of chronic treatment of rats with salbutamol on $G_{s\alpha}$ coupling to the βAR signal transduction cascade.*

To further investigate potential mechanisms that could account for the functional desensitisation described in section 3.2.1, the effects of chronic treatment of rats with salbutamol on G-protein coupling was studied. This was assessed pharmacologically by making use of cholera toxin (CTX), which directly activates $G_s$ by catalysing its ADP-ribosylation. After 7 day treatment with salbutamol, the ability of CTX to elevate cAMP in rat lung was reduced when compared to vehicle-treated animals (Figure 3.2.5). There was an apparently parallel 4.6-fold shift to the right of the concentration-response curve, and a significant increase in the $EC_{50}$ of CTX from 2.49 μg/ml to 11.3 μg/ml suggesting that the coupling between $G_s\alpha$ and adenylyl cyclase was impaired in the lung of "desensitised" animals.

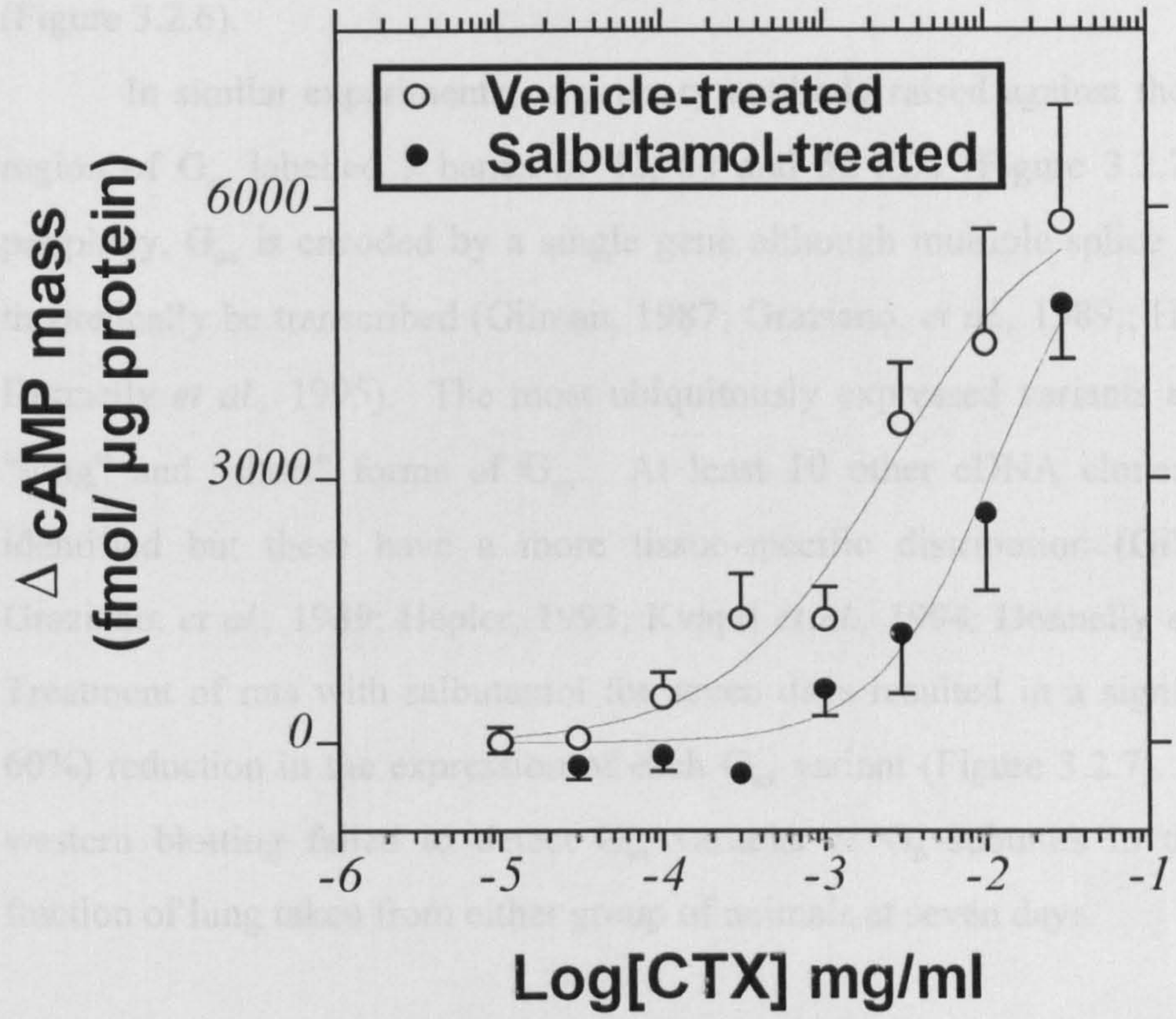



**Figure 3.2.5. Effect of chronic treatment of rats with salbuamol on CTX-induced cAMP accumulation in lung *ex vivo*.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle (○) or salbutamol (●; 40 µg/kg/h). After seven days, animals were sacrificed and the ability of CTX (10 ng/ml to 30 µg/ml) to elevate cAMP in lung tissue was determined as described in section 2.4. Each point represents the mean ± s.e.m. of six independent determinations from different animals.

### *3.2.4 Effect of chronic treatments of rats with salbutamol on the expression of $G_{s\alpha}$ and $G_{\beta}$ subunits.*

Western blotting was carried out on proteins extracted from salbutamol-treated rat lungs to assess whether the uncoupling of $G_s$ from the signal transduction cascade was associated with a reduction in the expression of $G_{s\alpha}$ or $G_{\beta}$ subunits. Using a primary antibody that recognises an epitope on the C-terminus of the common $G_{\beta}$-subunit, a 35 kDa band was labelled in the particulate fraction that was consistent with the reported size of the $G_{\beta}$-subunit (Gilman, 1987; Hepler, 1992; Tang *et al.*, 1992; Taussig *et al.*, 1993) (Figure 3.2.6).

Treatment of rats for one week with salbutamol did not alter the expression of $G_{\beta}$ (Figure 3.2.6).

In similar experiments, a primary antibody raised against the C-terminal region of $G_{s\alpha}$ labelled 3 bands of 42, 44 and 52 kDa (Figure 3.2.7A). In the periphery, $G_{s\alpha}$ is encoded by a single gene although multiple splice variants can theoretically be transcribed (Gilman, 1987; Graziano, *et al.,* 1989;; Hepler, 1993; Donnelly *et al.,* 1995). The most ubiquitously expressed variants are so-called "long" and "short" forms of $G_{s\alpha}$. At least 10 other cDNA clones have been identified but these have a more tissue-specific distribution (Gilman, 1987; Graziano, *et al.,* 1989; Hepler, 1993; Kvapil *et al.,* 1994; Donnelly *et al.,* 1995). Treatment of rats with salbutamol for seven days resulted in a significant (~40-60%) reduction in the expression of each $G_{s\alpha}$ variant (Figure 3.2.7). In contrast, western blotting failed to detect $G_{s\alpha}$ variants or $G_{\beta}$ subunits in the cytosolic fraction of lung taken from either group of animals at seven days.

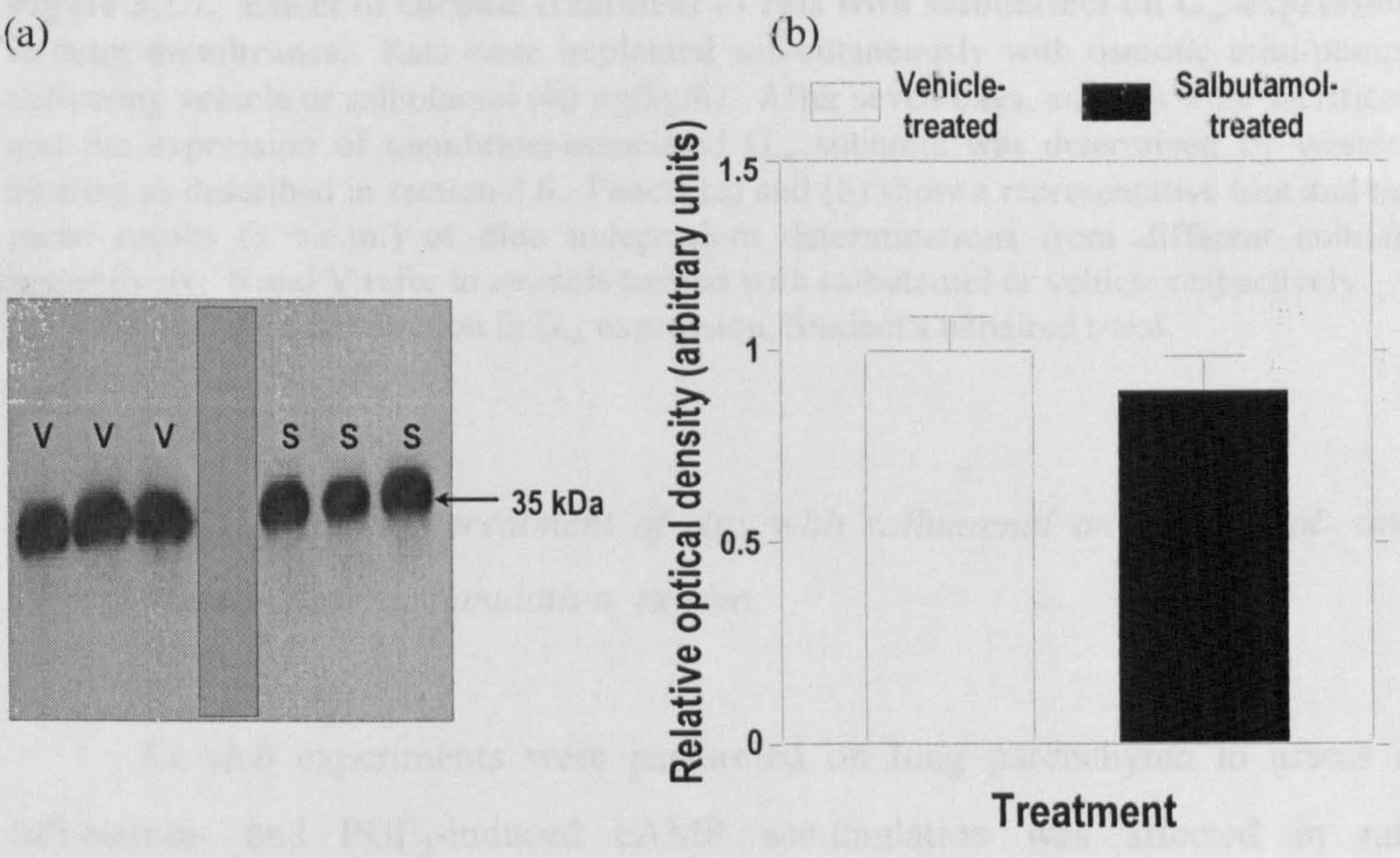



**Figure 3.2.6. Lack of effect of chronic treatment of rats with salbutamol on $G_{\beta}$ expression in lung membranes.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle or salbutamol (40 μg/kg/h) After seven days, animals were sacrificed and the expression of membrane-associated $G_{\beta}$ subunits was determined by western blotting as described in section 2.6. Panels (a) and (b) show a representative western blot and the mean results (± s.e.m.) of nine independent determinations from different animals respectively. S and V refer to animals treated with salbutamol or vehicle respectively.

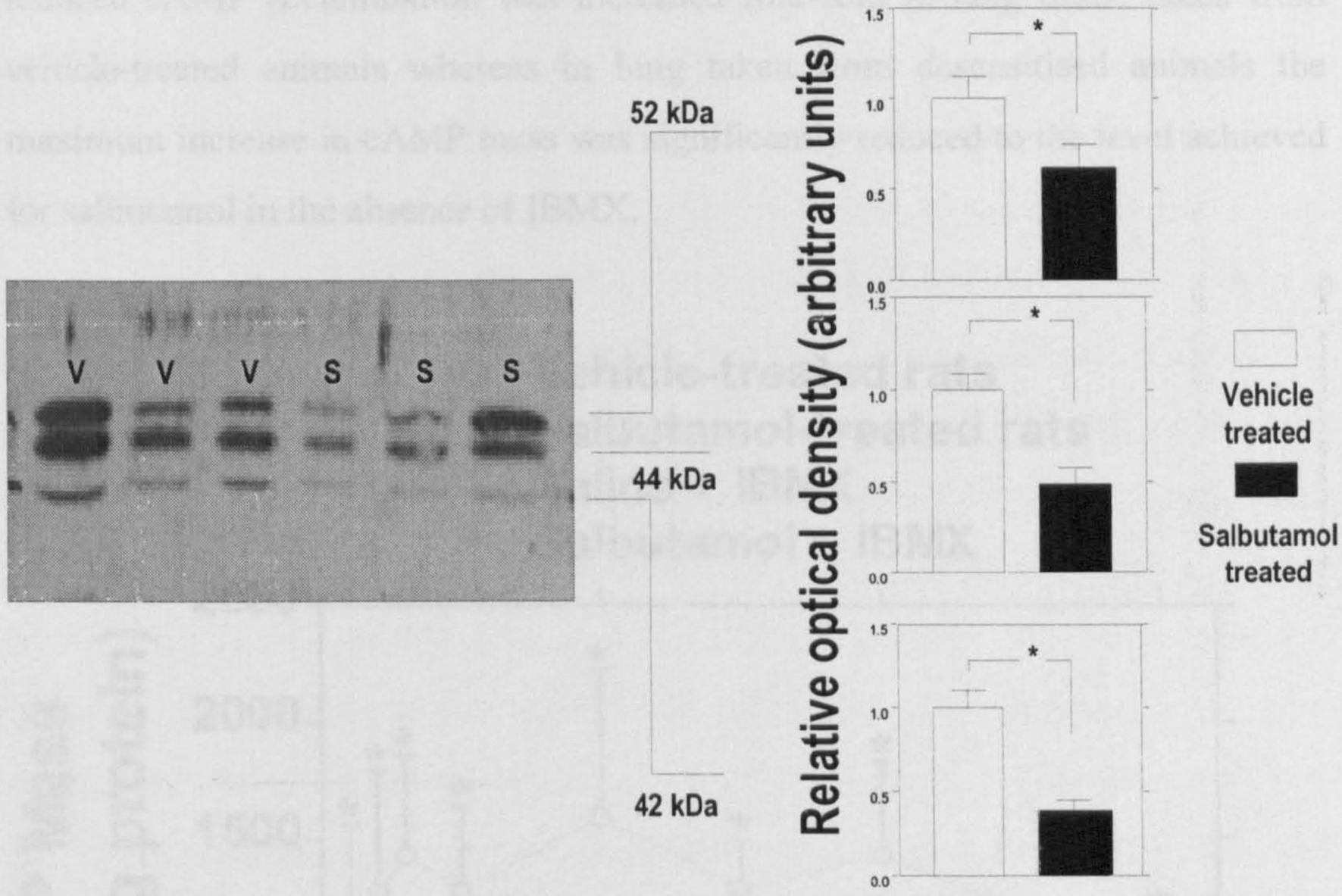



**Figure 3.2.7. Effect of chronic treatment of rats with salbutamol on $G_{s\alpha}$ expression in lung membranes.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle or salbutamol (40 μg/kg/h). After seven days, animals were sacrificed and the expression of membrane-associated $G_{s\alpha}$ subunits was determined by western blotting as described in section 2.6. Panels (a) and (b) show a representative blot and the mean results (± s.e.m.) of nine independent determinations from different animals respectively. S and V refer to animals treated with salbutamol or vehicle respectively. *P<0.05, significant reduction in $G_{s\alpha}$ expression, Student's unpaired t-test.

### *3.2.5 Effect of chronic treatment of rats with salbutamol on salbutamol- and $PGE_2$-induced cAMP accumulation, ex vivo.*

*Ex vivo* experiments were performed on lung parenchyma to assess if salbutamol- and $PGE_2$-induced cAMP accumulation was affected in rats desensitised to salbutamol. A time-response study revealed no change in the kinetics of salbutamol-induced cAMP accumulation between vehicle- and salbutamol-treated rats. In both groups of animals salbutamol (1 μM) increased cAMP mass rapidly that reached a maximum at about 5 minutes (Figure 3.2.8). However, the maximal response obtained with salbutamol was markedly attenuated (~90%) in lung harvested from rats treated chronically with salbutamol

(Figure 3.2.8). In the presence of IBMX (100 μM), the magnitude of salbutamol-induced cAMP accumulation was increased four-fold in lung tissue taken from vehicle-treated animals whereas in lung taken from desensitised animals the maximum increase in cAMP mass was significantly reduced to the level achieved for salbutamol in the absence of IBMX.

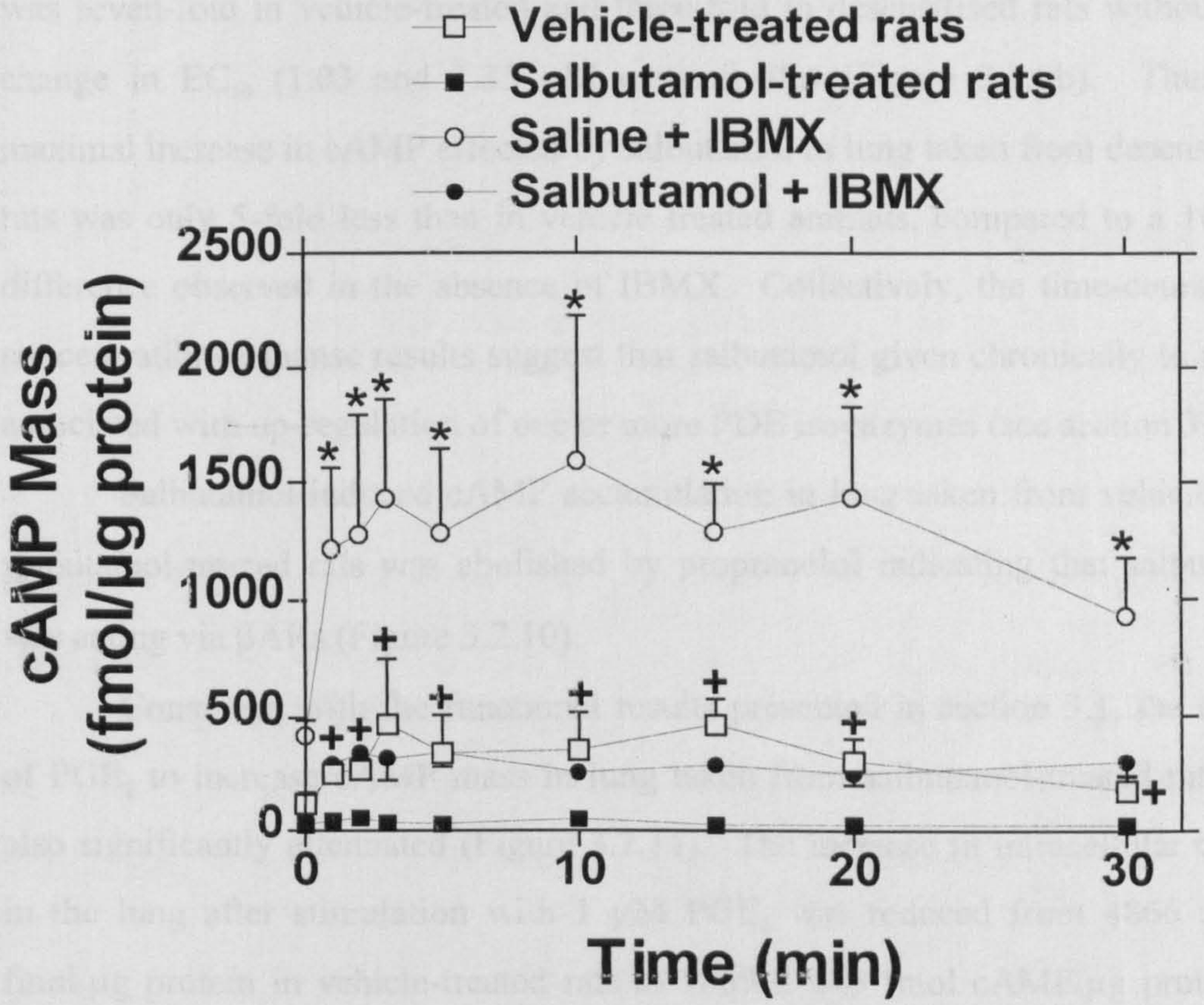



**Figure 3.2.8. Effect of chronic treatment of rats with salbutamol on the kinetics of salbutamol-induced cAMP accumulation in lung *ex vivo*.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle (open symbols) or salbutamol (filled symbols; 40 μg/kg/h). After seven days, animals were sacrificed and lung tissue was harvested from both groups of animals. The ability of salbutamol (1 μM) to increase cAMP mass then was determined in lung pre-incubated for 30 min with IBMX (100 μM; circles) or its vehicle (squares) as described in section 2.4. Each point represents the mean ± s.e.m. of six independent determinations.
$P<0.05$, significant difference in salbutamol-mediated intracellular cAMP elevation between salbutamol- and vehicle-treated rats in the (*) presence and (+) absence of IBMX, Student's unpaired t-test.

Identical results were obtained from concentration-response studies. Thus, in lung harvested from vehicle-treated rats, salbutamol evoked a concentration-dependent increase in cAMP mass (from 81 ± 75 to 502 ± 162 fmol/μg protein; 6-

fold elevation) with an $EC_{50}$ of 4 μM (Figure 3.2.9a), In contrast, the maximal increase in cAMP effected by salbutamol in lung taken from rats that were treated chronically with salbutamol was very modest (from 57 ± 36 to 98 ± 86 fmol/μg protein) and amounted to merely 9.7% of that seen in rats given vehicle under identical experimental conditions (Figure 3.2.9a). In the presence of IBMX, the maximum increase from basal in salbutamol-induced cAMP accumulation in lung was seven-fold in vehicle-treated and three-fold in desensitised rats without any change in $EC_{50}$ (1.03 and 1.33 μM respectively) (Figure 3.2.9b). Thus, the maximal increase in cAMP effected by salbutamol in lung taken from desensitised rats was only 5-fold less than in vehicle treated animals, compared to a 10-fold difference observed in the absence of IBMX. Collectively, the time-course and concentration-response results suggest that salbutamol given chronically to rats is associated with up-regulation of one or more PDE isoenzymes (see section 3.2.6).

Salbutamol-induced cAMP accumulation in lung taken from vehicle- and salbutamol-treated rats was abolished by propranolol indicating that salbutamol was acting via βARs (Figure 3.2.10).

Consistent with the functional results presented in section 3.1, the ability of $PGE_2$ to increase cAMP mass in lung taken from salbutamol-treated rats was also significantly attenuated (Figure 3.2.11). The increase in intracellular cAMP in the lung after stimulation with 1 μM $PGE_2$ was reduced from 4866 ± 394 fmol/μg protein in vehicle-treated rats to 1869 ± 243 fmol cAMP/μg protein in rats given salbutamol chronically ($P<0.05$, $n=6$), which was not significantly different from the basal cAMP level in vehicle-treated rats. Thus, chronic treatment of rats with salbutamol produced heterologous desensitisation at the level of cAMP accumulation (Figure 3.2.11).

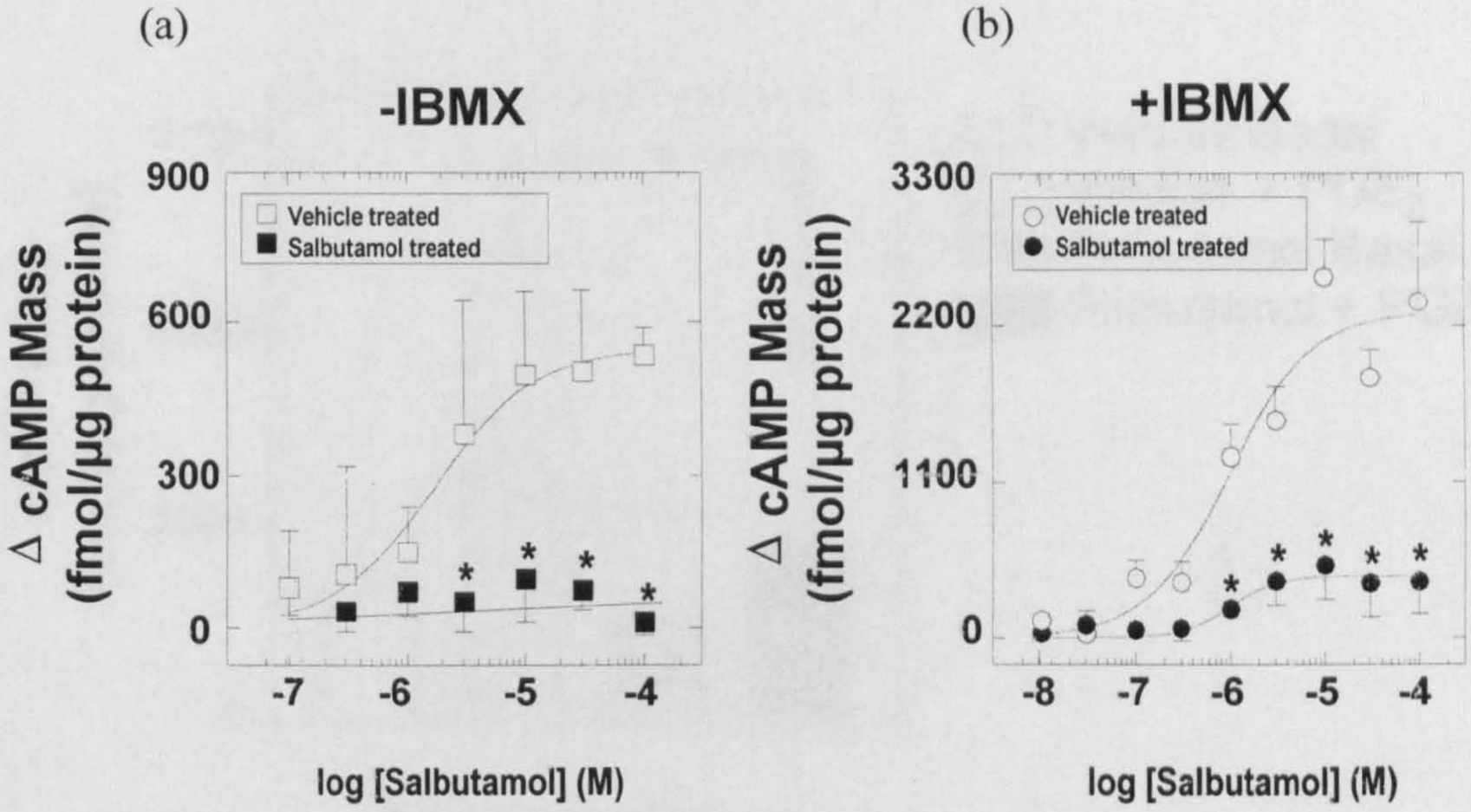



**Figure 3.2.9. Effect of treating rats chronically with salbutamol on the concentration-response relationship of salbutamol-induced cAMP accumulation in lung *ex vivo*.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle (open symbols) or salbutamol (filled symbols; 40 µg/kg/h). After seven days, animals were sacrificed and lung tissue was harvested from both groups of animals. The ability of salbutamol to increase cAMP mass then was determined in lung pre-incubated for 30 min with IBMX (100 µM; circles; panel b) or its vehicle (squares; panel a) as described in section 2.4. Each point represents the mean ± s.e.m. of eight independent determinations. * $P<0.05$ significant attenuation in cAMP mass obtained on stimulation with salbutamol, Student's unpaired t-test.

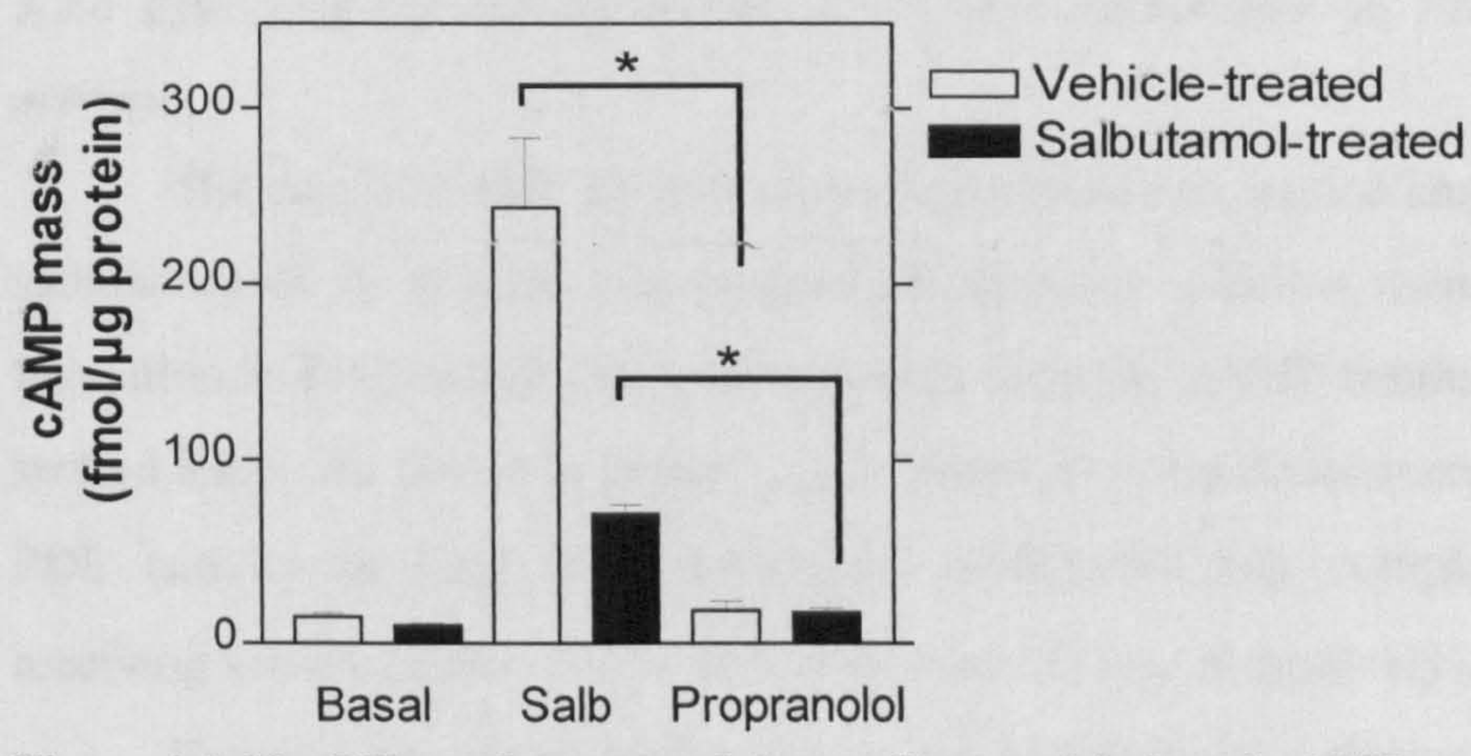



**Figure 3.2.10. Effect of propranolol on salbutamol-induced cAMP accumulation in salbutamol- and vehicle-treated rat lung *ex vivo*.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle (open bars) or salbutamol (filled bars; 40 µg/kg/h). After seven days, animals were sacrificed and lung tissue was harvested from both groups of animals. The ability of salbutamol (1 µM) to increase cAMP mass then was determined in lung pre-incubated for 30 min with propranolol (1 µM) or its vehicle as described in section 2.4. Each bar represents the mean ± s.e.m. of eight independent determinations. * $P<0.05$ significant attenuation in cAMP mass obtained on stimulation with salbutamol in propranolol pre-treated tissue compared to salbutamol-stimulated tissue, Student's unpaired t-test.

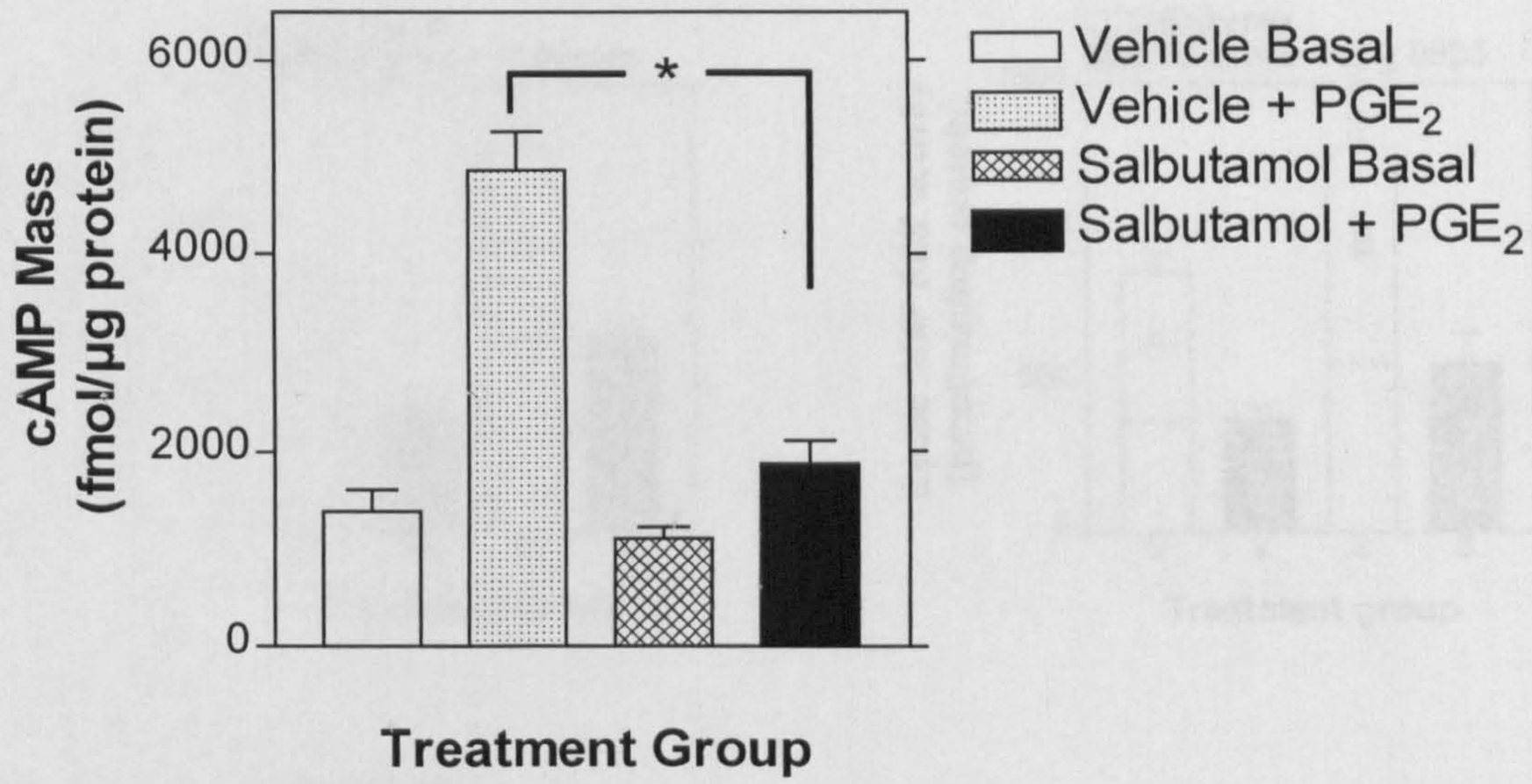



**Figure 3.2.11. Effect of treating rats chronically with salbutamol on $PGE_2$-induced cAMP accumulation in lung *ex vivo*.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle or salbutamol (40 µg/kg/h). After seven days, animals were sacrificed and lung tissue was harvested from both groups of animals. The ability of $PGE_2$ (1µM) to increase cAMP mass then was determined in lung pre-incubated for 30 min with IBMX (100 µM) as described in section 2.4. Each point represents the mean ± s.e.m. of eight independent determinations.
*$P<0.05$ significant attenuation in cAMP mass obtained on stimulation with $PGE_2$, Student's unpaired t-test.

*3.2.6 Effects of chronic treatment of rats with salbutamol on PDE isoenzyme activity.*

The rate of cAMP hydrolysis in lungs from rats treated chronically with salbutamol or its vehicle was assayed to determine whether there was an up-regulation in PDE activity as was suggested from the cAMP results described in section 3.2.5. As shown in figure 3.2.12, there was a significant increase in cAMP PDE activity in lung from salbutamol-desensitised rats compared to those receiving vehicle (1286 ± 91 vs 862 ± 60 pmol/min/mg respectively).

Experiments were performed in the presence of selective cAMP PDE inhibitors to determined which isoenzyme families were responsible for the increase in PDE activity described above. The PDE4 inhibitor, rolipram (30 µM), and the PDE3 inhibitor, Org 9935 (30 µM) (Shahid *et al.,* 1991), significantly attenuated cAMP hydrolysis in rat lung by 53% (vehicle) and 50% (salbutamol), and 57% (vehicle) and 57% (salbutamol) respectively (Figure 3.2.12). Quantification of this effect (B/A in Figure 3.2.12) indicated that PDE3 and PDE4

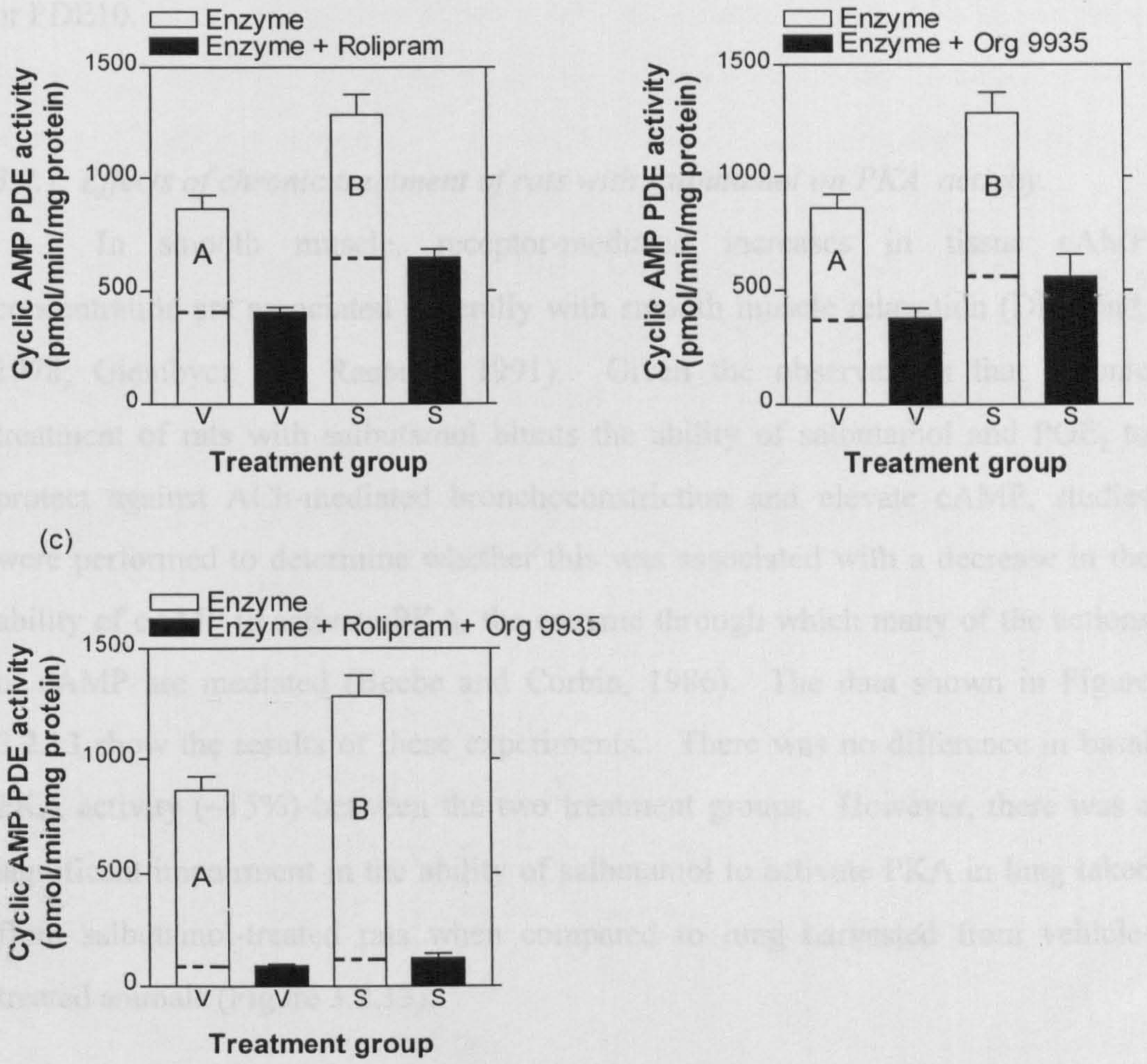



**Figure 3.2.12. Effect of chronic treatment of rats with salbutamol on PDE isoenzyme activity in lung *ex vivo*.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle or salbutamol (40 μg/kg/h). After seven days, animals were sacrificed and lung tissue was harvested from both groups of animals. The ability of rolipram (30 μM; panel a), Org 9935 (30 μM; panel b) and a combination of rolipram and Org 9935 (30 μM each; panel c) to suppress cAMP hydrolysis was then assessed. Open and filled bars show PDE activity in the absence and presence of PDE inhibitor (s) respectively. In each panel **B/A** corresponds to the fold-increase in activity attributable to the PDE isoenzyme defined with rolipram and Org 9935. Each bar represents the mean ± s.e.m. of 6 independent determinations.

were up-regulated 1.5- and 1.4-fold respectively. When used in combination, rolipram and Org 9935 suppressed PDE activity in lung from vehicle- and salbutamol-treated rats to the same level, indicating that the induction of PDE activity was due solely to an increase in activity of these two isoenzyme classes. The residual activity shown in figure 3.2.12c (91.6 ± 9.2 and 126 ± 20.8 pmol/min/mg in vehicle- and salbutamol-desensitised lung respectively) is

probably due to other cAMP-hydrolysing PDE isoenzymes such as PDE7, PDE8 or PDE10.

### *3.2.7. Effects of chronic treatment of rats with salbutamol on PKA activity.*

In smooth muscle, receptor-mediated increases in tissue cAMP concentration are associated generally with smooth muscle relaxation (Diamond, 1978; Giembycz and Raeburn, 1991). Given the observations that chronic treatment of rats with salbutamol blunts the ability of salbutamol and $PGE_2$ to protect against ACh-mediated bronchoconstriction and elevate cAMP, studies were performed to determine whether this was associated with a decrease in the ability of cAMP to activate PKA, the enzyme through which many of the actions of cAMP are mediated (Beebe and Corbin, 1986). The data shown in Figure 3.2.13 show the results of these experiments.. There was no difference in basal PKA activity (~15%) between the two treatment groups. However, there was a significant impairment in the ability of salbutamol to activate PKA in lung taken from salbutamol-treated rats when compared to lung harvested from vehicle-treated animals (Figure 3.2.13).

### *3.2.8. Effects of chronic treatment of rats with salbutamol on GRK activity and GRK2 protein expression.*

GRK2 (βARK1) is involved in desensitisation of βARs through its ability to phosphorylate the agonist-occupied form of the receptor (Benovic *et al.*, 1986, Kwatra *et al.*, 1987, Benovic *et al.*, 1989). To assess whether the functional desensitisation effected by salbutamol was accompanied by an increase in activity of GRK, a biochemical assay was used to measure GRK activity in lung taken from vehicle- and salbutamol-treated rats. As illustrated in figure 3.2.14, there was a significant increase (~55%) in cytosolic GRK activity in lung taken from salbutamol-treated rats when compared animals that received vehicle (Figure 3.2.14).

The increase in cytosolic GRK activity in salbutamol-treated rat lung was associated with a significant increase in GRK2 protein expression in cytosolic extracts as determined by western blotting. A primary antibody to the C-terminal region of GRK2 was used to probe for this protein, and recognised an 80 kDa band (Figure 3.2.15A), which is consistent with the size of GRK2 found in previous studies (Fredericks *et al.,* 1996). Quantification of the western blots by densitometry revealed a significant increase (~95%) in GRK2 protein the cytosolic fraction of lungs from rats treated chronically with salbutamol compared to vehicle-treated animals. In contrast, no change in GRK activity or GRK2 protein was detected in the particulate fraction of salbutamol-treated rats at seven days (data not shown).

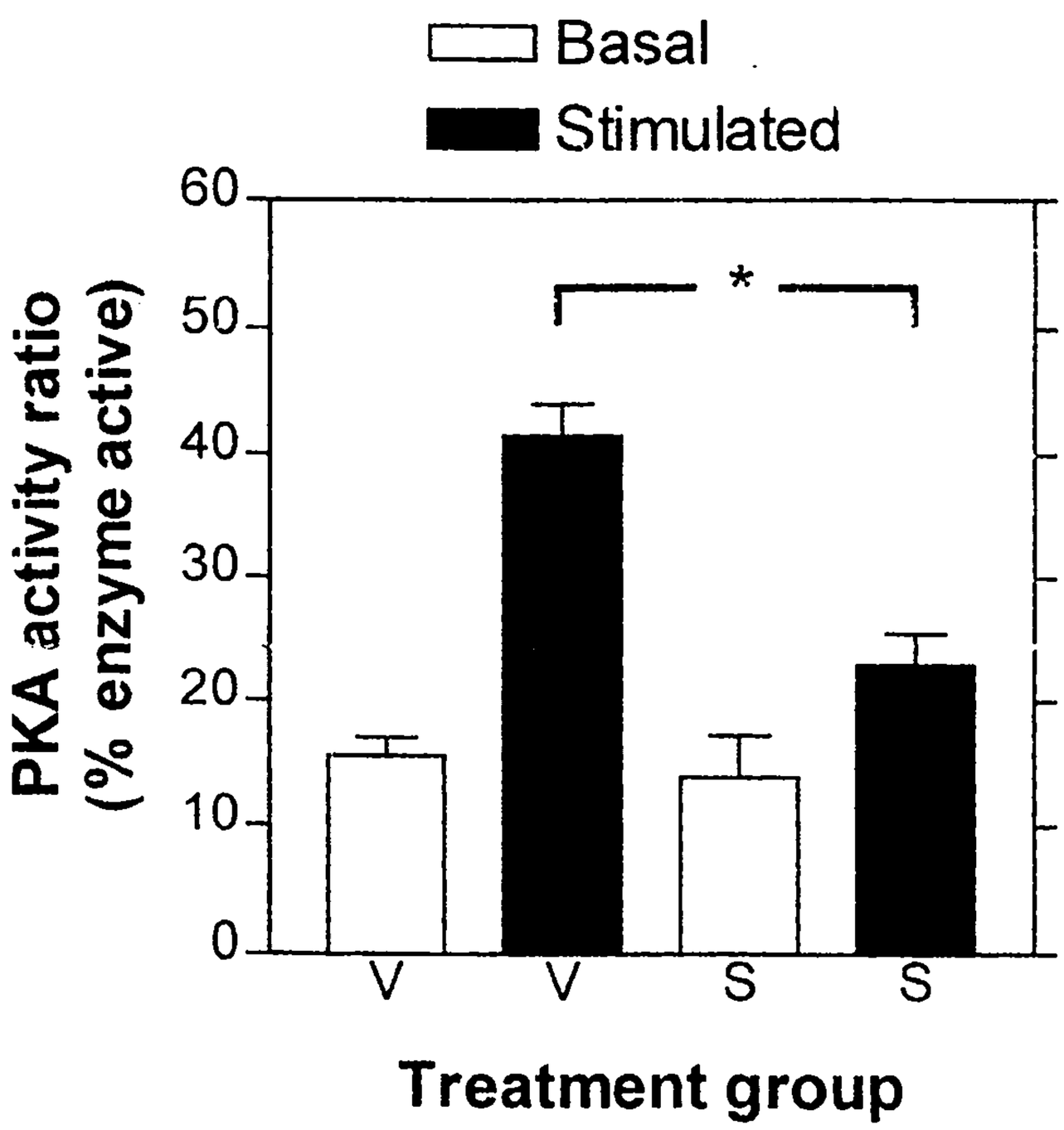



**Figure 3.2.13. Effect of chronic treatment of rats with salbutamol on salbutamol-induced PKA activition *ex vivo*.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle or salbutamol (40 μg/kg/h). After seven days, animals were sacrificed and lung tissue was harvested from both groups of animals. The ability of salbutamol to activate PKA in lung homogenates from salbutamol- or vehicle treated rats was assessed as described in section 2.8. Each bar represents the mean ± s.e.m. of six independent determinations measured in triplicate.
*$P<0.05$ significant attenuation in salbutamol-stimulated PKA activation, Student's unpaired t-test.

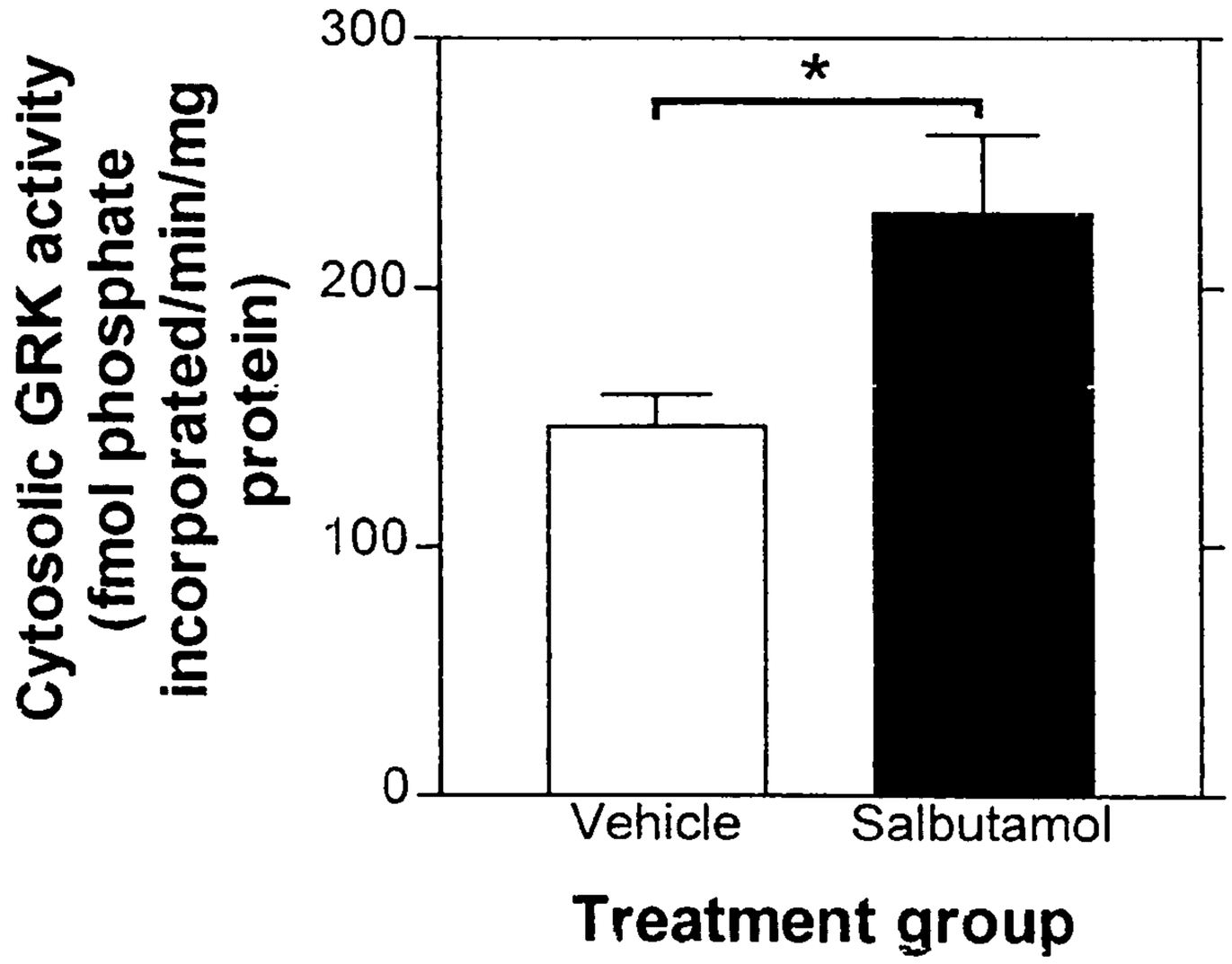



**Figure 3.2.14. Effect of chronic treatment of rats with salbutamol on cytosolic GRK activity in lung.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle or salbutamol (40 µg/kg/h). After seven days, animals were sacrificed and the activity of GRK in lung cytosol was measured by monitoring the phosphorylation of rhodopsin as described in section 2.7. Each bar represents the mean ± s.e.m. of 12 independent determinations.
*P<0.05 significant increase in GRK activity, Student's unpaired t-test.

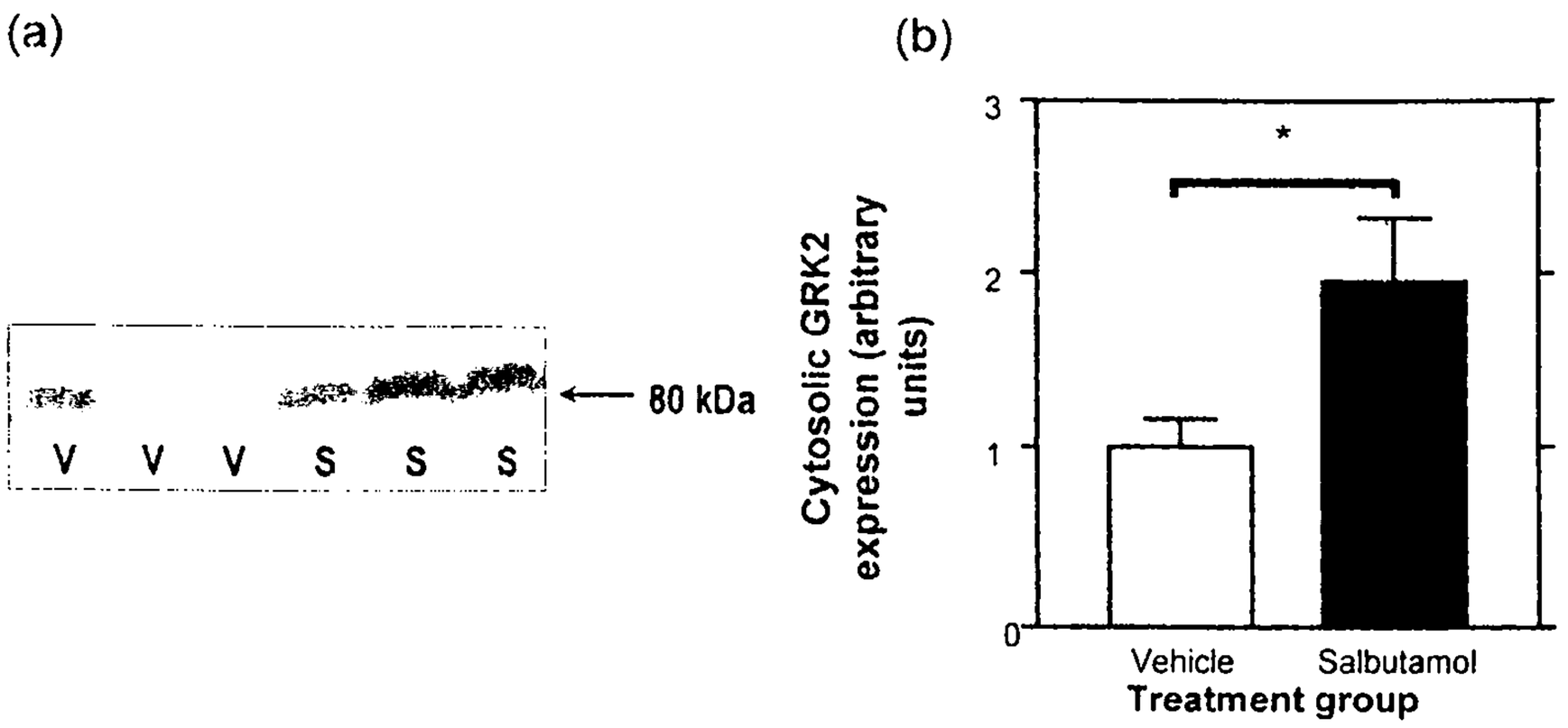



**Figure 3.2.15. Effects of chronic treatment of rats with salbutamol on GRK2 protein expression.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle or salbutamol (40 µg/kg/h). After seven days, animals were sacrificed and the expression of cytosol-associated GRK2 was determined by western blotting as described in section 2.6. Panels (a) and (b) show a representative blot and the mean results (± s.e.m.) of 12 independent determinations from different animals. S and V refer to animals treated with salbutamol or vehicle respectively.
*P<0.05, significant increase in GRK2 expression, Student's unpaired t-test.

*3.2.9. Effects of chronic treatment of rats with salbutamol on β-arrestin protein expression.*

As with GRK enzymes, the involvement of β-arrestin in the internalisation and subsequent desensitisation of βAR has been discussed previously (for review see Palczewski, 1994). To assess whether the biochemical and functional desensitisation demonstrated in sections 3.1 and 3.2 was associated with a change in β-arrestin expression, western blotting on lung extracted from rats chronically-treated with vehicle or salbutamol was undertaken using a primary antibody raised against the C-terminal region of the β-arrestin molecule. Using this technique, no change in the expression of β-arrestin protein in either total, membrane or cytosolic fractions was detected (Figure 3.2.16).

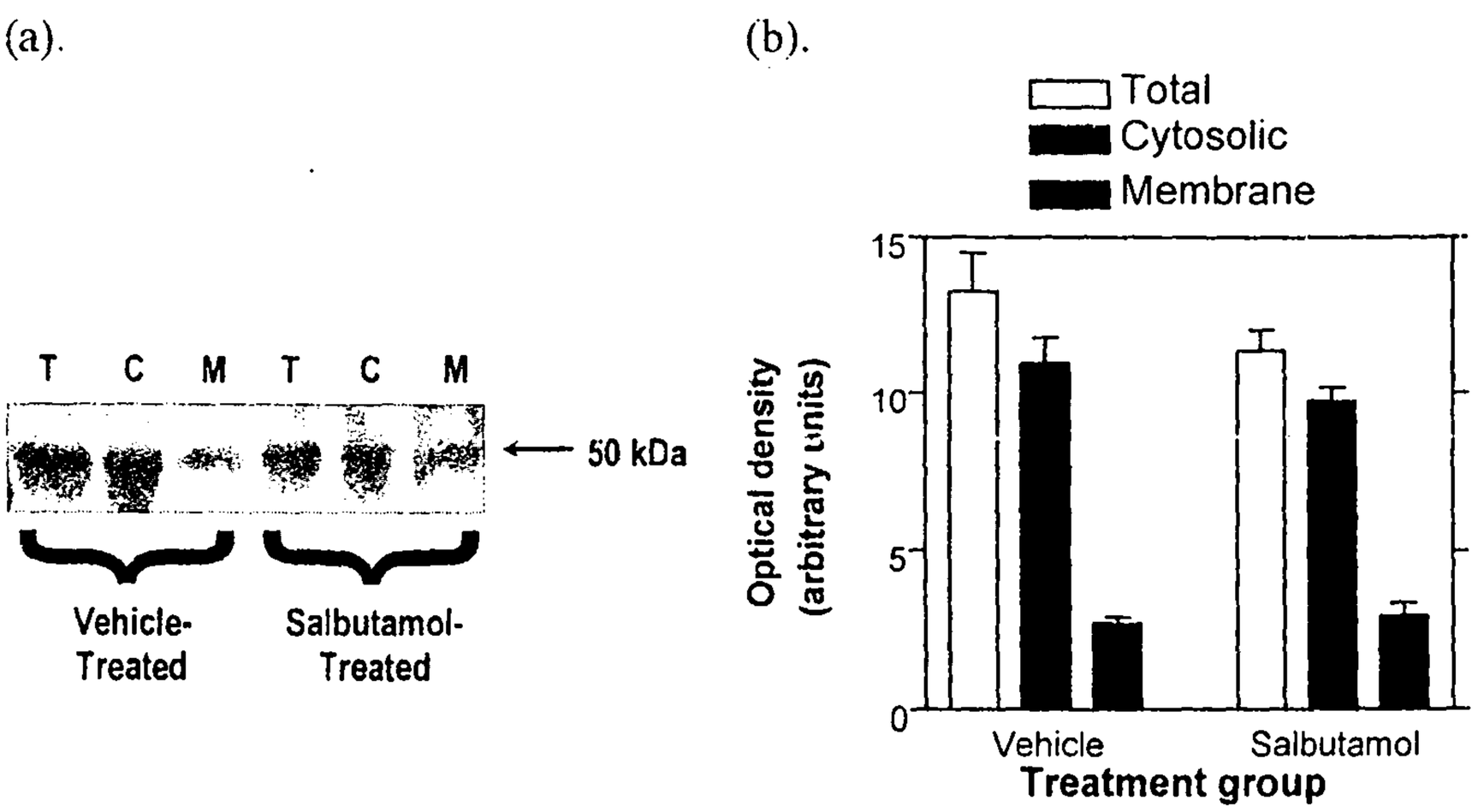



**Figure 3.2.16. Lack of effect of chronic treatment of rats with salbutamol on β-arrestin expression in lung membranes.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle or salbutamol (40 μg/kg/h) After seven days, animals were sacrificed and the expression of β-arrestin was determined by western blotting as described in section 2.6. Panels (a) and (b) show a representative western blot and the mean results (± s.e.m.) of eight independent determinations from different animals respectively. T, C and M refer to total, cytosolic and membrane protein fractions respectively.

### *3.2.10. Effects of chronic treatment of rats with salbutamol on salbutamol-induced cAMP elevation ex vivo, and $G_{s\alpha}/G_{\beta}$ protein expression in trachea.*

In the preceding sections, desensitisation to the bronchoprotective action of salbutamol and $PGE_2$ has been discussed. However, there is a discrepancy between the functional desensitisation, which reflects an action on airway smooth muscle *in vivo*, and the biochemical data, which was obtained using lung *ex vivo*. A limited number of additional experiments were, therefore, performed to determine if chronic treatment with salbutamol effected similar biochemical changes in the trachea.

Consistent with the results obtained with lung tissue, there was a significant reduction in the ability of salbutamol to elevate intracellular cAMP in tracheae excised from salbutamol-treated rats when compared to the same tissue excised from vehicle-treated animals (Figure 3.2.17). The basal intracellular cAMP concentration was not significantly different between the two treatment groups.

Western blotting was also performed to determine if changes in the expression of G protein subunits effected by salbutamol in lung (section 3.2.4) also occurred in tracheal membranes. As shown in figures 3.2.18 and 3.2.19, chronic treatment of rats with salbutamol did not affect $G_{\beta}$ levels, but significantly reduced the expression of all three $G_{s\alpha}$ variants by 50 to 70% when compared to their vehicle-treated counterparts.

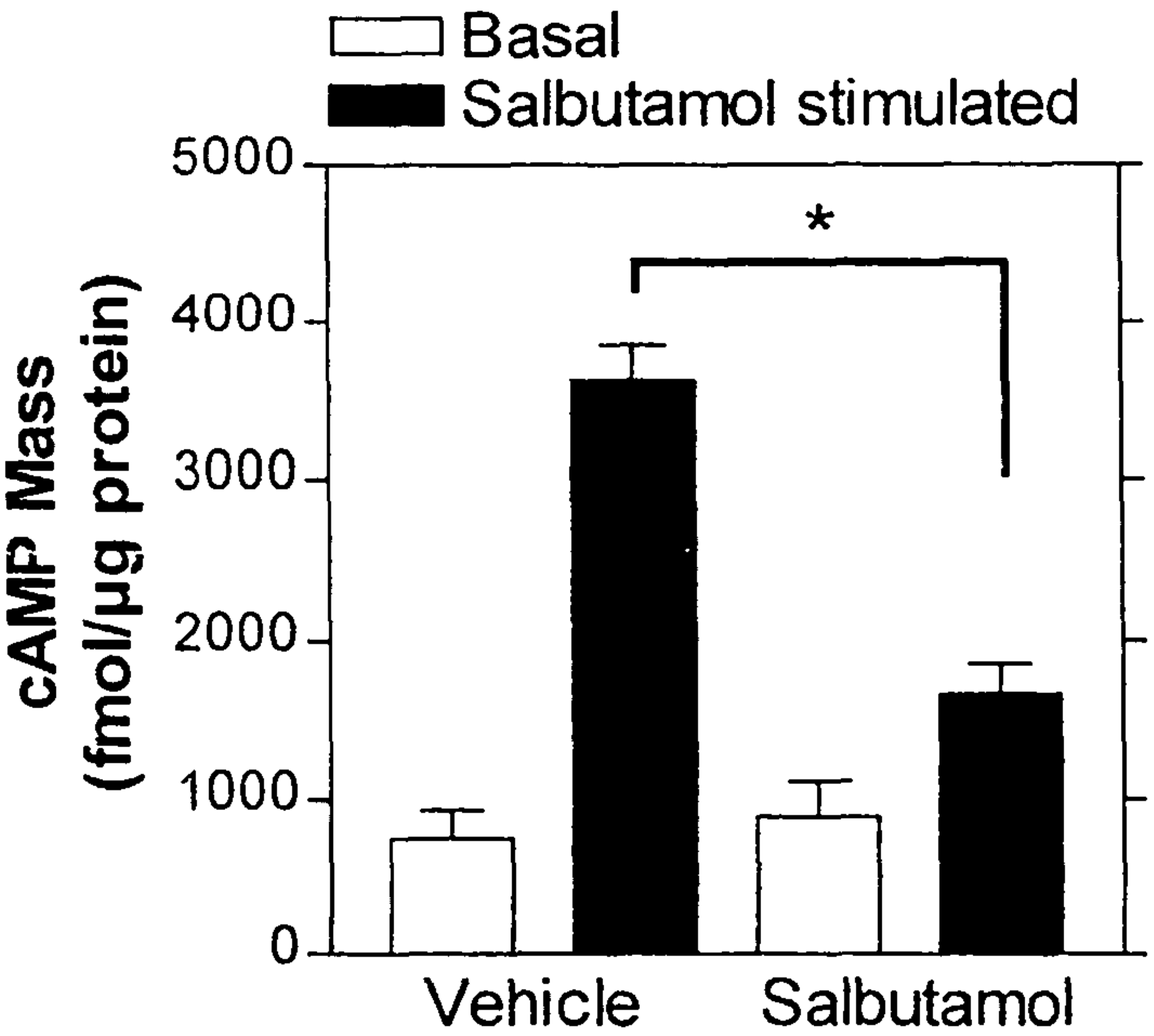



**Figure 3.2.17. Effect of treating rats chronically with salbutamol on salbutamol-induced cAMP accumulation in trachea *ex vivo*.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle or salbutamol (40 µg/kg/h). After seven days, animals were sacrificed and tracheal tissue was harvested from both groups of animals. The ability of salbutamol (1 µM) to increase cAMP mass then was determined in tracheae pre-incubated for 30 min with IBMX (100 µM) as described in section 2.4. Each bar represents the mean ± s.e.m. of six independent determinations in different animals. *P<0.05 significant attenuation in cAMP mass in trachea of salbutamol-treated rats, Student's unpaired t-test.

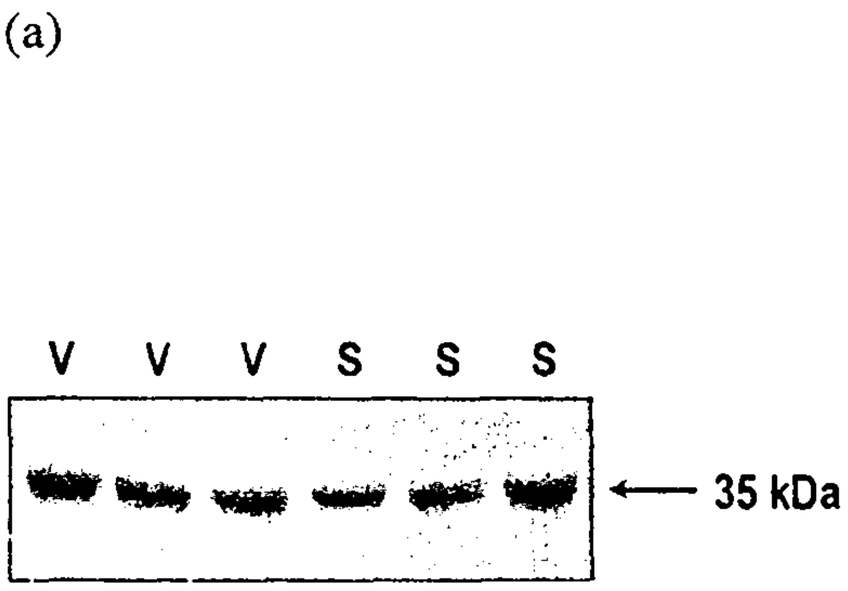



**Figure 3.2.18. Lack of effect of chronic treatment of rats with salbutamol on $G_\beta$ expression in tracheal membranes.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle or salbutamol (40 µg/kg/h). After seven days, animals were sacrificed and the expression of membrane-associated $G_\beta$ subunits was determined by western blotting as described in section 2.6. Panels (a) and (b) show a representative western blot and the mean results (± s.e.m.) of six independent determinations from different animals respectively. S and V refer to animals treated with salbutamol or vehicle respectively.

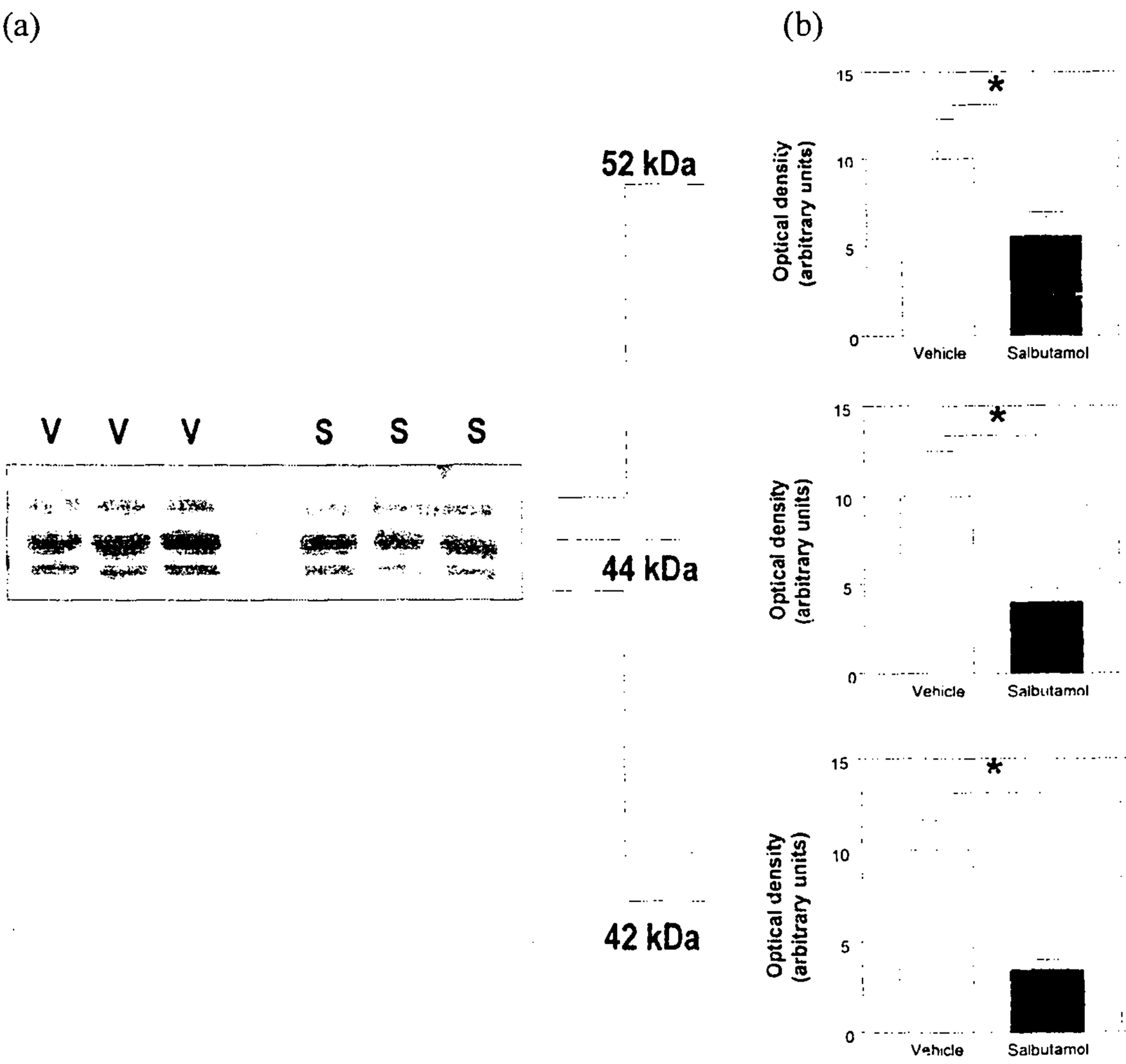



**Figure 3.2.19. Effect of chronic treatment of rats with salbutamol on $G_{s\alpha}$ expression in tracheal membranes.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle or salbutamol (40 µg/kg/h). After seven days, animals were sacrificed and the expression of membrane-associated $G_{s\alpha}$ subunits was determined by western blotting as described in section 2.6. Panels (a) and (b) show a representative blot and the mean results (± s.e.m.) of six independent determinations from different animals respectively. S and V refer to animals treated with salbutamol or vehicle respectively. *P<0.05, significant reduction in $G_{s\alpha}$ expression, Student's unpaired t-test.

## 3.2.11. *Discussion*

The experiments described in this chapter were performed to determine if desensitisation of the bronchoprotective effect of salbutamol *in vivo* was associated with (and could be explained by) changes in the $\beta_2$AR/$G_s$/AC/PKA cascade and/or in the expression and activity of GRK2 and β-arrestin. Receptor binding studies, in the presence of selective antagonists to enumerate $\beta_1$- and

$\beta_2$AR density, demonstrated the coexistence of both subtypes in rat lung that were expressed in a 1:2 ratio, which is similar to the ratio reported previously (Barnett *et al.,* 1978). After chronic treatment of rats with salbutamol there was a significant reduction in the density of $\beta_2$- but not $\beta_1$ARs without any change in the affinity of ICYP for either receptor subtype. Similar results have also been demonstrated in lung after chronic treatment of rats with terbutaline (Kudlacz *et al.,* 1990) and isoprenaline (Vassilev *et al.,* 1998). For the latter agonist Nishikawa *et al* (1993) found a reduced density of pulmonary $\beta_1$- and $\beta_2$AR in Wistar rats infused continually for 7 days with 10µg/kg/h isoprenaline. However, as isoprenaline is a non-selective βAR-agonist, it is not surprising that both subtypes were affected. Indeed, other groups have also found down-regulation of $\beta_1$- and $\beta_2$AR in cardiac ventricle and cerebral cortex from rats after prolonged *in vivo* exposure to isoprenaline (Beer *et al.,* 1987, Nanoff *et al.,* 1989).

Northern analyses were performed on lung parenchyma to assess if steady state $\beta_1$- and $\beta_2$AR mRNA expression was affected by chronic treatment of rats with salbutamol. At 7 days mRNA transcripts for both $\beta_1$- and $\beta_2$AR were unchanged between the treatment groups. These results are in contrast to the observations of Nishikawa *et al.,* (1993) who found a reduction in steady state $\beta_1$- and $\beta_2$AR message in isoprenaline-treated rats, but are nevertheless consistent with several *in vitro* studies, including those described by Kelsen *et al.,* (1997), who found that chronic catecholamine treatment induced $\beta_2$AR down-regulation in BEAS-2B cells (human bronchial epithelial cell line) without affecting steady-state levels of $\beta_2$AR mRNA.

A number of explanations could account for a reduction in $\beta_2$AR number without a commensurate decrease in $\beta_2$AR mRNA. Differential expression of pulmonary βAR subtypes in different rat strains (Van Liefde *et al.,* 1994) and even cell types within the lung (Danner and Lohse, 1997) could explain the differences between the current study and that performed by Nishikawa *et al.,* (1993). Another possibility is that Nishikawa *et al.,* (1993) used isoprenaline, which is a full, non-selective βAR agonist in most tissues, whereas salbutamol, a partial agonist at $\beta_2$ARs, was used in this study. However, perhaps the most likely explanation is that the reduction in $\beta_2$AR binding sites at 7 days is the result of a

down-regulation of $\beta_2$AR mRNA transcripts that occured at an earlier time-point, and which had recovered at day 7. This interpretation would be consistent with the idea that the down-regulation of $\beta_2$ARs is due to destabilisation of $\beta_2$AR mRNA via βARB.

After chronic treatment of rats with salbutamol, lung was used in *ex vivo* studies to investigate the ability of salbutamol and $PGE_2$ to elevate cAMP mass. Chronic treatment with salbutamol did not affect the kinetics of cAMP accumulation between treatment groups, but the maximal increase in cAMP obtained after stimulation with salbutamol was significantly reduced in salbutamol-treated rats when compared to rats treated with vehicle. Similarly, the concentration-response curve that described salbutamol-induced cAMP accumulation was also markedly depressed; although there was no significant change in the $EC_{50}$ values between the two groups. There was also an attenuation of the ability of $PGE_2$ to increase cAMP in lung taken from desensitised rats, demonstrating heterologous desensitisation at the biochemical level. The reduction in salbutamol- and $PGE_2$-induced cAMP accumulation is consistent with an uncoupling of $G_{s\alpha}$ from AC and/or down-regulation of $G_{s\alpha}$, but also appears to be partly caused by an increase in PDE activity. Evidence for this conclusion was the finding that IBMX, a non-selective PDE inhibitor, partially restored the sensitivity of lung taken from desensitised animals to salbutamol. Moreover, inhibitor studies implicated both PDE3 and PDE4 in this effect.

However, whether the enhanced rate of cAMP metabolism contributed to the loss of anti-spasmogenic activity of salbutamol and $PGE_2$ at day seven is unclear given that the protection afforded by forskolin against ACh-induced bronchoconstriction was preserved in both groups of animals. These results might indicate that the increase in PDE is functionally irrelevant or occurring in non-airway smooth muscle cells. Alternatively, since forskolin can evoke huge increases in cAMP, the up-regulation of PDE may be insufficient to attenuate the level of cAMP such that the activity of PKA is reduced. The idea that desensitisation of a $G_s$-coupled receptor could be due, at least in part, to the up-regulation of PDE isoenzymes in relevant cells and a concomitant increase in their ability to degrade cAMP is a largely ignored hypothesis (Giembycz, 1996).

However, the data presented herein imply that there could be many factors affected by chronic salbutmol treatment including up-regulation of PDE isoenzymes, which would have a significant effect on signalling through the βAR pathway. Conceivably, PDE up-regulation may be functionally relevant in the present model at earlier time points when the cAMP/PKA cascade is not impaired. This would be in agreement with studies performed by Seybold *et al.*, (1998) and Torphy *et al.*, (1995) who reported that PDE3 and PDE4 isoenzymes were rapidly up-regulated in leukocytes exposed to salbutamol, and contributed to $\beta_2$AR desensitisation.

Data presented by Penn *et al.*, (1998) suggests that human airway smooth muscle $\beta_2$ARs are highly susceptible to rapid desensitisation by multiple agents and identified PKA as a mediator of this acute effect. However, in the present study, there was a significant impairment of the ability of salbutamol to activate PKA in lung taken from salbutamol-treated rats at seven days when compared to tissue excised from vehicle-treated animals, which was consistent with compromised cAMP formation. Roth *et al.*, (1991) showed that PKA-mediated uncoupling of βAR to cAMP formation is very rapid, with a $t_{½}$ of 3.5 minutes, and was reversible within 15 minutes. Therefore, PKA-mediated phosphorylation of the $\beta_2$AR is unlikely to be functionally relevant at 7 days. In addition to $\beta_2$AR desensitisation, PKA also is involved in producing the functional responses evoked by $\beta_2$AR agonists. As mentioned in section 1.2.3 there are several actions of PKA which can mediate smooth muscle relaxation including inhibition of inositol phosphate hydrolysis (Madison and Brown, 1988; Hall *et al.*, 1989), stimulation of calcium uptake by internal stores (Mueller and Van Breeman, 1979), inactivation of myosin light chain kinase (Silver and Stull, 1982) and activation of cell membrane ion channels and transporters such as $K^+$ channels and the $Na^+/K^+$ ATPase. (Kume *et al.*, 1989; Gunst and Stropp, 1988; Knox and Brown 1991). The net effect of these processes would be to reduce intracellular calcium and contractile protein phosphorylation, thereby producing relaxation. Thus, a reduction in activity of PKA could reduce the functional response to $\beta_2$AR stimulation and blunt relaxation of smooth muscle. It is important to emphasize that the reduction in salbutamol-stimulated PKA activity at seven days does not

preclude the possibility that PKA phosphorylated residues on the $\beta_2$AR, thereby uncoupling the receptor from the signal transduction cascade at an earlier time point.

One of the important mechanisms involved in $\beta_2$AR desensitisation is receptor phosphorylation and subsequent internalisation. Phosphorylation of the $\beta_2$AR produces rapid desensitisation of the cAMP response to a $\beta_2$AR-agonist by impairing receptor-$G_{s\alpha}$ interaction. The two classes of enzymes involved in $\beta_2$AR phosphorylation are PKA, which was discussed in the preceeding paragraph, and GRKs (Hausdorf *et al.,* 1989). The relative importance of the two kinases in mediating $\beta_2$AR desensitisation is cell-type specific (Shih and Malbon, 1994). GRK2 (or βARK1), the first cloned member of a multigene family of related GRKs (Inglese *et al.,* 1993, Premont, *et al.,* 1995), phosphorylates $\beta_2$ARs only in the active configuration induced by ligand binding. Subsequent uncoupling of the receptor from the G protein by stearic hindrance is produced by the β-arrestin protein that binds to the phosphorylated form of the receptor thereby producing homologous (agonist-specific) desensitisation. GRK2, but not GRK3, is expressed in the respiratory tract (McGraw and Liggett, 1997).

Assuming the importance of receptor phosphorylation and sequestration in $\beta_2$AR desensitisation, the effects of chronic treatment of rats with salbutamol on expression and enzyme activity of GRK was assessed. The extent to which GRK2 is involved in $\beta_2$AR desensitisation in the *in vivo* model described herein is unclear since membrane-bound GRK activity was unchanged after seven days of treatment with salbutamol. However, translocation of GRK2 to the plasma membrane is rapid and transient, occurs concurrently with receptor phosphorylation and precedes desensitisation and receptor sequestration. Thus, the absence of elevated GRK activity in lung membranes is not surprising. GRKs display considerable substrate specificity (Krupnick and Benovic, 1998) despite the fact that a single family member can phosphorylate multiple GPCRs (see Chuang *et al.,* 1996). Therefore, it seems unlikely that activation of GRK2 by salbutamol represents a mechanism of *in vivo* pulmonary $\beta_2$AR desensitisation for it cannot account for the impairment of $PGE_2$-induced airway and biochemical responses in this model. Equally, the ability of E-series prostaglandins to activate

GRK (Strasser *et al.,* 1986) fails to explain how chronic treatment of rats with $PGE_2$ desensitises the airways to the anti-spasmogenic action of salbutamol. Presumably, specificity is conferred by the inability of GRKs to phosphorylate receptors that are not occupied by high concentrations of activating ligands. Unexpectedly, the activity of GRK2 in lung cytosol from salbutamol-treated rats was significantly increased compared to vehicle-treated animals. Moreover, this effect was associated with increased protein expression suggesting that GRK2 mRNA stability and/or gene transcription had been increased. This interpretation is supported by the results of a recent study in which long term *in vivo* infusion of mice with isoprenaline increased mRNA, protein and activity of GRK2 in the heart by a mechanism that was attributed to a cAMP-independent increase in GRK2 gene transcription (Iaccorino *et al.*, 1998).

The role of β-arrestin in $\beta_2$AR desensitisation has also been reported many times (Gurevich *et al.*, 1995; Söhleman *et al.*, 1995; Ferguson *et al.*, 1996). It is the binding of the arrestin to the phosphorylated receptor that appears to play a primary role in quenching signal transduction, apparently via directly disrupting interaction between the receptor and G proteins (Benovic *et al.*, 1987; Lohse *et al.*, 1990, 1992). Western blotting using proteins extracted from excised lungs from rats treated chronically with salbutamol and vehicle established that there was no difference in expression of β-arrestin protein between the two treatment groups. There is increasing evidence that β-arrestin has a more complex role than just acting as an "adapter" protein for phosphorylated receptors. Indeed, several groups have published data implicating β-arrestin in targeting internalised receptors and parts of the tyrosine-kinase signalling pathway together in intracellular compartments (van Biesen *et al.*, 1995, Luttrell *et al.*, 1997, Daaka *et al.*, 1997). As β-arrestin has other roles apart from acting as an adaptor protein, it is therefore unlikely that the desensitisation of a single receptor system would affect the expression of an increasingly multifunctional protein.

The ability of salbutamol to promote heterologous desensitisaton *in vivo* implicates processes other than GRKs and receptor down-regulation. Hence, the ability of CTX to elevate cAMP in salbutamol-treated rat lung was studied to assess if uncoupling of $G_{s\alpha}$ to $\beta_2$AR or down-regulation of $G_s$ could account for

the functional *in vivo* desensitisation described in section 3.1. After chronic treatment of rats with salbutamol, the ability of CTX to elevate cAMP was reduced at all concentrations examined when compared to its effect in lung taken from vehicle-treated rats. Indeed, there was almost a five-fold shift to the right of the CTX concentration response curve without an apparent change in the maximum response. These results imply that $G_s$ has become uncoupled from AC. Kume and Takagi (1997) looked at the reduction of mechanical responsiveness to isoprenaline after continuous or repeated exposure to the same agonist in guinea pig tracheal smooth muscle. It was found that the inhibitory action of isoprenaline on contractions induced by methacholine (MCh) were suppressed after incubation with isoprenaline for an hour, and that the effects of isoprenaline on MCh-induced contraction gradually decreased after repeated application at intervals of 1 h. The reduced responsiveness to isoprenaline was not mimicked by forskolin, consistent with the *in vivo* results reported in the present study, supporting the supposition that the desensitisation is mediated upstream of AC. Significantly, Kume and Takagi, (1997), also found that brief pre-exposure of guinea pig tracheal strips to CTX prevented the desensitisation, a finding that indirectly supports the suggestion that a decrease in the activity of $G_s$ is a central mechanism involved in the reduction in response to repeated stimulation of tissue with βAR agonists.

The reduced coupling of $G_s$ to AC in lung taken from salbutamol-treated rats was associated with a concordant reduction in $G_{s\alpha}$ expression but no effect on the β-subunit of $G_s$. All three variants of $G_{s\alpha}$ detected using western blotting were down-regulated (~50% reduction) in salbutamol-treated rat lung. Although the mechanism by which salbutamol promoted this effect was not investigated, it has previously been reported that persistent activation of $G_{s\alpha}$ with analogues of GTP promotes its removal from the plasma membrane, demonstrating that $G_{s\alpha}$ need not remain closely associated with the plasma membrane after activation (Milligan and Unson, 1989). Moreover, several groups of investigators have shown that sustained exposure of neuroblastoma cells to either isoprenaline or iloprost results in a substantial decrease in cellular levels of the α-subunit of $G_s$ (Kim and Milligan 1994, Adie and Milligan 1994, McKenzie and Milligan, 1990, Adie *et al.,* 1992). In those systems, desensitisation was associated with a reduction in

$\beta_2$AR binding sites and a reduction in the expression (and therefore coupling) of $G_{s\alpha}$ which could have a significant impact on the responsiveness of the cells to agonist (Kim and Milligan 1994, Adie and Milligan 1994, McKenzie and Milligan, 1990, Adie *et al.*, 1992). The down-regulation of $G_{s\alpha}$ in lung membranes from salbutamol-treated rats provides a compelling explanation for heterologous desensitisation in this model. Indeed, down-regulation $G_{s\alpha}$ would blunt the ability of salbutamol, and other agonists that utilise the same pool of $G_{s\alpha}$ (e.g. $PGE_2$), to activate AC and protect the airways against ACh-induced bronchoconstriction. There are at least three explanations that could account for this effect: decreased $G_{s\alpha}$ gene transcription, destabilisation of pre-existing $G_{s\alpha}$ mRNA transcripts or redistribution/degradation of membrane-associated $G_{s\alpha}$ isoforms. McKenzie and Milligan (1990) have reported that down-regulation of $G_{s\alpha}$ after long-term treatment of NG108-15 cells (a neuroblastoma/glioma hybrid cell line) with $PGE_1$ is not associated with a reduction in $G_{s\alpha}$ mRNA or prevented by cycloheximide. Thus, in those studies it seems unlikely that the reduction of $G_{s\alpha}$ is transcriptionally regulated or reflects a reduction in mRNA stability. Concordant with this interpretation is the observation that isoprenaline (and other cAMP-elevating agonists) promotes the translocation of $G_{s\alpha}$ from the membrane to the cytosolic fraction in a number of different cells (Ransnas *et al.*, 1989; Wedegaerter *et al.*, 1996). This redistribution of $G_{s\alpha}$ occurs rapidly and is followed, after chronic exposure, by degradation of $G_{s\alpha}$ in the cytosolic pool (Milligan, 1993). In the present study, $G_{s\alpha}$ was not detected in the cytosol of lung taken from salbutamol-treated rats despite a significant reduction in membrane expression, suggesting that the chronicity of agonist exposure had effected degradation of $G_{s\alpha}$.

The functional *in vivo* desensitisation following chronic treatment of rats with salbutamol related to the $\beta_2$ARs located on airway smooth muscle, whereas the biochemical studies were performed with lung parenchyma. To validate a link between the biochemical mechanisms observed in lungs to the functional observations in the airways, a limited number of *ex vivo* experiments were repeated using tracheal smooth muscle. The attenuation in the ability of salbutamol and $PGE_2$ to elevate intracellular cAMP in lung after acute treatment

of rats with salbutmol was also observed in trachea, together with down-regulation of membrane-associated $G_{s\alpha}$ expression. Although not conclusive, these data imply that the biochemical mechanisms highlighted in this chapter that are associated with desensitisation of the $\beta_2$AR signal transduction cascade, are responsible, at least in part, for the desensitisation of the bronchoprotective action of salbutamol and $PGE_2$ discussed in chapter 3.1.

In summary, the rat model of *in vivo* pulmonary $\beta_2$AR desensitisation was used to investigate the biochemical mechanisms that could be involved in this phenomenon. The data presented in this chapter demonstrate that chronic exposure of rats to salbutamol is associated with a significant loss in $\beta_2$AR binding sites on the surface of the cells, a reduction in $G_{s\alpha}$ and an associated uncoupling from AC. Together, these changes contributed to the impaired ability of salbutamol and $PGE_2$ to increase cAMP and activate PKA. Other aspects of the signal transduction cascade affected were PDE3 PDE4 isoenzymes, the activity of which were induced in rat lung after chronic salbutamol-treatment although this effect was not apparently functionally relevant at day 7 of treatment. There was also a significant increase in GRK2 activity associated with an increase in protein expression in salbutamol-treated rat lung, which may result in increased phosphorylation and internalisation of agonist-bound $\beta_2$ARs. However, while this process may have contributed to $\beta_2$AR desensitisation it can only account for homologous desensitisation as GRKs only phosphoryiate the agonist-occupied receptor. Thus, the most likely biochemical mechanism involved in attenuated $\beta_2$AR signalling and accounting for *in vivo* pulmonary heterologous desensitisation is the down-regulation of $G_{s\alpha}$. Figure 3.2.20 illustrates possible sites in the $\beta_2$AR signal transduction cascade where desensitisation can be effected.

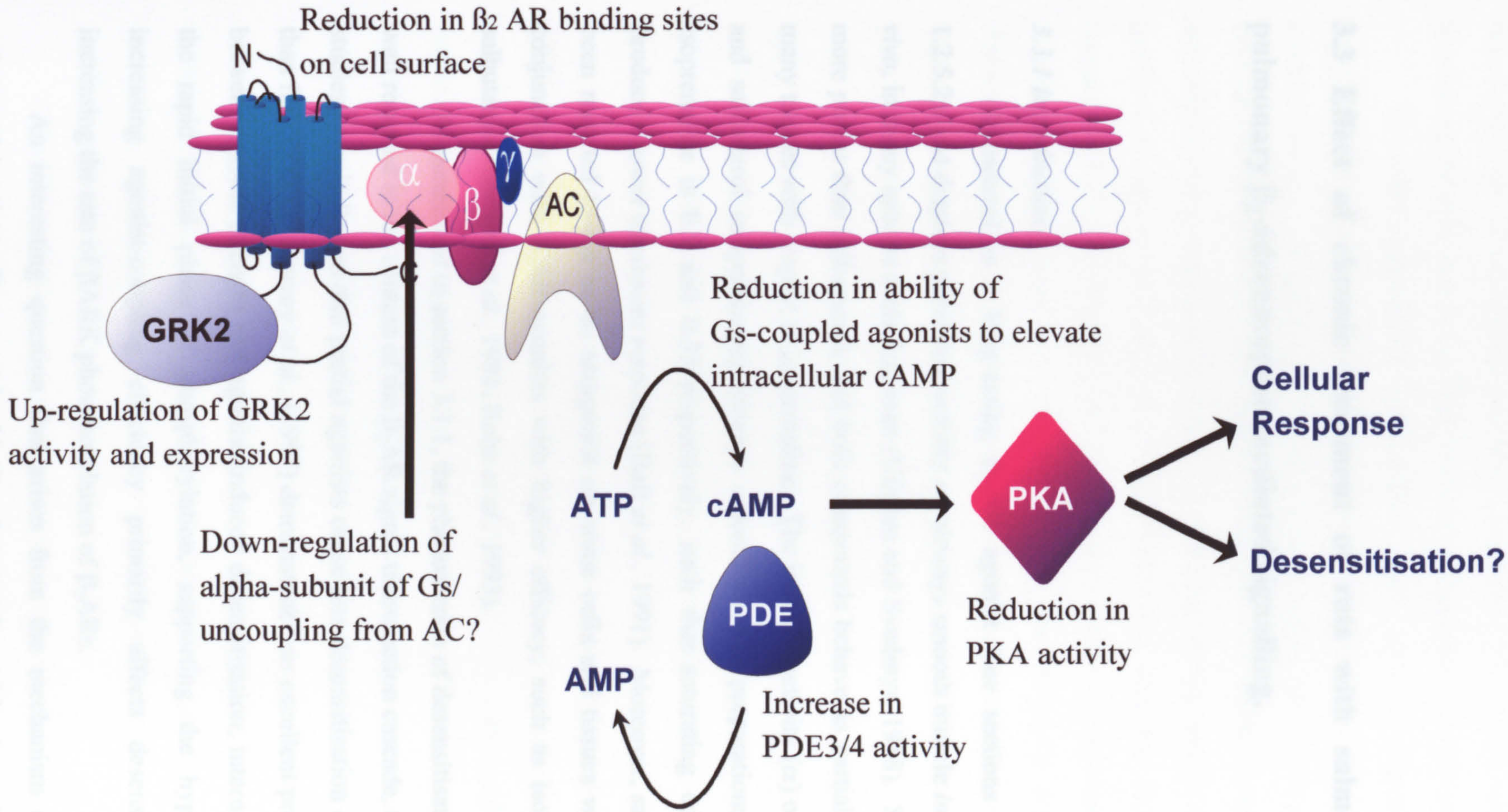



Figure 3.2.20 Biochemical effects of chronic treatment of rats with salbutamol on the β2-adrenoceptor signal transduction cascade *ex vivo*.

## 3.3 Effect of chronic treatment of rats with salmeterol on pulmonary $\beta_2$-adrenoceptor-mediated signalling.

### *3.3.1 Introduction.*

Salmeterol is a long-acting $\beta_2$-AR agonist (see sections 1.2.5.1 and 1.2.5.2), and displays persistent activity on airways smooth muscle *in vitro* and *in vivo*, in many species including man (Ullman and Svedmyr, 1988). Salmeterol is more potent than salbutamol, and both compounds behave as partial agonists on many tissues with respect to isoprenaline. The intrinsic activity ($\alpha$) of salbutamol and salmeterol in guinea-pig airways smooth muscle preparations relative to isoprenaline is 0.5 and 0.35 respectively, such that saturating concentrations produce a lower maximum response (Ball *et al.*, 1991). Moreover, salmeterol has been reported to act as an antagonist on some cells and tissues when used in conjunction with $\beta_2$AR-agonists with higher efficacy, such as isoprenaline or salbutamol (Dougall *et al.*, 1991; Rabe *et al.*, 1993).

As mentioned in section 3.1.1, the phenomenon of desensitisation has been well reported in the context of the $\beta_2$AR signal transduction cascade, and previous studies have indicated that partial agonists cause less desensitisation of the $\beta_2$-AR than full agonists. January *et al.*, (1997) demonstrated an excellent proportionality between agonist strength and agonist-induced desensitisation, internalisation and the rapid initial phase of phosphorylation, supporting the hypothesis that increasing agonist-coupling efficiency primarily affects desensitisation by increasing the rate of $\beta$ARK phosphorylation of $\beta_2$ARs.

An interesting question that arises from the mechanism of action of salmeterol is what effects exosite binding has on desensitisation. Does the fact

that the active head of salmeterol, which is in close proximity to the agonist site of the receptor for a longer period of time than shorter acting $\beta_2$AR agonists, mean that there is an increased risk of desensitisation despite its lower efficacy.

As with salbutamol, and other $\beta_2$AR agonists, agonist-mediated desensitisation has been studied extensively *in vitro* and the biochemical mechanisms are well defined. Although clinical studies have been performed to evaluate the safety of salmeterol, and work has been undertaken to assess both the anti-inflammatory properties of salmeterol in animal models and the desensitising potential of salmeterol in cell lines, there have been very few biochemical or functional studies investigating the consequences of chronic exposure of animals or humans to salmeterol *in vivo*. To this end, the rat model developed and discussed in section 2.1.12 was used to determine the effect of salmeterol, given chronically, to rats on $\beta_2$AR signalling *in vivo* and some of the *ex vivo* markers described in section 3.2.

### *3.3.2 Effect of chronic treatment of rats with salmeterol on the ability of salmeterol and $PGE_2$, given acutely, to protect against ACh-induced bronchoconstriction.*

The ability of salmeterol (100μg/kg i.v.) to protect the airways against ACh-induced bronchoconstriction in vehicle- and salmeterol-treated rats was examined (see Figure 3.3.1 for representative traces). In salmeterol-treated rats, administration of salmeterol had no significant effect on ACh-induced bronchoconstriction (Table 3.3; Figure 3.3.2) whereas in vehicle-treated animals salmeterol reduced ACh-induced bronchoconstriction by 44% (Fig 3.3.2; Table 3.3). These data demonstrate that a functional *in vivo* desensitisation had occurred. Unexpectedly, the maximum protection afforded by salmeterol in vehicle-treated rats persisted only for 10 minutes before lung function began to recover ($t_{1/2}$ offset ~25min). In other words, salmeterol behaved as a short-acting $\beta_2$AR agonist (see Fig 3.3.3 section 3.3.4).

The effect of $PGE_2$ was also examined in vehicle- and salmeterol-treated rats to determine whether the functional desensitisation was homologous or

heterologous (Figure 3.3.4). In salmeterol-treated rats, administration of $PGE_2$ had no effect on ACh-induced bronchoconstriction but significantly protected (by ~33%) the airways against ACh-mediated bronchoconstriction in vehicle-treated rats (Figure 3.3.4). These results thus, indicate that the functional desensitisation observed in salmeterol-treated rats is heterologous in nature.

**Table 3.3:** Summary of the *in vivo* bronchoprotection investigation using rats treated chronically with vehicle and salmeterol.

| **Acute Treatment Group** | **Vehicle-Treated** | | | **Salmeterol-Treated** | | |
|---|---|---|---|---|---|---|
| | Pre- ($cm\ H_2O$) | Post- ($cm\ H_2O$) | Percentage Protection | Pre- ($cm\ H_2O$) | Post- ($cm\ H_2O$) | Percentage Protection |
| Salmeterol | 9.3 ± 0.2 | 5.6 ± 0.8* | 43.5 ± 8.6 | 11.3 ± 1.7 | 10 ± 1.2 | 11.2 ± 3.6 |
| $PGE_2$ | 10.3 ± 1.1 | 7 ± 1.1* | 32.3 ± 3.8 | 12.3 ± 0.9 | 12.3 ± 1.1 | 6.4 ± 3.7 |
| Salbutamol (+) | - | - | - | 11.9 ± 1.6 | 10.8 ± 1.4 | 9 ± 3.6 |
| Salbutamol (-) | - | - | - | 11 ± 0.8 | 8.6 ± 0.8 | 22.3 ± 2.6 |

Shown is the bronchoconstriction (in $cm\ H_2O$) obtained upon ACh-exposure and the percentage protection afforded by the respective drug. (+) and (-) indicate the presence or absence of mini-osmotic pump in the animal during experiment respectively. Data represents the mean ± s.e.m. of four determinations from different rats. * $P<0.05$ significant protection against ACh-induced bronchoconstriction, Student's paired t-test.

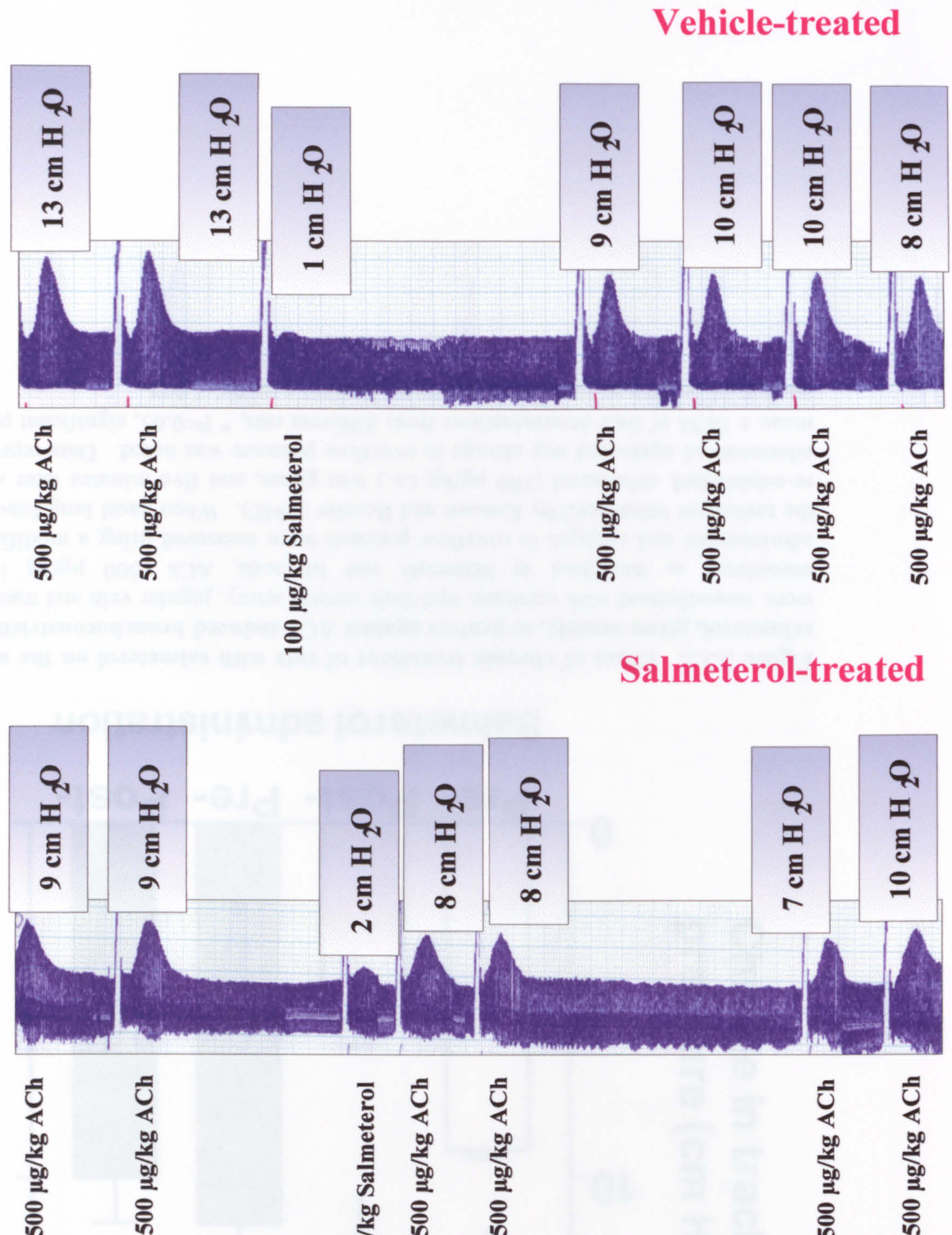



**Figure 3.3.1** Represenative trace of airway resistance experiments. Rats were anaesthetised with urethane, and their carotid artery, jugular vein and trachea were cannulated as described in Materials and methods. ACh (500 µg/kg i.v.) was administered and changes in overflow pressure were measured using a modification of the technique introduced by Konsett and Rossler (1940). When basal lung function was re-established, salmeterol (100 µg/kg i.v.) was given, and five munutes later ACh was administered again and any change in overflow pressure was noted.

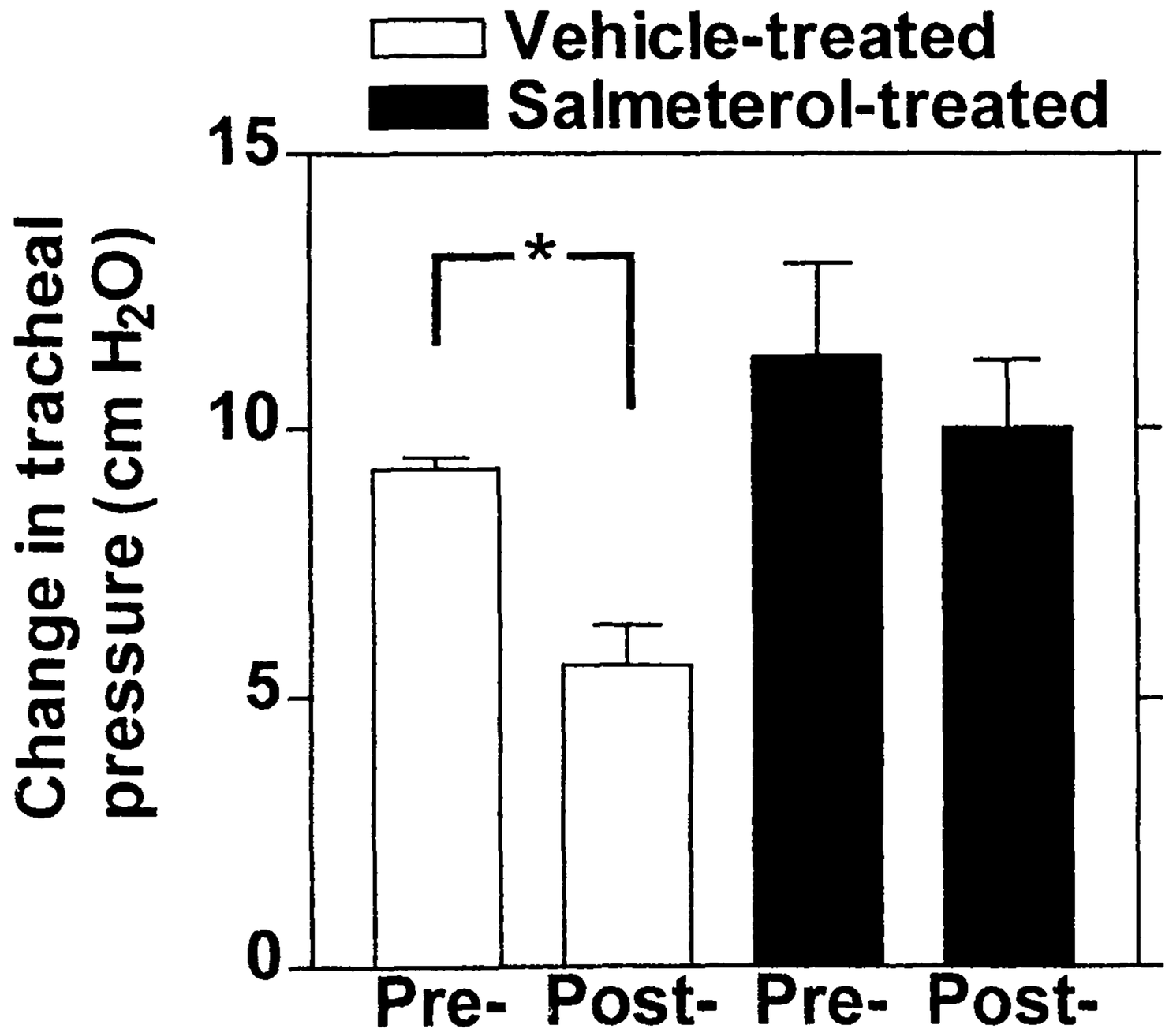



**Figure 3.3.2. Effect of chronic treatment of rats with salmeterol on the ability of salmeterol, given acutely, to protect against ACh-induced bronchoconstriction.** Rats were anaesthetised with urethane, and their carotid artery, jugular vein and trachea were cannulated as described in Materials and Methods. ACh (500 μg/kg i.v.) was administered and changes in overflow pressure were measured using a modification of the technique introduced by Konsett and Rossler (1940). When basal lung function was re-established, salmeterol (100 μg/kg i.v.) was given, and five minutes later ACh was administered again and any change in overflow pressure was noted. Data represent the mean ± SEM of four determinations from different rats, * $P<0.05$, significant protection against ACh-induced bronchoconstriction, Student's paired T-test.

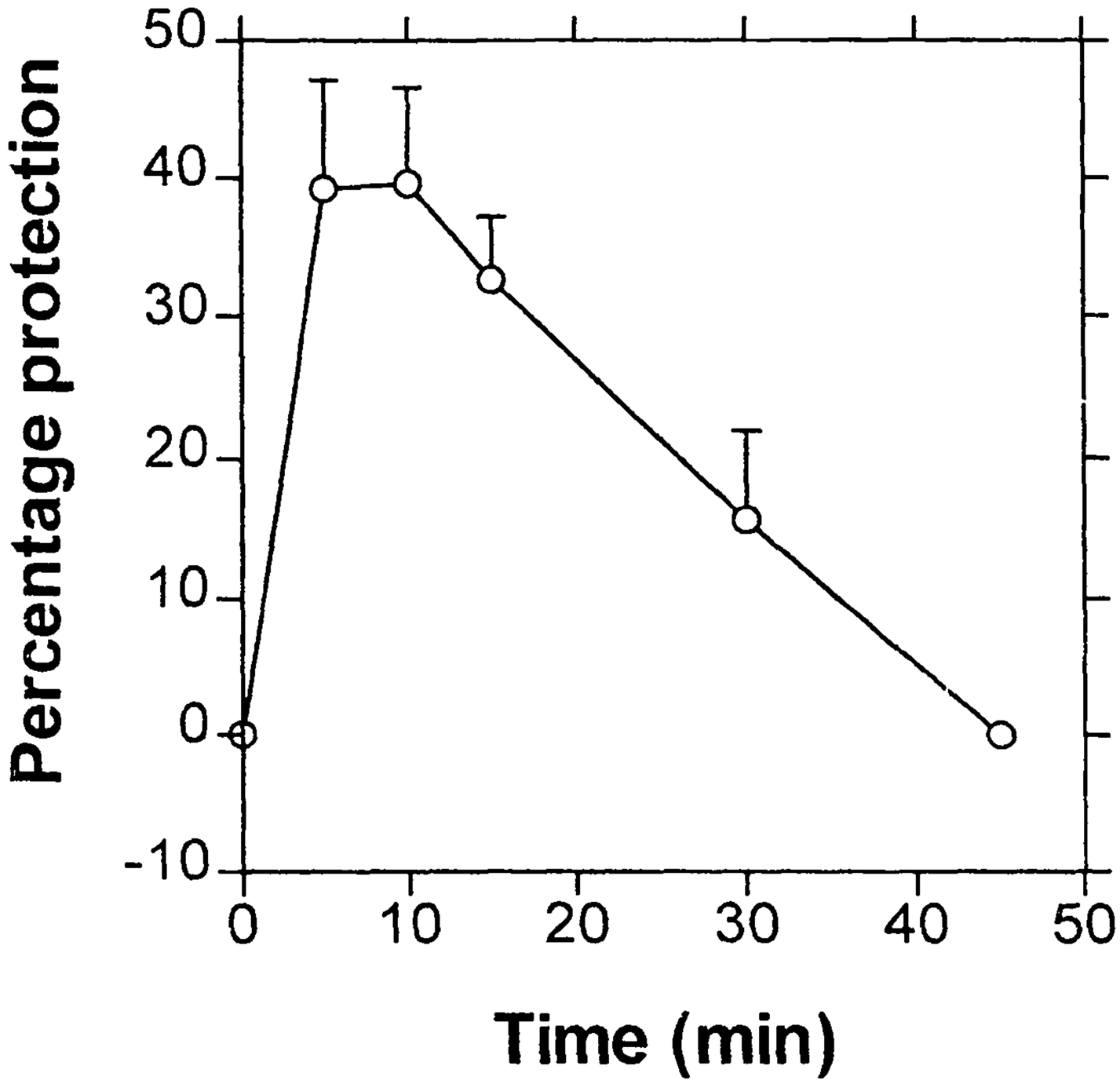



**Figure 3.3.3. Time-response of salmeterol-mediated protection against ACh-induced bronchoconstriction.** Rats were anaesthetised with urethane, and their carotid artery, jugular vein, and trachea were cannulated as described in Materials and Methods. ACh (500 µg/kg i.v.) was administered and changes in overflow pressure were measured using a modification of the technique introduced by Konsett and Rossler (1940). When basal lung function was re-established, salmeterol (100 µg/kg i.v.) was given, and five minutes later ACh was administered again and any change in overflow pressure was noted. ACh was administered at further time points until the spasmogenic response had returned to original value.

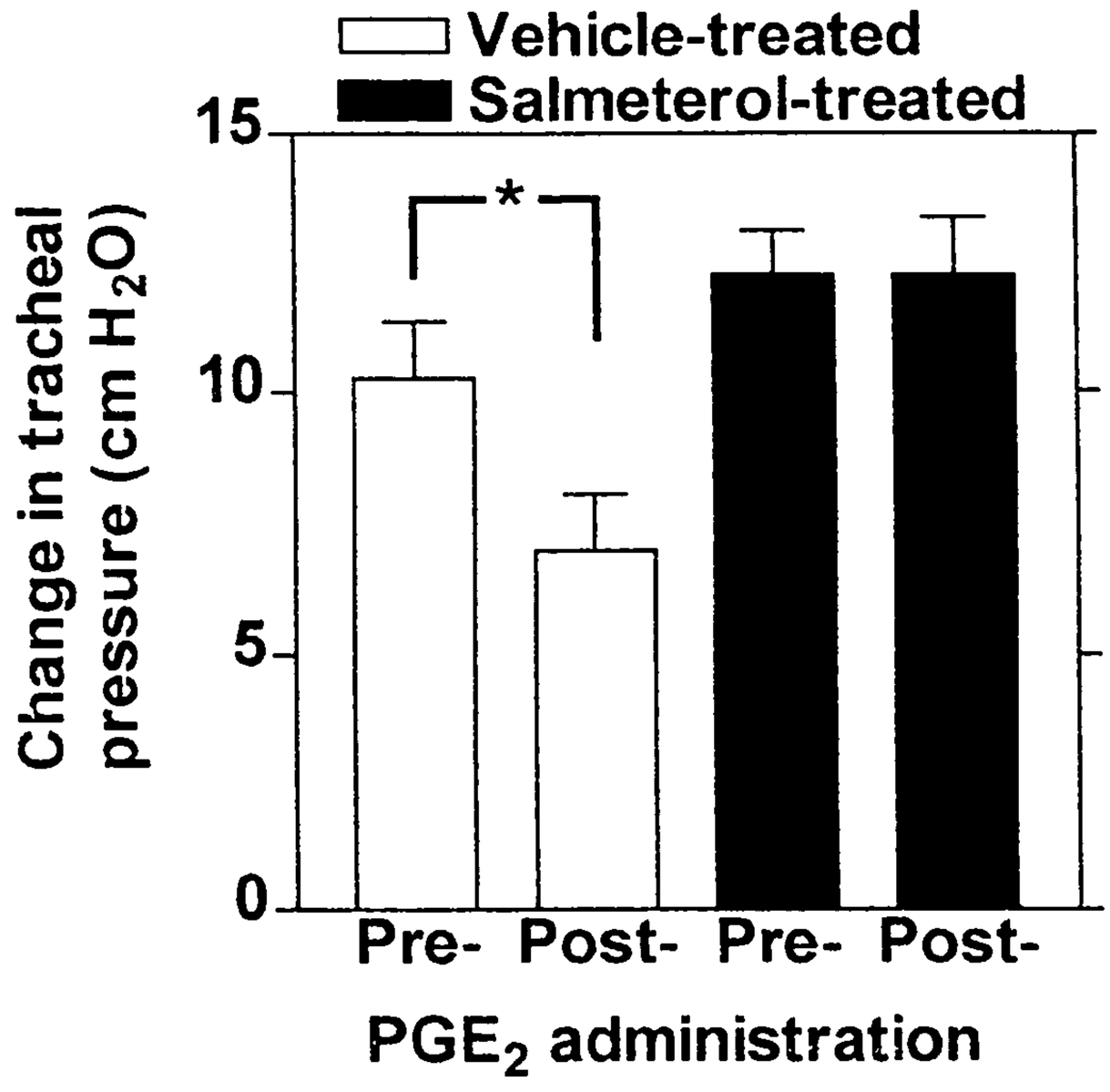



**Figure 3.3.4. Effect of chronic treatment of rats with salmeterol on the ability of $PGE_2$, given acutely, to protect against ACh-induced bronchoconstriction.** Rats were anaesthetised with urethane, and their carotid artery, jugular vein, and trachea were cannulated as described in Materials and Methods. ACh (500 μg/kg i.v.) was administered and changes in overflow pressure were measured using a modification of the technique introduced by Konsett and Rossler (1940). When basal lung function was re-established, $PGE_2$ (300 μg/kg i.v.) was given, and five minutes later ACh was administered again and any change in overflow pressure was noted. Data represent the mean ± SEM of four determinations from different rats, *$P<0.05$, significant protection against ACh-induced bronchoconstriction, Student's paired T-test.

*3.3.3.1 Effect of chronic treatment of rats with salmeterol on the ability of salbutamol, given acutely, to protect against ACh-induced bronchoconstriction.*

The ability of salbutamol (100μg/kg i.v.) to protect the airways against ACh-induced bronchoconstriction in vehicle- and salmeterol-treated rats was examined to determine if an agonist with higher efficacy could overcome the desensitisation produced by salmeterol. In salmeterol-treated rats, with the osmotic mini-pumps still implanted, salbutamol had no significant effect on ACh-induced bronchoconstriction (See Table 3.3, Figure 3.3.5). Similarly, when the mini-pumps were surgically removed from the rats, and after 1h "clearance" time

(an acute i.v. bolus of salmeterol lasted ~35 min in a vehicle-treated rat and, 1 h was considered sufficient to clear any residual salmeterol from the osmotic mini-pump), salbutamol still failed to protect the airways against ACh-induced bronchoconstriction (Table 3.3, Figure 3.3.5). These data indicate that salbutamol could not overcome the desensitisation effected by salmeterol despite being of higher efficacy.

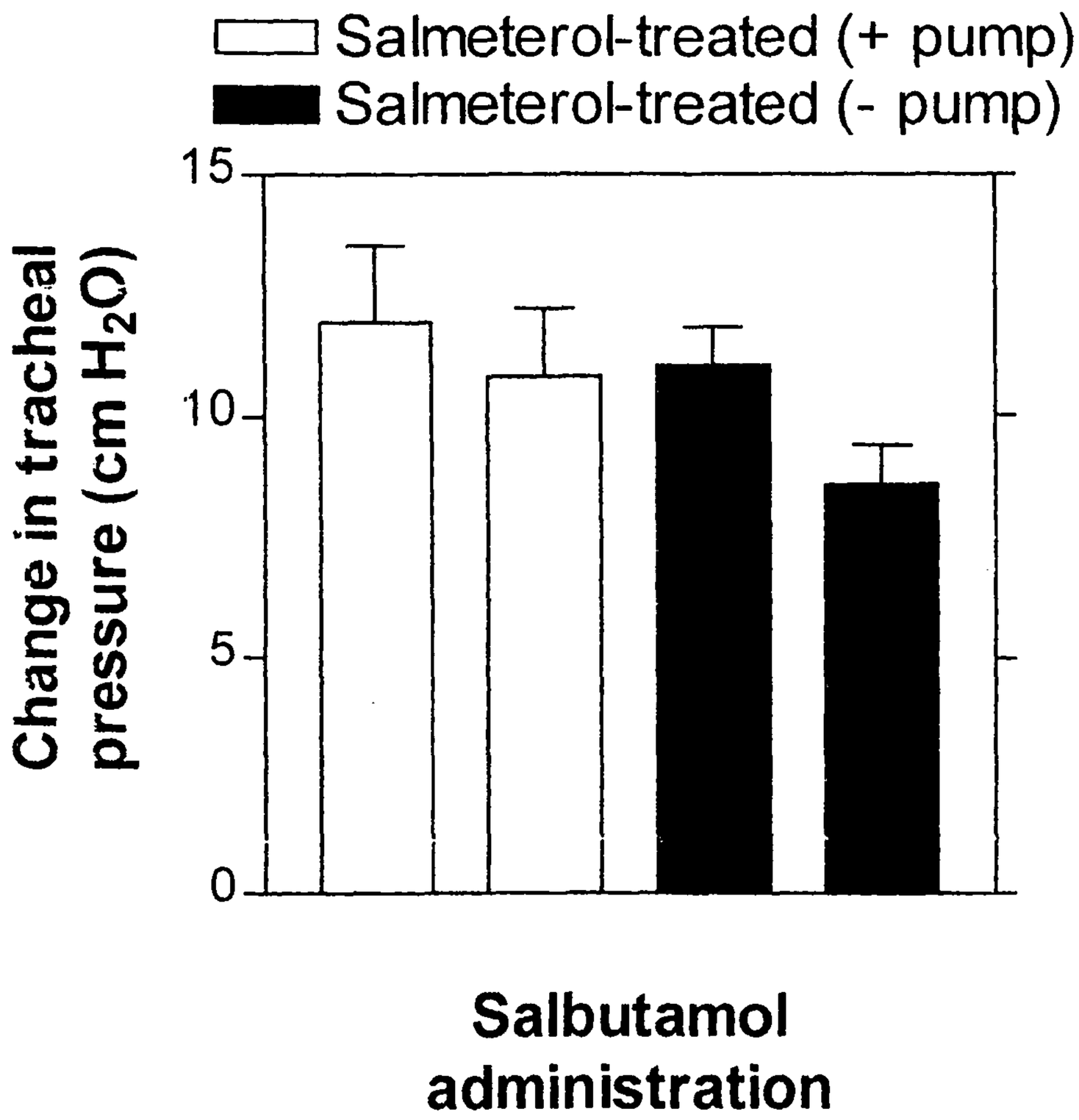



**Figure 3.3.5. Effect of chronic treatment of rats with salmeterol on the ability of salbutamol, given acutely, to protect against ACh-induced bronchoconstriction.** Rats were anaesthetised with urethane, and their carotid artery, jugular vein and trachea were cannulated as described in Materials and Methods. ACh (500 μg/kg i.v.) was administered and changes in overflow pressure were measured using a modification of the technique introduced by Konsett and Rossler (1940). When basal lung function was re-established, salbutamol (100 μg/kg i.v.) was given, and five minutes later ACh was administered again and any change in overflow pressure was noted. Data represent the mean ± SEM of four determinations from different rats, * $P<0.05$, significant protection against ACh-induced bronchoconstriction, Student's t-test.

*3.3.3.2 On the mechanism of the short-acting behaviour of salmeterol.*

A logical explanation of the short-acting behaviour of salmeterol (see section 3.3.2 and Figure 3.3.3) when given i.v. to rats is rapid metabolism to a shorter-acting metabolite that retains affinity and efficacy at βARs. This was investigated by giving rats a bolus of salmeterol and looking for potential metabolites (see section 2.1.6). Unfortunately, the concentration of metabolites in the circulation was very low, and none of them could be detected quantitatively. Consequently, a more sensitive method of selected ion monitoring was used, which involved choosing a limited number of ions for detection rather than scanning over a wide range. The ions corresponding to salmeterol and four of its known metabolites (GR72438 and GR37052 [both products of O-dealkylation], the phenolic glucuronide product and the aliphatic oxidation product) in the rat were selected. Again, due to the limited volume of sample, the results obtained were qualitative. However, salmeterol and a peak consistent with the alcohol formed by O-dealkylation with loss of butyl benzene was observed. The peak areas recorded for the 5 and 20 min samples indicated that the concentration of circulating salmeterol dropped rapidly; the peak areas more than halved in this time. This was probably due to the drug being both rapidly metabolised and being distributed into tissues. The volume of distribution of salmeterol has been recorded as 42 ml/min/kg suggesting that the compound was widely distributed into tissues (Manchee *et al.*, 1993).

*3.3.4 β-Adrenoceptor density and mRNA expression.*

One explanation for the loss in protection by salmeterol against ACh-induced bronchoconstriction is down-regulation of $\beta_2$AR number. Indeed, chronic treatment of rats with salmeterol effected a significant reduction (69%) in the density of $\beta_2$ARs in lung parenchyma (Figures 3.3.6, and 3.3.8), without a significant change in the affinity of [$^{125}$I]ICYP (Table 3.4). Unexpectedly, the $\beta_1$-subtype was also down-regulated by ~49% in the salmeterol-treated group without a significant change in the affinity if [$^{125}$I]ICYP (Figures 3.3.6 and 3.3.7; Table 3.4).

*In vitro* experiments were also performed on lung parenchyma to assess whether chronic treatment of rats with salmeterol affected the steady-state level of βAR mRNAs in lung. After the 7 day treatment period, the steady-state $\beta_1$- and $\beta_2$AR mRNA level, expressed as ratio to the housekeeping gene GAPDH, was unchanged in lung taken from desensitised rats when compared to vehicle-treated animals (Figure 3.3.9).

**Table 3.3.4. Effect of chronic treatment of rats with salmeterol on the density of cell surface β-adrenoceptors on rat lung membranes and on the affinity of [$^{125}$I] ICYP.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle or salmeterol (10 μg/kg/hr). After seven days, lung membranes were prepared and total, $\beta_1$- and $\beta_2$AR density and the affinity of [$^{125}$I]ICYP was determined as described in section 2.2. Data represent the mean ± s.e.m of eight independent observations.

| | Total βARs | | $\beta_1$ARs* | | $\beta_2$ARs** | |
|---|---|---|---|---|---|---|
| | Kd (pM) | Bmax (fmol/mg protein) | Kd (pM) | Bmax (fmol/mg protein) | Kd (pM) | Bmax (fmol/mg protein) |
| Vehicle | 5.5 ± 2.5 | 119 ± 14 | 24.2 ± 4.3 | 61 ± 7 | 4.9 ± 0.7 | 94 ± 10 |
| Salmeterol | 5.8 ± 0.7 | 36 ± 8† | 39.2 ± 11.3 | 31 ± 7+ | 5.9 ± 0.8 | 29 ± 6† |

†, $P < 0.01$, significant reduction in Bmax compared to vehicle-treated rats, Student's paired t-test.
+, $P < 0.05$, significant reduction in Bmax compared to vehicle-treated rats, Student's paired t-test.
*$\beta_1$AR subtype defined in the presence of the selective $\beta_2$AR antagonist, ICI 118551 (0.1 μM)
**$\beta_2$AR subtype defined in the presence of the selective $\beta_1$AR antagonist, CGP 20712A (0.1 μM).

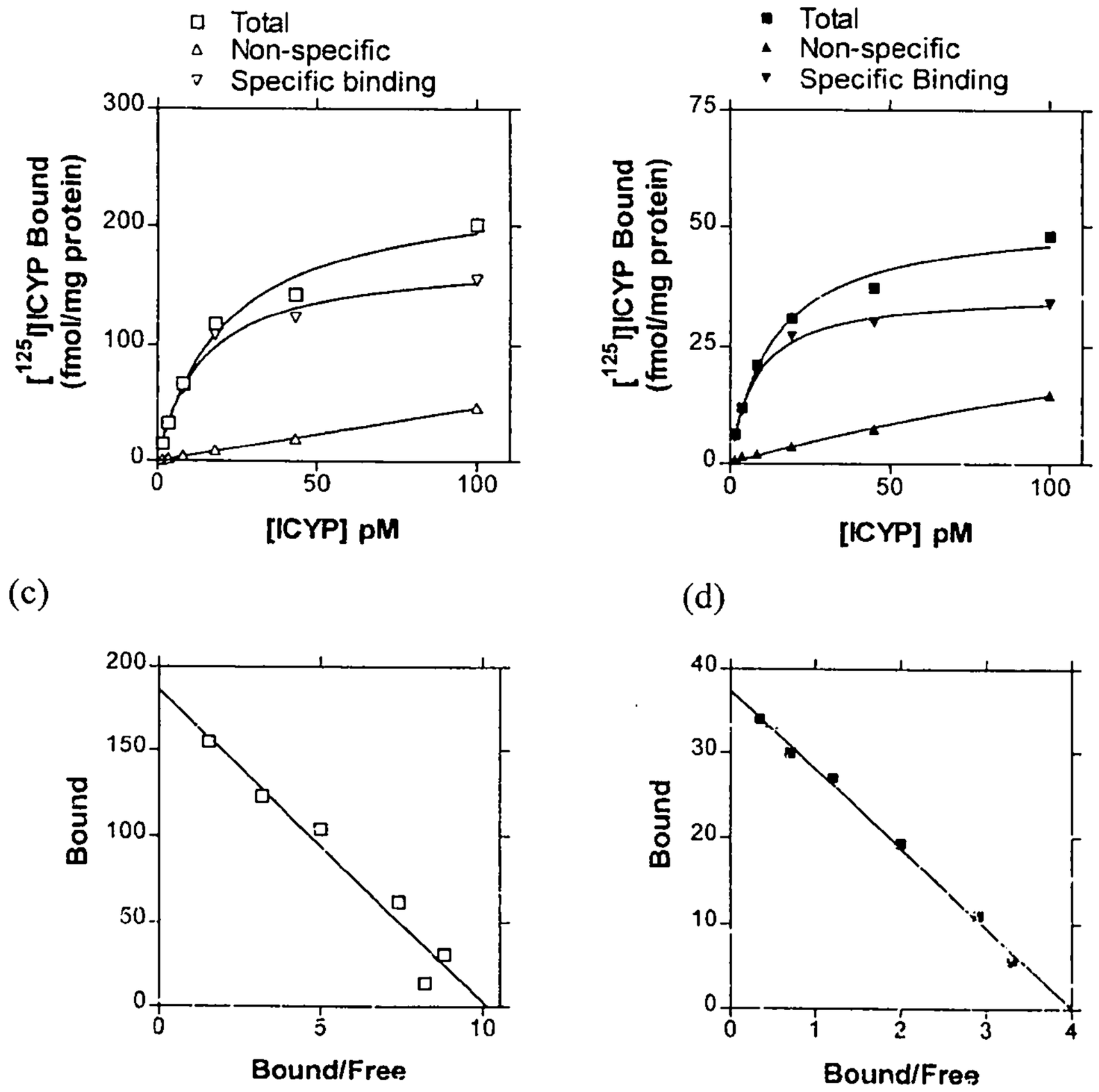



**Figure 3.3.6. Binding of [$^{125}$I]ICYP to total β-adrenoceptors in lung membranes prepared from vehicle- and salmeterol-treated rats.** Panels (a) and (b) show representative saturation isotherms for [$^{125}$I]ICYP binding to the lung of vehicle- (open symbols) and salmeterol-treated (closed symbols) rats respectively. Experiments were performed at 37°C in 25 mM Tris buffer containing 154 mM NaCl and 1 mM ascorbic acid (pH 7.4) using a protein concentration of 10 µg/tube. Non-specific binding was defined with 200 µM isoprenaline. Panels (c) and (d) show Scatchard transformations of the data in (a) and (b) respectively. Results are representative of eight determinations using tissue from different animals. See section 2.2 for further details.

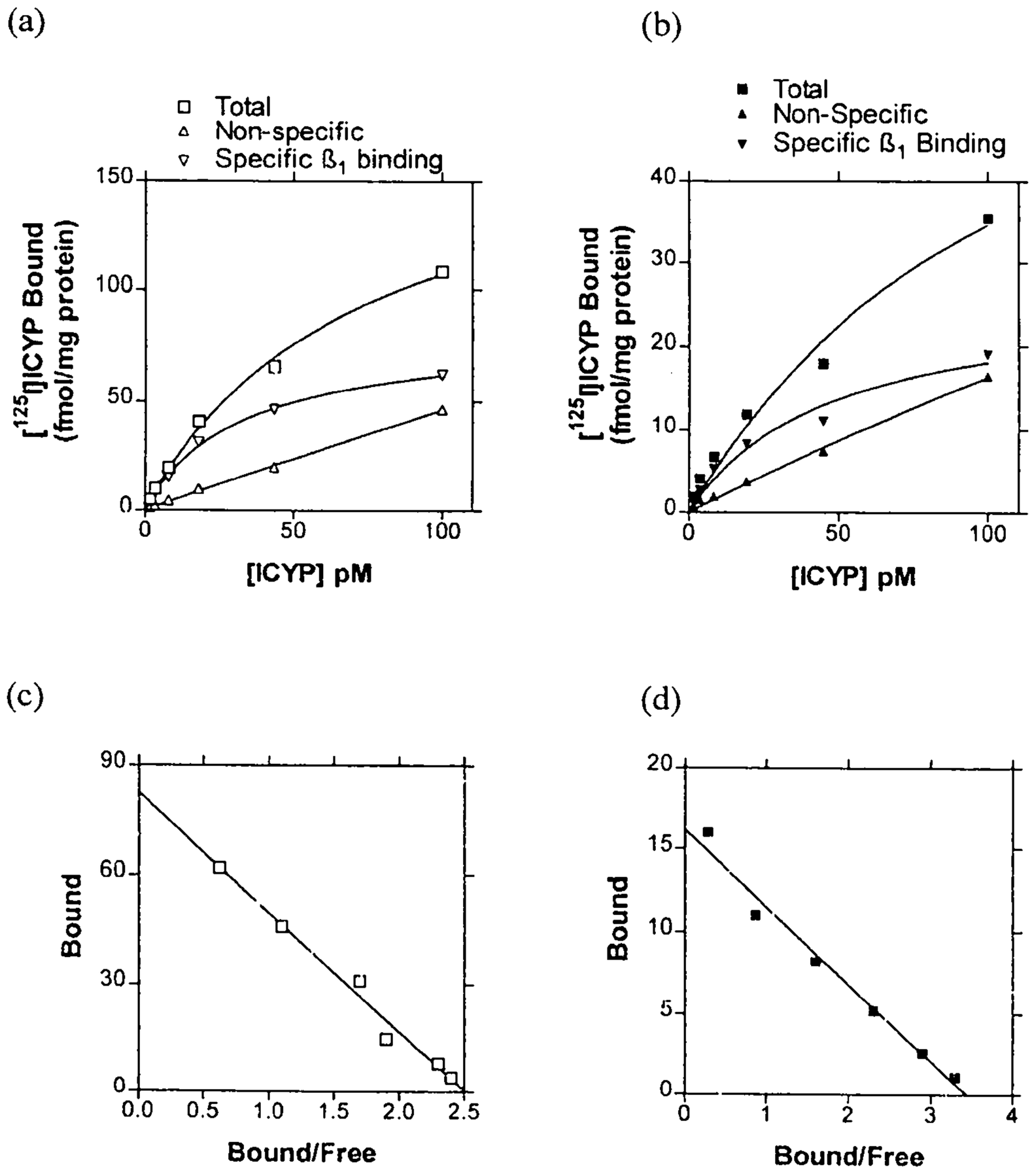



**Figure 3.3.7. Binding of [$^{125}$I]ICYP to $\beta_1$-adrenoceptors in lung membranes prepared from vehicle- and salmeterol-treated rats.** Panels (a) and (b) show representative saturation isotherms for [$^{125}$I]ICYP binding to the lung of vehicle- (open symbols) and salmeterol-treated (closed symbols) rats respectively. Experiments were performed at 37°C in 25 mM Tris buffer containing 154 mM NaCl and 1 mM ascorbic acid (pH 7.4) using a protein concentration of 10 µg/tube. The density of $\beta_1$ARs was analysed by [$^{125}$I]ICYP binding in the presence of 0.1 µM ICI 118551. Non-specific binding was defined with 200 µM isoprenaline. Panels (c) and (d) show Scatchard transformations of the data in (a) and (b) respectively. Results are representative of eight determinations using tissue from different animals. See section 2.2 for further details.

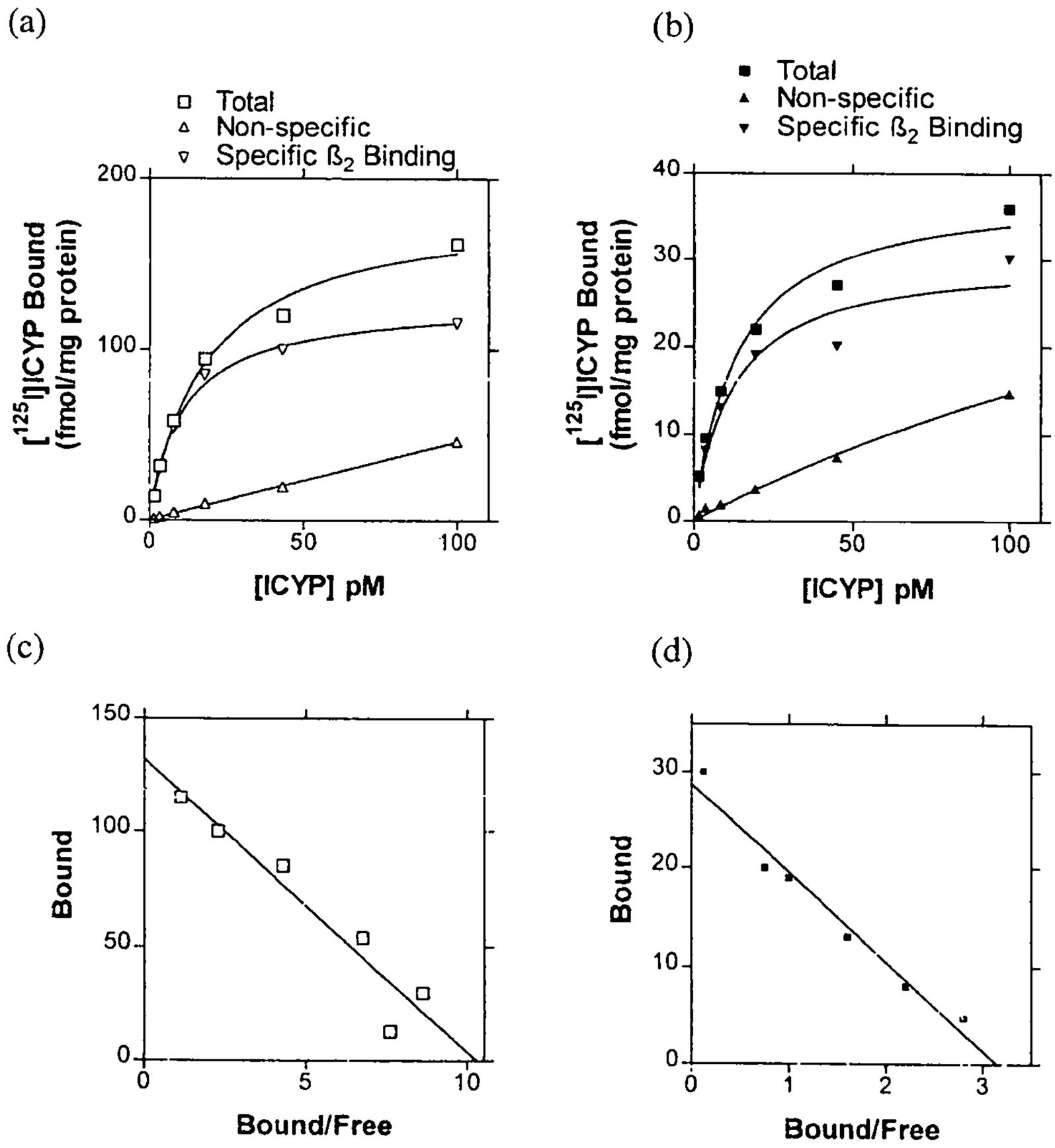



**Figure 3.3.8. Binding of [$^{125}$I]ICYP to $\beta_2$-adrenoceptors in lung membranes prepared from vehicle- and salmeterol-treated rats.** Panels (a) and (b) show representative saturation isotherms for [$^{125}$I]ICYP binding to the lung of vehicle- (open symbols) and salmeterol-treated (closed symbols) rats respectively. Experiments were performed at 37°C in 25 mM Tris buffer containing 154 mM NaCl and 1 mM ascorbic acid (pH 7.4) using a protein concentration of 10 µg/tube. The density of $\beta_2$ARs was analysed by [$^{125}$I]ICYP binding in the presence of 0.1 µM CGP 20712 A. Non-specific binding was defined with 200 µM isoprenaline. Panels (c) and (d) show Scatchard transformations of the data in (a) and (b) respectively. Results are representative of eight determinations using tissue from different animals. See section 2.2 for further details.

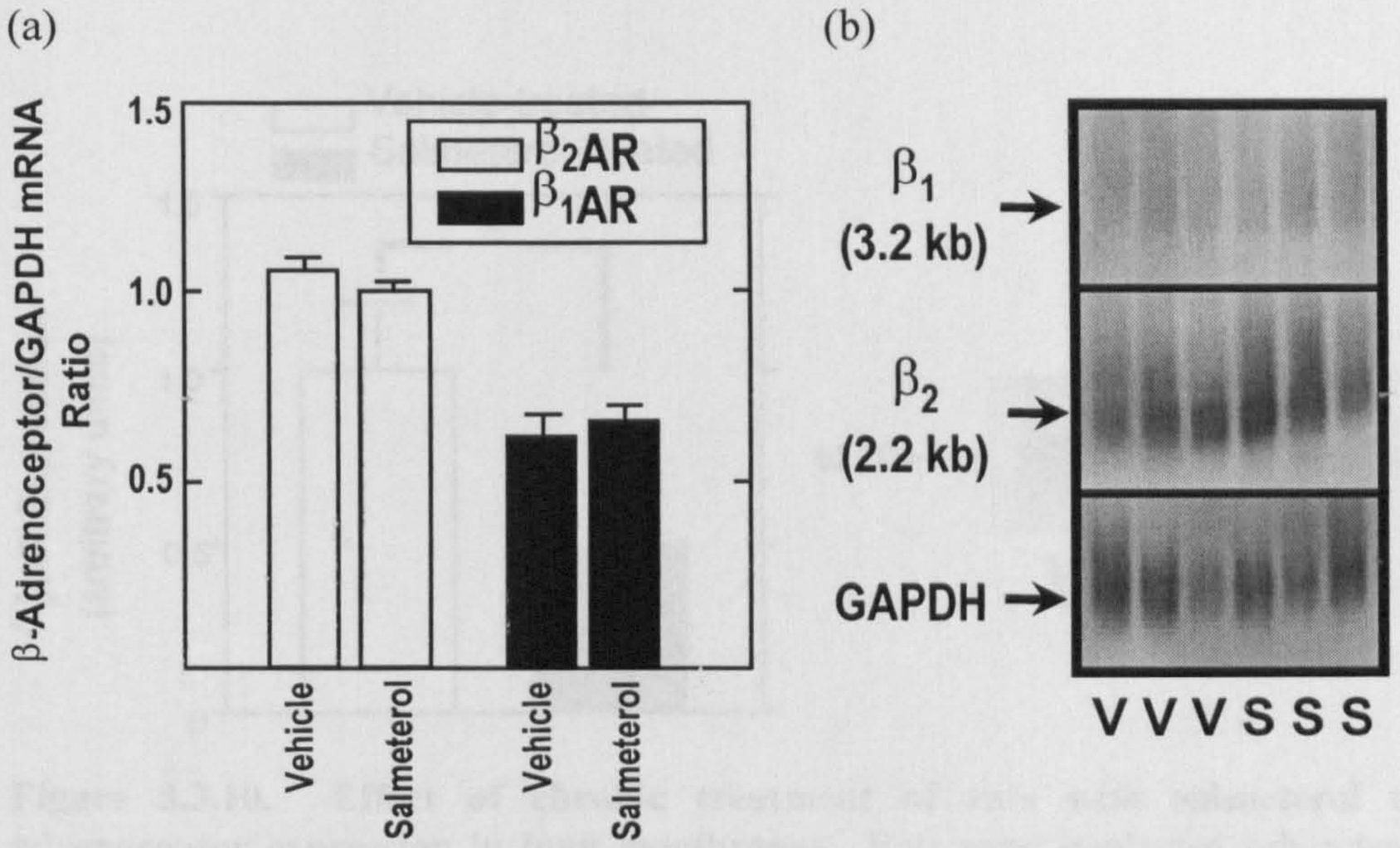



**Figure 3.3.9. Effect of chronic treatment of rats with salmeterol on the steady-state levels of $\beta_1$- and $\beta_2$-adrenoceptor mRNA.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering salmeterol (10 μg/kg/h) or vehicle (1 x PBS/0.1% glacial acetic acid). After seven days, lung was excised and $\beta_1$- and $\beta_2$AR mRNA was extracted and levels estimated by Northern analysis. Panel (a) shows the mean results of six independent determinations taken from different animals and are expressed relative to the "house-keeping" gene GAPDH. In panel (b), a representative autoradiogram is shown where S and V refer to salmeterol- and vehicle-treated animals respectively.

*3.3.5 Effect of chronic treatment of rats with salmeterol on the expression of $\beta_2$-adrenoceptor protein measured by western blotting.*

To determine whether the binding of salmeterol to an "exosite" within the $\beta_2$AR could have affected the radioligand binding results described above, western blotting was performed using an antibody that recognises the $\beta_2$AR protein. After 7 days of treatment with salmeterol there was a significant reduction in pulmonary $\beta_2$AR protein expression when compared to tissue harvested from vehicle-treated rats (Figure 3.3.10). Although these data do not address whether exosite binding of salmeterol changes the conformation of the receptor to affect the binding of [$^{125}$I]ICYP, the reduction in receptor protein suggests that the decrease in $\beta_2$AR binding sites detected with [$^{125}$I]ICYP is a true observation.

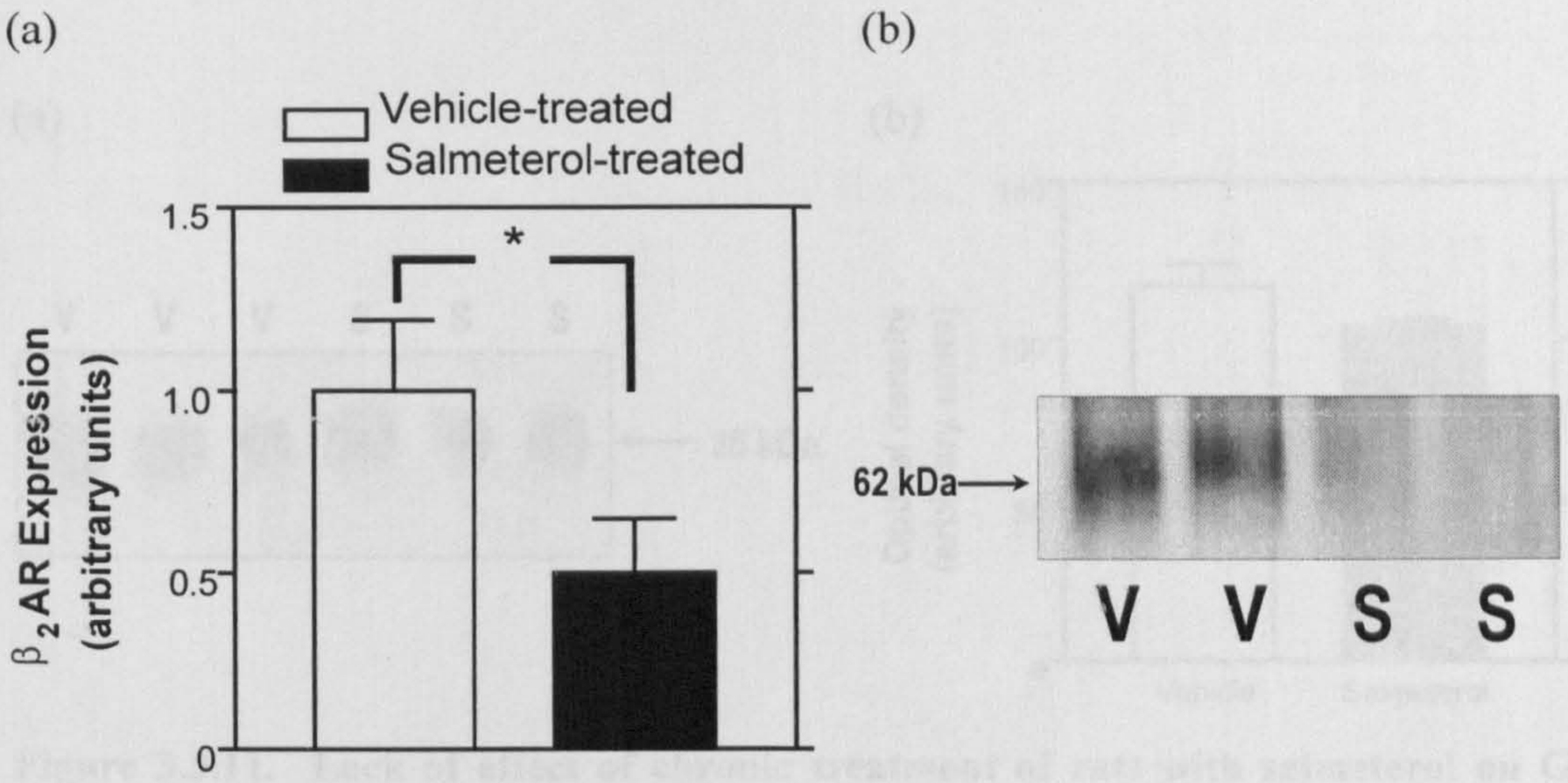



**Figure 3.3.10. Effect of chronic treatment of rats with salmeterol on $\beta_2$-adrenoceptor expression in lung membranes.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle or salmeterol (10 μg/kg/h). After seven days, animals were sacrificed and the expression of $\beta_2$ARs was determined by western blotting as described in section 2.6. Panels (a) and (b) show the mean results (± s.e.m. of six independent determinations from different animals) and representative blots respectively. S and V refer to animals treated with salmeterol or vehicle respectively. *P<0.05, significant reduction in $\beta_2$-adrenoceptor expression, Student's unpaired T-test.

### *3.3.6 Effect of chronic treatment of rats with salmeterol on the expression of $G_{s\alpha}$ and $G_\beta$.*

Western blotting was performed on lung proteins extracted from salmeterol-treated rats to assess whether the functional desensitisation was associated with a reduction in expression of either of the $G_{s\alpha}$ or $G_\beta$ subunits. Using a primary antibody that recognises an epitope on the C-terminus of the common $G_\beta$-subunit, a 35 kDa band was labelled, consistent with the reported size of $G_\beta$. Treatment of rats for one week with salmeterol did not alter the expression of $G_\beta$ (Figure 3.3.11).

In similar experiments, a primary antibody raised against the C-terminal region of $G_{s\alpha}$ labelled 3 bands in the lung of vehicle-treated rats of 42, 44 and 52 kDa (see section 3.2.4) (Figure 3.3.12). After treatment of rats with salmeterol for seven days, expression of each variant was significantly (~40-70%) reduced (Figure 3.2.12).

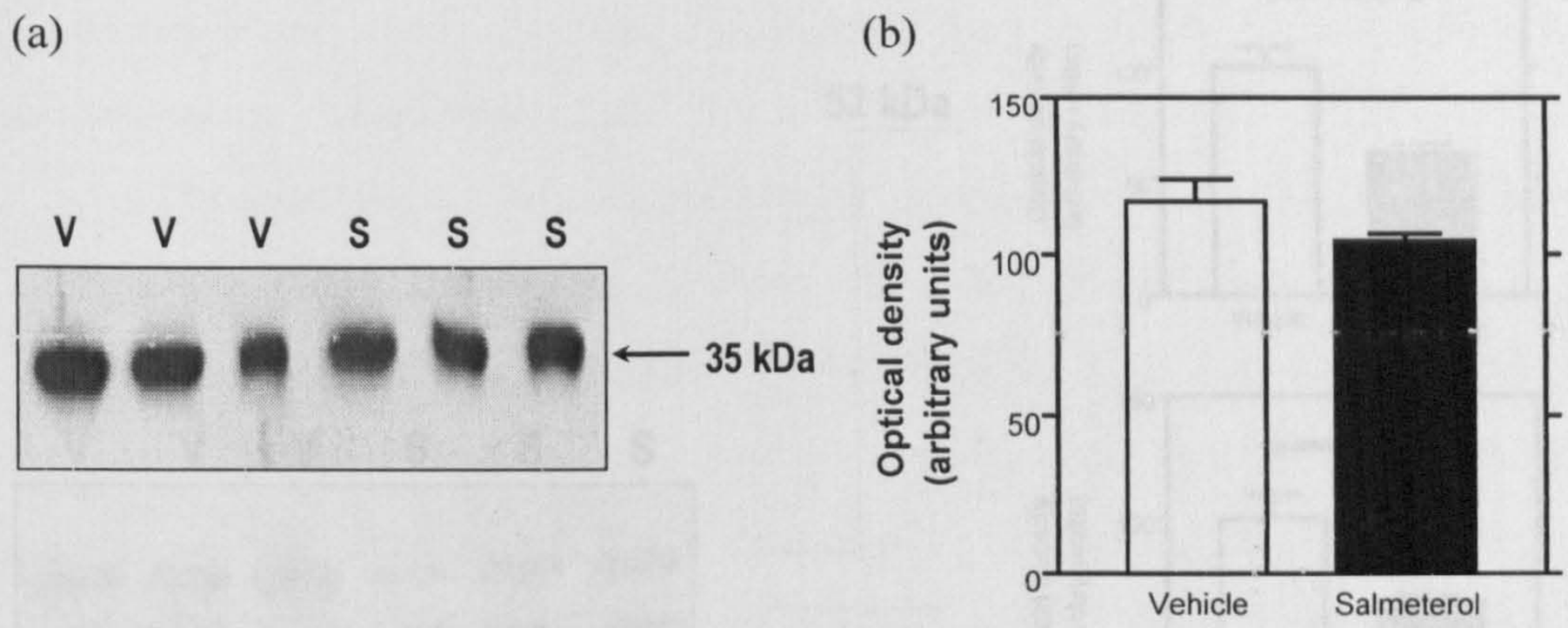



**Figure 3.3.11. Lack of effect of chronic treatment of rats with salmeterol on $G_\beta$ expression in lung membranes.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle or salmeterol (10 µg/kg/h) After seven days, animals were sacrificed and the expression of membrane-associated $G_\beta$ subunits was determined by western blotting as described in section 2.6. Panels (a) and (b) show representative western blots and the mean results (± s.e.m. of nine independent determinations from different animals) respectively. S and V refer to animals treated with salmeterol or vehicle respectively.

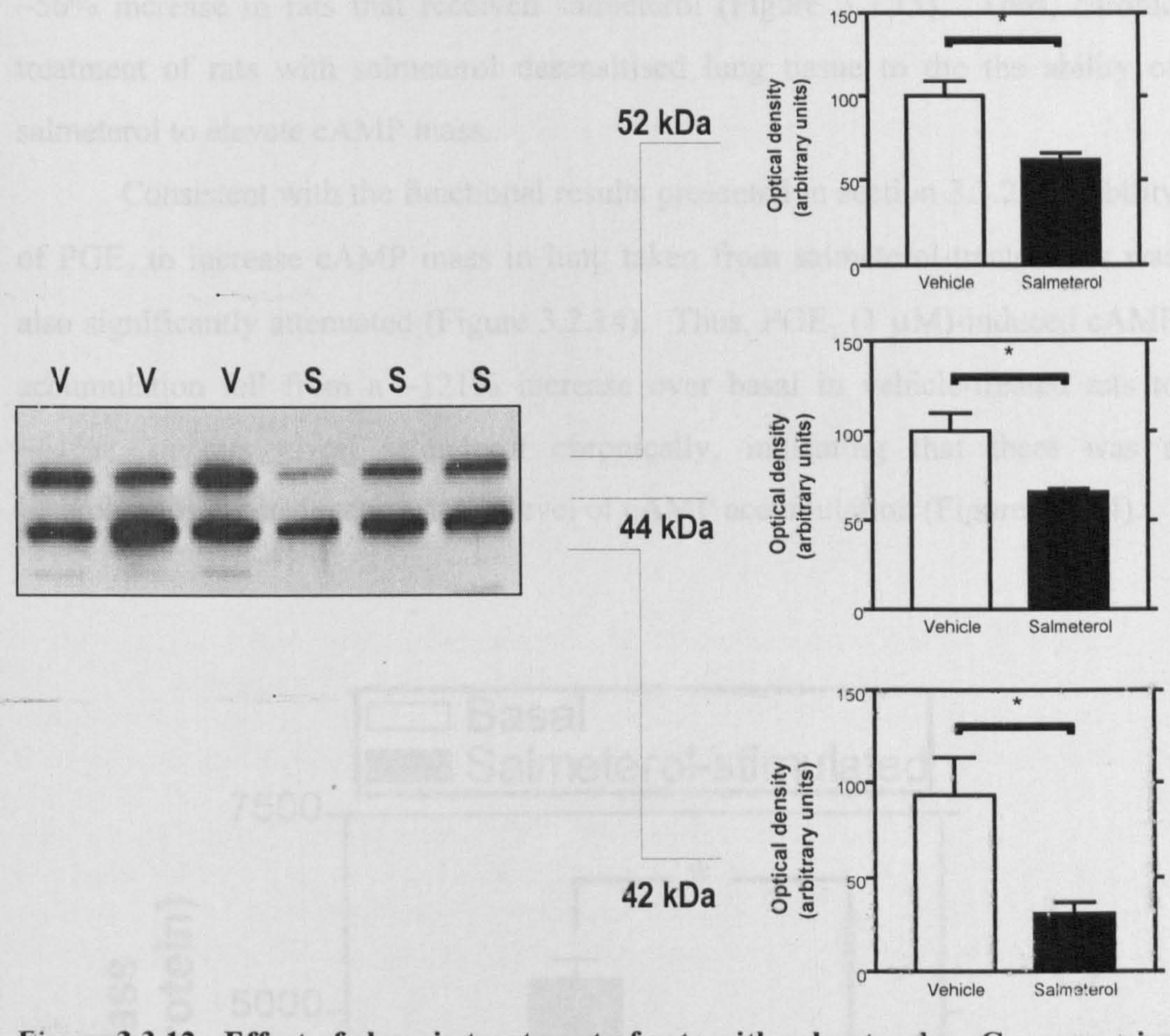



**Figure 3.3.12. Effect of chronic treatment of rats with salmeterol on $G_{s\alpha}$ expression in lung membranes.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle or salbutamol (10 μg/kg/h). After seven days, animals were sacrificed and the expression of membrane-associated $G_{s\alpha}$ subunits was determined by western blotting as described in section 2.6. Panels (a) and (b) show a representative blot and the mean results (± s.e.m. of nine independent determinations from different animals) respectively. S and V refer to animals treated with salmeterol or vehicle respectively. *P<0.05, significant reduction in $G_{s\alpha}$ expression, Student's unpaired T-test.

### *3.3.7 Effect of chronic treatment of rats with salmeterol on the ability of salmeterol and $PGE_2$ to elevate the intracellular cAMP content in rat lung ex vivo.*

*Ex vivo* experiments were performed on lung parenchyma to assess if salmeterol- and $PGE_2$-induced cAMP accumulation was affected in $\beta_2$AR desensitised rats. No change in the basal intracellular cAMP level was observed between treatment groups. However, there was a significant attenuation in the ability of salmeterol to elevate intracellular cAMP in lung taken from salmeterol-treated rats when compared to animals that received vehicle. This amounted to a

~174% increase in cAMP mass over basal for vehicle-treated rats compared to ~56% increase in rats that received salmeterol (Figure 3.3.13). Thus, chronic treatment of rats with salmeterol desensitised lung tissue to the the ability of salmeterol to elevate cAMP mass.

Consistent with the functional results presented in section 3.3.2, the ability of $PGE_2$ to increase cAMP mass in lung taken from salmeterol-treated rats was also significantly attenuated (Figure 3.2.14). Thus, $PGE_2$ (1 μM)-induced cAMP accumulation fell from a ~121% increase over basal in vehicle-treated rats to ~61% in rats given salmeterol chronically, indicating that there was a heterologous desensitisation at the level of cAMP accumulation (Figure 3.3.14).

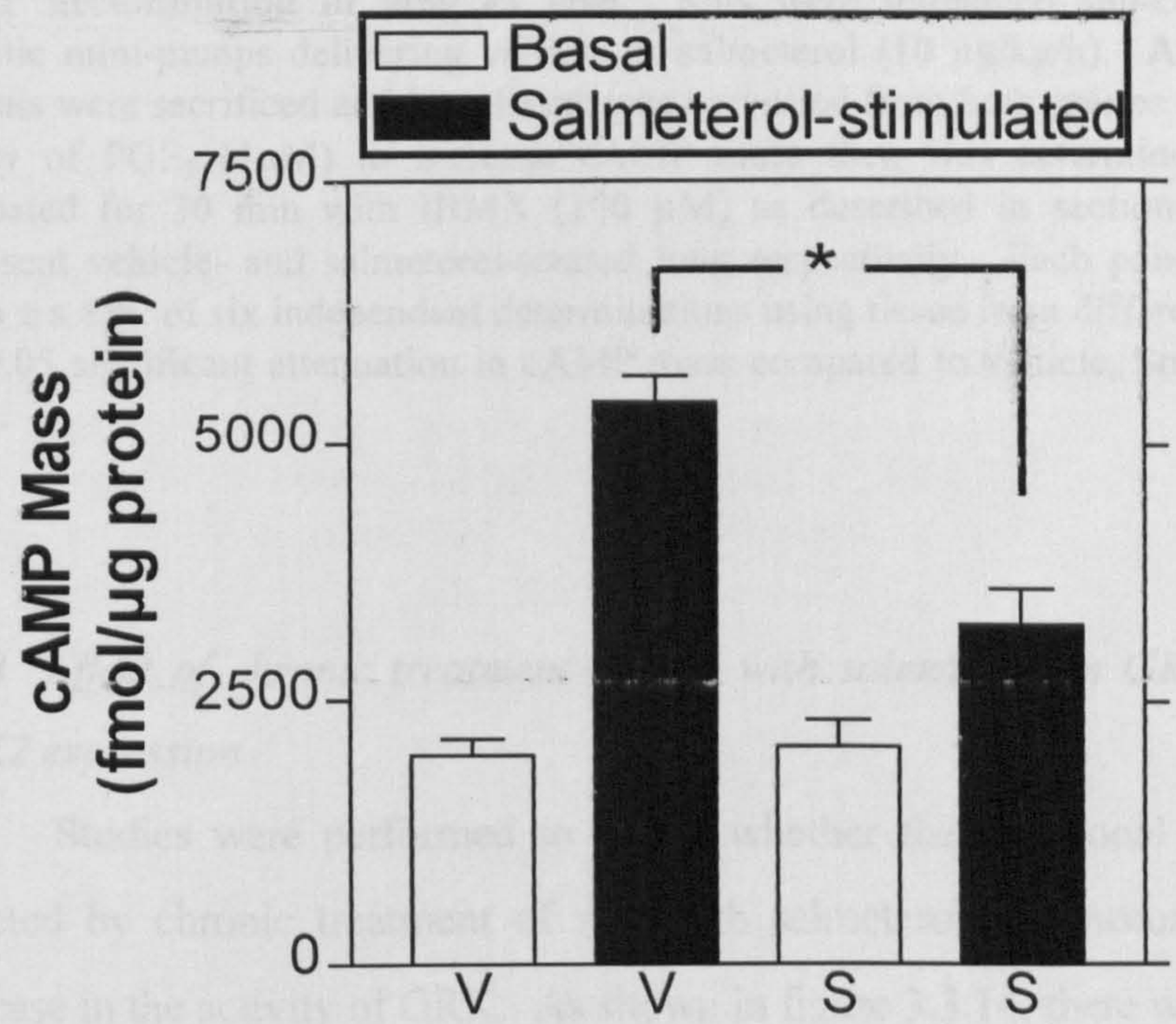



**Figure 3.3.13. Effect of chronic treatment of rats with salmeterol on salmeterol-induced cAMP accumulation in lung *ex vivo*.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle or salmeterol (10 μg/kg/h). After seven days, animals were sacrificed and lung tissue was harvested. The ability of salmeterol (1μM) to increase cAMP mass then was determined in lung pre-incubated for 30 min with IBMX (100 μM) as described in section 2.4. V and S represent vehicle- and salmeterol-treated rat lung respectively. Each point represents the mean ± s.e.m. of six independent determinations using tissue from different animals.
*$P<0.05$ significant attenuation in cAMP mass compared to vehicle, Student's unpaired t-test.

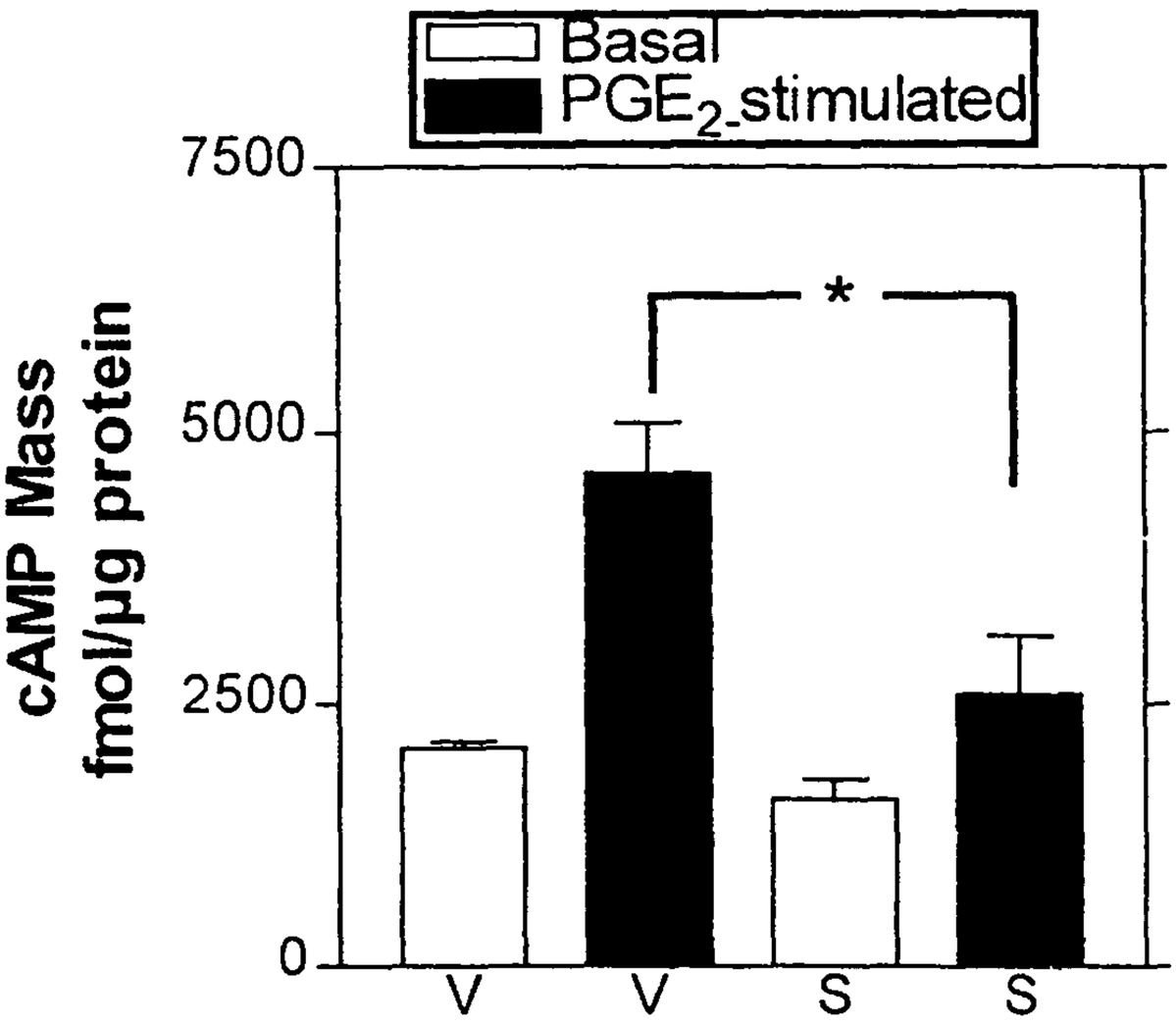



**Figure 3.3.14. Effect of chronic treatment of rats with salmeterol on $PGE_2$-induced cAMP accumulation in lung *ex vivo*.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle or salmeterol (10 µg/kg/h). After seven days, animals were sacrificed and lung tissue was harvested from both groups of animals. The ability of $PGE_2$ (1µM) to increase cAMP mass then was determined in lung pre-incubated for 30 min with IBMX (100 µM) as described in section 2.4. V and S represent vehicle- and salmeterol-treated lung respectively. Each point represents the mean ± s.e.m. of six independent determinations using tissue from different animals. *P<0.05 significant attenuation in cAMP mass compared to vehicle, Student's unpaired t-test.

### *3.3.8 Effect of chronic treatment of rats with salmeterol on GRK activity and GRK2 expression.*

Studies were performed to assess whether the functional desensitisation effected by chronic treatment of rats with salmeterol was accompanied by an increase in the activity of GRK. As shown in figure 3.3.14, there was a significant (~204%) increase in GRK activity in the lung cytosol of salmeterol-treated rats compared to animals that received vehicle. A primary antibody raised against the C-terminal region of GRK2 was used to probe for this molecule in cytosolic extracts, and recognised an 80 kDa band (Figure 3.3.15a), consistent with the size of GRK2 found in previous studies (Fredericks *et al.*, 1996). However, in contrast to the results obtained with salbutamol-treated rats (section 3.2.8), quantification of the Western blots by densitometry revealed that there was no significant

difference in the expression of GRK2 in the lungs of vehicle and salmeterol-treated animals (Figure 3.3.16) despite levels being elevated by ~40%. No change in activity or protein expression was detected in the particulate fraction of lung harvested from salmeterol- or vehicle-treated rats (data not shown).

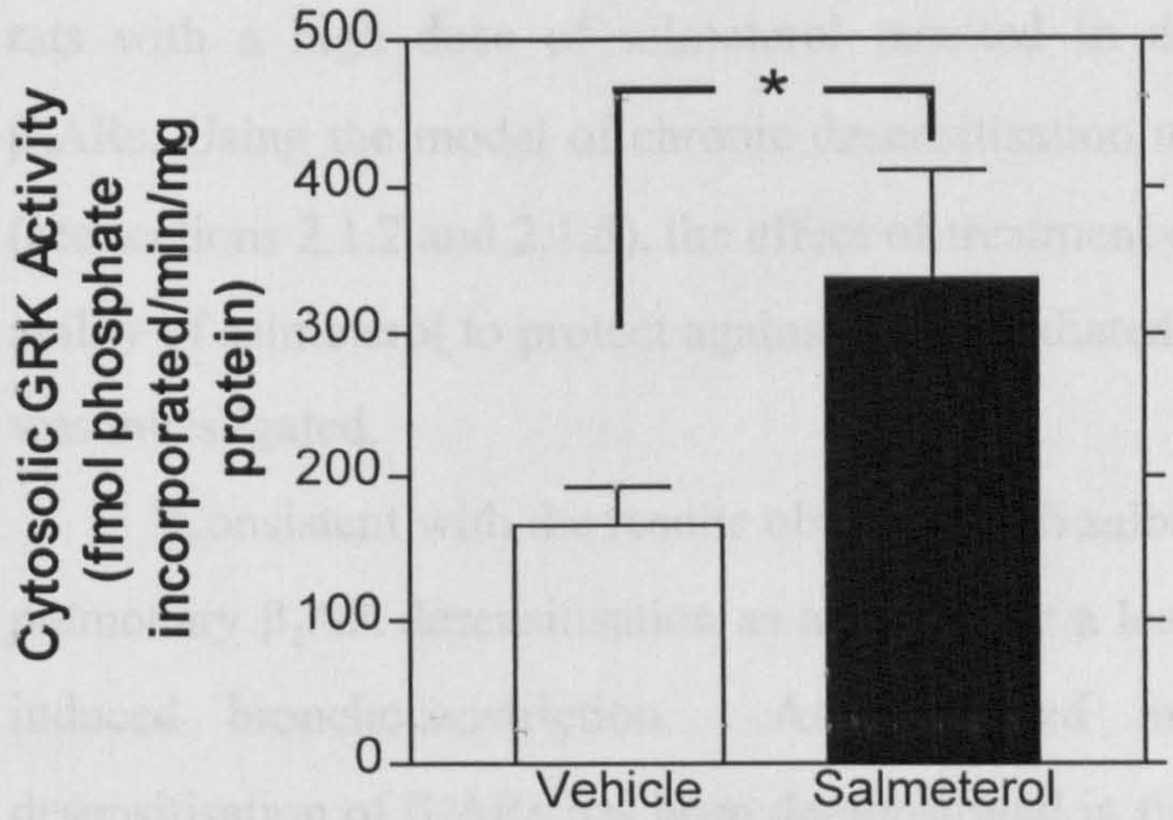



**Figure 3.3.15. Effect of chronic treatment of rats with salmeterol on cytosolic GRK activity in lung.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle or salmeterol (10 μg/kg/h). After seven days, animals were sacrificed and activity of GRK in lung cytosol extracted from salmeterol- or vehicle-treated rats was assessed as described in section 2.7. Each point represents the mean ± s.e.m. of 12 independent determinations using tissue from different animals.
*P<0.05 significant increase in GRK activity, Student's unpaired T-test.

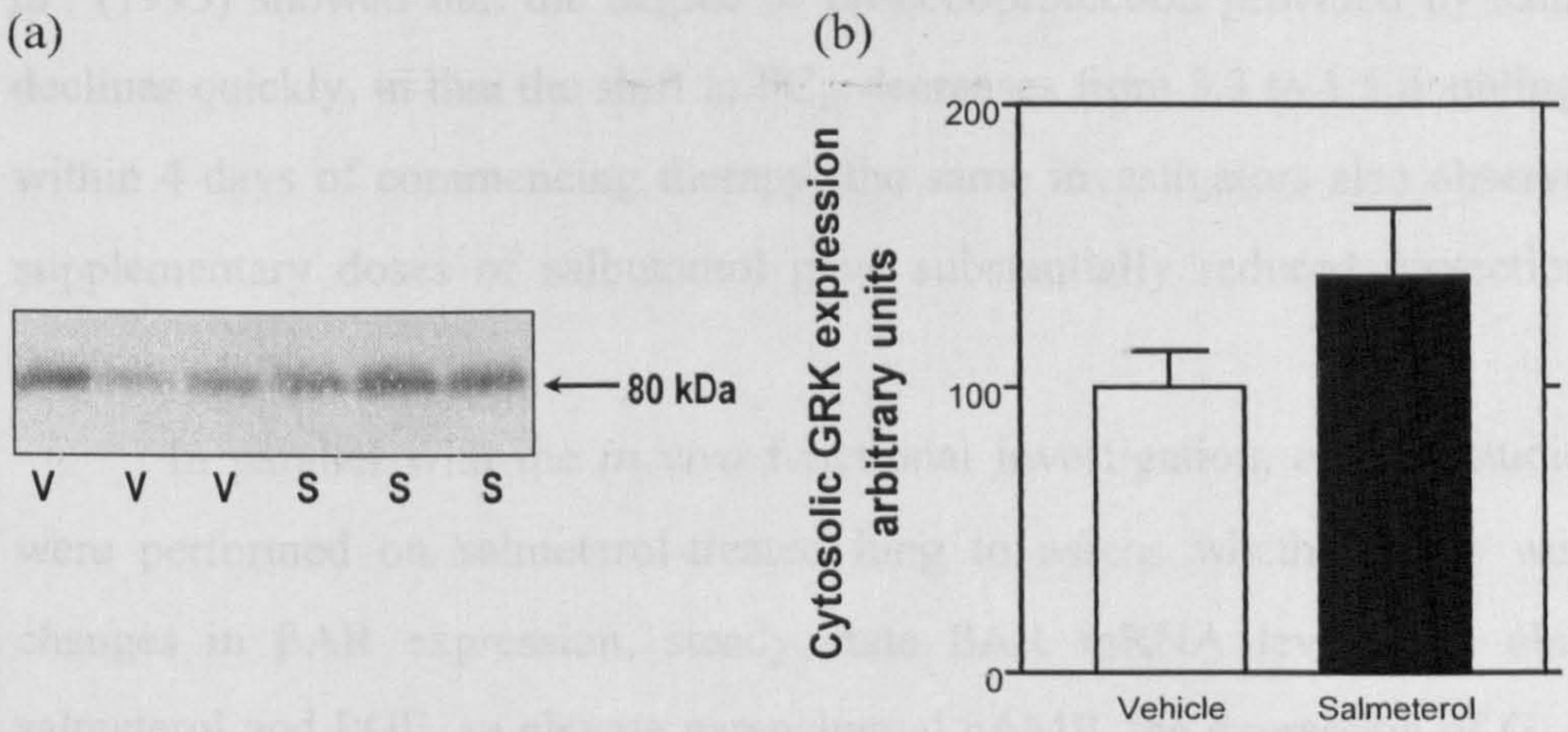



**Figure 3.3.16. Effect of chronic treatment of rats with salmeterol on GRK2 protein expression.** Rats were implanted sub-cutaneously with osmotic mini-pumps delivering vehicle or salmeterol (10 μg/kg/h). After seven days, animals were sacrificed and the expression of cytosol-associated GRK2 was determined by western blotting as described in section 2.6. Panels (a) and (b) show representative blots and the mean results (± s.e.m. of 12 independent determinations from different animals) respectively. S and V refer to animals treated with salmeterol or vehicle respectively.

### *3.3.9 Discussion*

The present study was undertaken to assess whether chronic treatment of rats with a high dose of salmeterol resulted in desensitisation of pulmonary $\beta_2$ARs. Using the model of chronic desensitisation used in the salbutamol study, (see sections 2.1.2 and 2.1.5), the effect of treatment of rats with salmeterol on the ability of salmeterol to protect against ACh-mediated bronchoconstriction initially was investigated.

Consistent with the results obtained with salbutamol, salmeterol produced pulmonary $\beta_2$AR desensitisation as assessed by a loss in protection against ACh-induced bronchoconstriction. As discussed in section 3.1.10, *ex vivo* desensitisation of β-ARs has been demonstrated in tracheae from rats treated with isoprenaline and terbutaline, demonstrating that functional desensitisation can occur after chronic βAR agonist treatment (Aviner and Noland, 1978; Taylor, 1987). Similarly, in humans, regular use of salmeterol leads to tolerance with respect to protection against bronchoconstrictor stimuli such as inhaled methacholine (Cheung *et al.*, 1992) or exercise (Ramage *et al.*, 1992). Bhagat *et al.*, (1995) showed that the degree of bronchoprotection provided by salmeterol declines quickly, in that the shift in $PC_{20}$ decreases from 3.3 to 1.5 doubling doses within 4 days of commencing therapy; the same investigators also observed that supplementary doses of salbutamol gave substantially reduced protection after treatment with salmeterol

In parallel with the *in vivo* functional investigation, *ex vivo* studies also were performed on salmeterol-treated lung to assess whether there were any changes in βAR expression, steady state βAR mRNA levels, the ability of salmeterol and $PGE_2$ to elevate parenchymal cAMP, the expression of $G_s$ and $G_\beta$ subunits and the expression and activity of GRK.

In lung harvested from rats treated chronically with salmeterol there was a highly significant reduction in the density of $\beta_2$ARs and a smaller but, nevertheless, significant, decrease in $\beta_1$AR number with no change in the affinity

of [$^{125}$I]ICYP for either subtype. These results are similar to a study by Nishikawa *et al.,* (1993) who found that chronic infusion of the non-selective βAR agonist, isoprenaline, into rats for 7 days was associated with a reduction in both $\beta_1$- and $\beta_2$AR receptor density. Other groups also have found down-regulation of $\beta_1$- and $\beta_2$AR after prolonged exposure to isoprenaline (Beer *et al.,* 1987, Nanoff *et al.,* 1989) although this is the first report with salmeterol *in vivo*. The reduction in $\beta_1$ARs is surprising as salmeterol has been shown to be highly selective for $\beta_2$ARs in lung and heart tissue (Roux *et al.*, 1996). However, one is forced to conclude that the dose of salmeterol, chronicity of treatment coupled with its highly lipophilic nature, may have lead to enough stimulation of the $\beta_1$AR to cause down-regulation. An alternative explanation may be that salmeterol is metabolised when given by the subcutaneous route (see section 3.3.3.2 and below) to a shorter-acting agonist that has lost selectivity towards the $\beta_2$-AR subtype. Although not formally investigated in this thesis, this possibility could be tested by using selective $\beta_1$AR antagonists given that relaxation is also mediated by the $\beta_1$AR subtype in this tissue (Anderson, 1995).

To determine whether that the reduction in cell surface $\beta_2$AR binding sites measured using radioligand binding was due to salmeterol antagonising the binding of [$^{125}$I]ICYP, $\beta_2$AR expression was determined by western blotting using an antibody raised against the C-terminal region of the protein. The observation that there was a significant reduction in $\beta_2$AR protein expression in membranes extracted from salmeterol-treated rats, implies that the reduction in $\beta_2$AR density detected by radioligand binding was, indeed, due to down-regulation of $\beta_2$AR number.

Northern analyses were performed to assess if steady state $\beta_1$- and $\beta_2$AR mRNA expression was affected in salmeterol-desensitised lung. After 7 days of chronic treatment of rats with salmeterol, the steady state mRNA level for both $\beta_1$- and $\beta_2$AR was unchanged between the treatment groups. Although these results are consistent with the salbutamol study (section 3.2.2) they, nevertheless, contrast with those reported by Nishikawa and co-workers who found that treatment of rats with a non-selective βAR agonist (isoprenaline) caused a reduction in steady-state mRNA levels in rat lung (Nishikawa *et al.,* 1993). Differences in rat strains (as

presented by Van Liefde *et al.,* 1994) or even cell type (as discussed by Danner and Lohse, 1996) could explain the differences between the current study and those performed by Nishikawa *et al.,* (1993). As discussed in section 3.2.11, there also may be a temporal separation between changes in steady state message and down-regulation of βAR binding sites on the cell surface; that is, reduction in message could have occurred at an earlier time point and returned to pre-stimulation levels by seven days. Indeed, *in vitro*, Nishikawa *et al.*, (1996) reported that 3 h treatment of human lung with salmeterol resulted in reduced $\beta_2$AR density and mRNA expression, findings that support the supposition that salmeterol can induce a rapid reduction $\beta_2$AR density. Cycling of receptors from the cell surface to cytoplasmic compartments and back again has been extensively discussed in the literature (see Koenig *et al.,* 1997 for review). An increase in receptor cycling away from the membrane could reduce cell surface receptor binding sites. Similarly, an increase in $\beta_2$AR degradation could reduce cellular $\beta_2$AR protein without a change in $\beta_2$AR message.

The next step in the study was to assess whether the functional $\beta_2$AR desensitisation produced by salmeterol was homologous (agonist-specific) or heterologous (non-agonist specific) (Chuang *et al.*, 1996). This was investigated by assessing the ability of salmeterol and $PGE_2$ to protect against ACh-mediated bronchoconstriction (see sections 3.1.2-3.1.5). After 7 days constant infusion of salmeterol, the ability of both GPCR agonists to protect against ACh-mediated bronchoconstriction was abolished indicating that the desensitisation was heterologous in nature.

To assess whether the *in vivo* functional heterologous desensitisation observed after chronic treatment of rats with salmeterol was mediated by similar mechanisms to those identified for salbutamol, the expression of $G_{s\alpha}$ and $G_\beta$ proteins was measured by western blotting. In agreement with the salbutamol study, no change in $G_\beta$ subunits was detected whereas all three $G_{s\alpha}$ variants were significantly down-regulated and could account, in part, for the desensitisation.

As mentioned in section 3.2.11, it has been shown previously that sustained exposure of cells to agonists that couple through $G_s$ results in a substantial decrease in cellular levels of $G_{s\alpha}$ (Gun-Do and Milligan 1994; Adie

and Milligan 1994; McKenzie and Milligan; 1990, Adie *et al.*, 1992); similarly, $G_{s\alpha}$ is removed from the plasma membrane if persistently stimulated with analogues of GTP (Milligan and Unson, 1989). Kume and Takagi, (1997) reported that pre-exposure of guinea pig tracheal strips to cholera toxin, which directly stimulates $G_{s\alpha}$, prevented the reduced responsiveness that resulted from repeated treatment with βAR-agonists. This observation indirectly supports the suggestion that a decrease in the activity of $G_s$ accounts for the reduction in response to repeated stimulation of tissues with βAR-agonists. Thus, down-regulation of $\beta_2$AR binding sites on the surface of the cell, and of $G_{s\alpha}$ protein in lung, could have a significant inhibitory effect on the $\beta_2$AR signal transduction cascade, and could explain totally the reduced anti-spasmogenic effects of salmeterol and $PGE_2$ seen in salmeterol-treated rats.

A prediction, based on the *in vivo* results and the reduced expression of $\beta_2$ARs and $G_{s\alpha}$, is a reduction in the ability of the transduction cascade to signal efficiently. This prediction was supported by the finding that the ability of salmeterol and $PGE_2$ to elevate intracelluar cAMP was significantly attenuated in lung taken from salmeterol-treated rats when compared to animals that received vehicle. Thus, the tissue exhibited a biochemical heterologous desensitisation that mirrored the *in vivo* findings. A reduction in the ability of salmeterol to increase cAMP mass would also reduce the functional bronchodilator response, and reduce the bronchoprotective action of both salmeterol and $PGE_2$ observed after chronic treatment using both drugs. Extemporising from the observation that there is a reduced ability of acute salmeterol and $PGE_2$ to elevate intracellular cAMP in lung taken from salbutamol-treated rats, and comparing the results obtained with salmeterol to those obtained in tissue from rats treated chronically with salbutamol (sections 3.2.7 and 3.2.12), one would predict the activity of PKA to be concomitantly reduced, further reducing the efficiency of the signal transduction cascade.

The relative importance of the GRKs and PKA in mediating $\beta_2$AR desensitisation is cell-type specific (Shih and Malbon, 1994). It has been demonstrated that desensitisation attributed to GRK phosphorylation/β-arrestin binding *in vitro* is strongly dependent on occupancy by full agonists (the $EC_{50}$ for

GRK2 phosphorylation approximates the $K_d$ for agonist binding) (Perkins *et al.*, 1991, Palczewski *et al.*, 1990, Premont *et al.*, 1995, Clark *et al.*, 1988, Hausdorff *et al.*, 1989). Indeed, Benovic *et al.*, (1988) showed that the ability of partial agonists to stimulate *in vitro* GRK2 phosphorylation of βAR reconstituted into liposomes was reduced relative to full agonists and that the reduction was closely correlated with the efficacy of agonists stimulating of AC. Thus, it was of interest to assess the effect of chronic salmeterol-treatment of rats on the activity and expression of GRK2 in lung tissue. In agreement with the data obtained with salbutamol, the activity of GRK2 in the lung of salmeterol-treated rats was significantly increased compared to lung taken from vehicle-treated animals, and this was associated with a trend towards an increase in protein expression. The increase in activity of GRK2 might be expected given the significant reduction in $\beta_2$AR density and the fact that GRK2 activity reflects an increased sequestration of $\beta_2$AR from the membrane into endosomes in the cell. The observation that GRK2 expression and activity is increased in excised lung from rats chronically treated with salmeterol, where surface receptor binding sites in lung tissue are reduced, underlies a possible role of GRK in $\beta_2$AR desensitisation. However, as GRKs only phosphorylate agonist-occupied receptors this mechanism could only implicate GRK in homologous desensitisation of βARs.

Another interesting point observed in this study was that in vehicle-treated rats a protective effects of salmeterol persisted for only 30-40 minutes after i.v. administration. This is surprising considering the well documented long-acting nature of salmeterol. Similar results have been reported by Mohammed *et al.*, (1996) in guinea pigs. In that study, the reason for the discrepancy relates to the route of administration. Thus, when salmeterol was given by aerosol the resulting bronchoprotection lasted for several hours. In contrast, salmeterol given i.v. resulted in a bronchoprotection that lasted for only 30 minutes, consistent with the results of the present study.

One explanation for the short bronchoprotective activity of salmeterol when given by the i.v. route is that it is metabolised to shorter-acting, but still biochemically active, metabolites. This would be in contrast to an aerosol where salmeterol's high lipophilicity would ensure that it is absorbed rapidly into the

plasma membrane and retained, enabling the "tail" to interact with the exosite of the $\beta_2$AR moiety. Although the saligenin head of salmeterol confers resistance to metabolism by catechol-*o*-methyltransferase (discussed in section 1.2.6.1), the aliphatic side chain is not resistant to metabolism.

When the blood of rats given a bolus of salmeterol was analysed by LC-MS, the concentration of salmeterol in the plasma fell rapidly due to metabolism and a very high volume of distribution. The clearance of salmeterol was predominantly by metabolism to a number of species, with the major route being glucuronidation. In a similar study by Manchee *et al.*, (1993), several species of animal, including Sprague-Dawley rats, were given a dose of salmeterol either orally (2-200 mg/kg of [$^{14}$C]-labelled salmeterol) or i.v. (2 mg/kg) and the absorption, distribution, metabolism and excretion of salmeterol were monitored. In all of the species studied, most of the salmeterol was excreted in bile or faeces, with unchanged salmeterol accounting for <5% of the excreted dose in all species. Rat bile contained one major metabolite (see IV in Figure 3.3.16) and a number of minor metabolites. Metabolite IV co-chromatographed with authentic phenolic salmeterol glucuronide and was hydrolysed by β-glucuronidase with the appearance of unchanged salmeterol. These findings indicated that metabolite IV was a phenolic glucuronide conjugate of parent compound. Two minor metabolites (I and II), excreted predominantly in rat urine, were identified by GC/MS analysis as GR37052 and GR72438 products of O-deaikylation; however the activity of these metabolites at the $\beta_2$AR is unknown. These metabolites were also present in urine as their corresponding glucuronide conjugates. In compound IV the side chain is intact, and it is possible that if it is an active compound at $\beta_2$ARs it may be long-acting. Compounds I and II are more likely to be short-acting since the length of the side chains are significantly reduced. If active at $\beta_2$ARs, compound I and II may produce the short-acting effect described above. The data presented in section 3.3.4 agree with the findings of Manchee *et al.*, (1993) in that the major route of clearance is by glucuronidation.

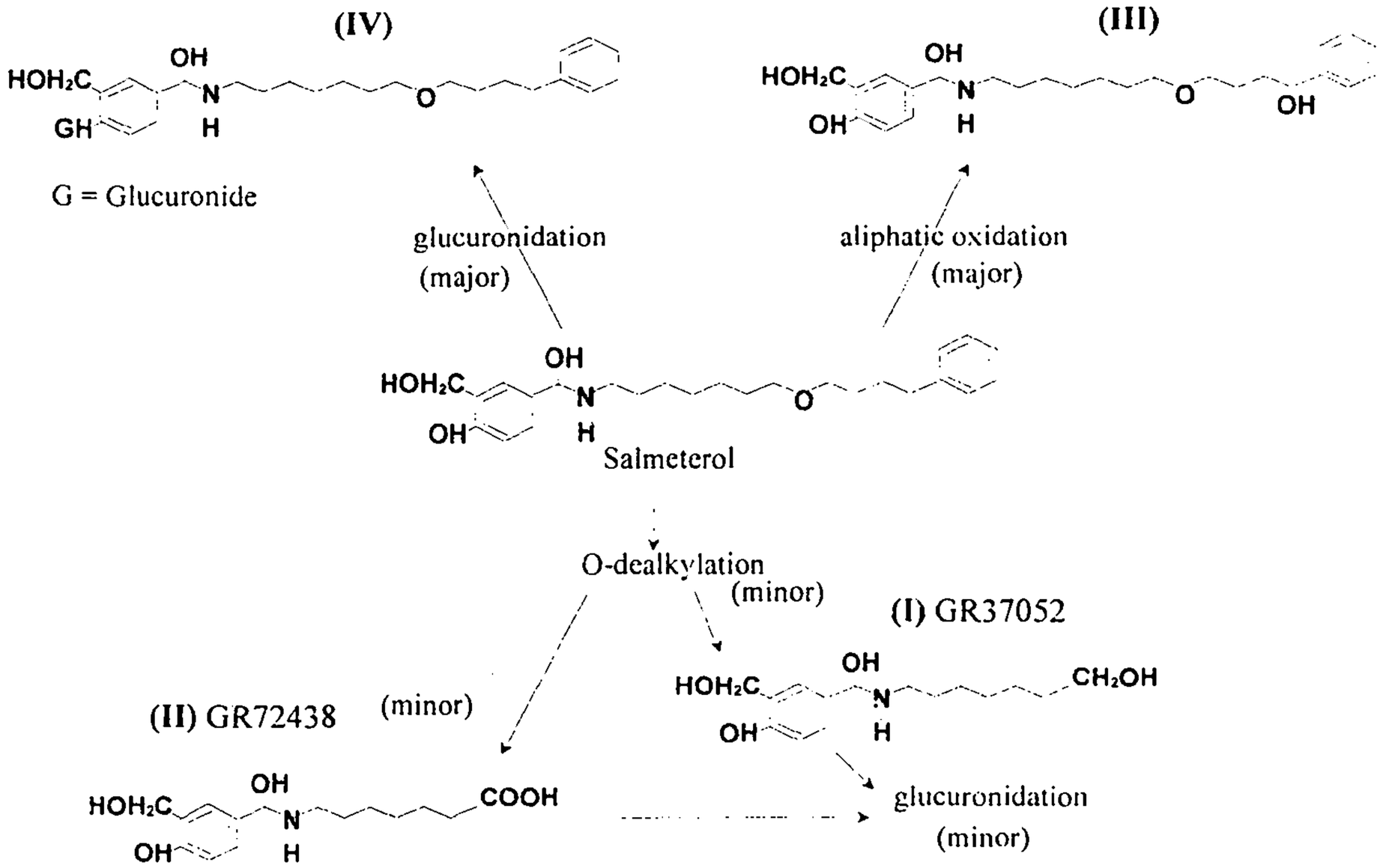



**Figure 3.3.16. Major and minor metabolites of salmeterol in the rat** (Adapted from Manchee *et al.*, 1993).

As salbutamol is often used as a "rescue therapy" for asthmatic subjects, the effect of using salbutamol in salmeterol-desensitised rats was investigated. In vehicle treated rats, salbutamol and salmeterol afforded 50% and 39% protection of ACh-induced bronchoconstriction respectively, whereas in salmeterol-desensitised rats salmeterol afforded an 11% protection. Salbutamol administration in rats, where the osmotic mini-pumps were left in the animal, afforded only 10% protection against ACh-mediated bronchoconstriction, whereas if the pumps were removed and 1 h clearance time given (1 h being in excess of the time required for ACh-induced bronchoconstriction to recover after an acute dose of salmeterol in vehicle-treated rats) the protection afforded by salbutamol increased to 22%. This implies that either salmeterol is antagonising the effect of salbutamol, by blocking salbutamol entry into the active site of the $\beta_2$AR, or that the animals were recovering from the desensitisation due to chronic salmeterol treatment. Salmeterol has been shown to act as an antagonist both to the inhibitory effect of eformoterol on $H_2O_2$ production in eosinophils (Rabe *et al.*, 1993), and also to antagonize the relaxation of human contracted bronchi induced by the short-acting $\beta_2$AR agonists isoprenaline, terbutaline, fenoterol and

salbutamol (Molimard *et al.,* 1998). However, as the anti-spasmogenic activity of salmeterol was short-acting, it is unlikely that it acts as an antagonist in this system. Thus, limited resensitisation of the $\beta_2$AR signal transduction cascade may have occurred.

In summary, the rat model used to assess desensitisation responses to chronic salbutamol treatment was also used to study the effects of chronic treatment with the long-acting $\beta_2$AR agonist salmeterol. The data presented above demonstrate that chronic exposure of rats to salmeterol is associated with a significant loss in protection for both salmeterol and $PGE_2$ against ACh-induced bronchoconstriction. This is a demonstration of functional heterologous desensitisation identical to that observed in rats treated chronically with salbutamol.

In conclusion, components of the $\beta_2$AR signal transduction cascade in lung affected by treating rats chronically with salmeterol appear similar to those affected by salbutamol under identical experimental conditions. The data presented in this study demonstrate that chronic exposure of rats to salmeterol is associated with a significant loss in $\beta_1$- and $\beta_2$AR binding sites on the surface of the cells, with no change in affinity of ligand for receptor. Supporting the radioligand binding data was a significant reduction in $\beta_2$AR membrane protein expression. The stimulatory GTP binding protein, $G_s$, was also affected by chronic salmeterol treatment, with a significant loss in expression of all three observed species of the $\alpha$-subunit. Taken together, these changes are presumably responsible for the significant attenuation in the ability of acute salmeterol and $PGE_2$ to elevate intracellular cAMP in salmeterol-treated rat lung and to protect the airways against contractile stimuli such as ACh. These processes can, therefore, satisfactorily account for the heterologous desensitisation. There was also a significant increase in GRK2 activity and a trend towards an increase in protein expression in salmeterol-treated rat lung, which inevitably have the potential to increase phosphorylation and internalisation of agonist-bound $\beta_2$ARs. However, this mechanism will only enhance $\beta$AR desensitisation without affecting other $G_s$-coupled receptors and is likely to be of limited significance.

# Chapter 4

# General Discussion and Future Directions

Since the cloning and expression of GPCRs there has been a massive increase in our understanding of many aspects of their function. The use of molecular biology to engineer and express mutant receptors has made it possible to assign a function for single amino acids within the receptor. Thus, we are beginning to define, at the molecular level, the exact nature of conformational changes in the receptor during the binding of an agonist and subsequent interaction with G proteins. With regard to the biochemical function of GPCRs, it has become clear that the comfortable assignment of any particular receptor into a single signalling cascade is naive. There is now a large body of evidence showing that GPCRs do not signal by activating one G protein to generate one signal. Instead, different G proteins are activated by one receptor to generate multiple intracellular signals. The use of molecular probes has also enabled us to specifically detect the expression of individual receptor subtypes within tissues. In conjunction with the use of increasingly selective agonists and antagonists, this has allowed the definition of a role for these receptors *in vivo*.

The AC-coupled βAR signal transduction cascade has served as a model system for the study of the phenomenon of desensitisation of GPCRs (Perkins *et al.*, 1991; Dixon *et al.*, 1988; Palzcewski and Benovic, 1990; Clark, 1986;

Premont *et al.*, 1995). Desensitisation is functionally defined as an attenuation of hormonal responsiveness upon agonist stimulation. A number of molecular mechanisms operating at the receptor level have been implicated in desensitisation (Klein *et al.*, 1989). Examination of the kinetics of the desensitisation process suggest that in several systems it is biphasic, i.e. there is a rapidly evolving desensitisation that is essentially complete after exposure of cells to micomolar agonist levels for 30 min, and after several hours there is a more conclusive attenuation of signalling involving down-regulation of receptor protein (Bouvier *et al.*, 1988; Su *et al.*, 1979; Homburger *et al.*, 1980). Two major patterns of rapid desensitisation have been characterised, namely agonist-specific or homologous desensitisation and agonist-nonspecific or heterologous desensitisation (Lohse, 1995). Further desensitisation of response requires much more prolonged exposure to agonist, in the order of several hours. Processes such as the agonist-induced loss of total cellular receptor number, or down-regulation, which would be expected to alter βAR responsiveness, cannot be responsible for rapid diminishment of signal transduction cascade signalling because they evolve too slowly. Down-regulation appears to be a process biochemically distinct from sequestration, in that receptors are apparently destroyed.

There has been controversy regarding the excessive use of $\beta_2$AR-agonists and their potential contribution to asthma morbidity and mortality. Epidemics of asthma deaths in the United Kingdom in the 1960's and New Zealand in the late 1970's have implicated regular or excessive use of short acting $\beta_2$AR-agonists (O'Donnell *et al.*, 1989). Several studies have suggested that overuse of these agents is associated with an increased risk of death from asthma (Spitzer *et al.*, 1992). The mechanism(s) underlying the possible adverse effects of $\beta_2$AR-agonists remain to be clarified and are subject to much discussion. Although numerous studies have identified impairment of $\beta_2$AR-mediated functions in human and non-human airways, and in cell systems, there is a paucity of knowledge about the regulation of the airway $\beta_2$AR-adenylyl cyclase signal transduction cascade *per se*. Mechanisms of acute regulation of $\beta_2$AR signalling identified in other systems have shown important roles for phosphorylation by intracellular kinases (GRKs, PKA) in modulating the responsiveness of this

system. However, the extent to which these mechanisms are used appears to be highly cell-specific (Shih and Malbon, 1994). In contrast to the advances in our understanding of the structure and function of the βAR and other GPCRs, and the knowledge of the mechanics of desensitisation, little is known about how this comes about *in vivo* in response to chronic stimulation. Thus, it was the aim of this thesis to consider the effect of treating Sprague-Dawley rats for 7 days with a high dose of salbutamol and salmeterol on the *in vivo* airways responsiveness to agents that act through GPCRs and on a number of *ex vivo* biochemical indices that could account for desensitisation.

The model proved useful in that $\beta_2$AR desensitisation was reproducibly elicited *in vivo* and that several mechanisms were identified that could account for this change in functional responsiveness. The results obtained are different to those reported in the vast majority of *in vitro* studies in isolated cells and tissues where only a single mechanism is usually highlighted. Moreover, these data also demonstrate that *in vivo* desensitisation is regulated in a complex manner that is likely to be strongly influenced by the GPCR agonist, receptor density/efficiency of coupling on target cells and duration of exposure.

It has been shown that chronic stimulation of cells with ligands that activate GPCRs coupled negatively to AC (e.g. μ-, δ-, κ-opioid receptors and m2- and m4-muscarinic receptors) can lead to an increase in cAMP levels (Sunahara *et al.*, 1996; Birnbaumer *et al.*, 1990; Guderman *et al.*, 1996). This phenomenon, which is particularly manifest upon withdrawal of the inhibitory agonist, is referred to as AC superactivation (Sharma *et al.*, 1975; Ammer and Schulz, 1993., Avidor-Reiss *et al.*, 1995a,b., Law *et al.*, 1994., Thomas, *et al.*, 1987, 1988, 1996; Wang *et al.*, 1994.). AC superactivation is believed to play a role in the development of tolerance and withdrawal upon prolonged opiate exposure (Sharma *et al*, 1975; Avidor-Reiss *et al.*, 1995). AC superactivation was first described in NG108-15 neuroblastoma × glioma hybrid cells that had been chronically treated with agonists of opioid, muscarinic, $\alpha_2$-adrenergic, or somatostatin receptors (Sharma *et al.*, 1975; Traber *et al.*, 1975; Sabol and Nirenberg, 1979; Thomas and Hoffman, 1992), but has also been described in

non-neuronal cell lines such as rat adipocytes (Parsons *et al.*, 1986) and S49 mouse lymphoma cells (Thomas and Hoffman, 1992).

Two groups of investigators showed, using transiently transfected cell-lines, that when morphine was chronically applied to AC-I -, ACV-, ACVI- and ACVIII-transfected cells, an increase in cAMP levels was observed following withdrawal of the agonist - demonstrating AC superactivation (Avidor-Reiss *et al.*, 1997. Thomas and Hoffman, 1996). Superactivation of ACI and ACV did not depend on the method of stimulation (hormone receptor activation, FS, TPA, or increased $Ca^{2+}$ concentration), but conversely, ACII, ACIV and ACVII activities were increased by chronic morphine stimulation (Avidor-Reiss *et al.*, 1997).

Regarding the mechanisms involved in AC superactivation, it has been shown that ACII and ACIV in membrane preparations of transfected Sf9 cells are stimulated by purified bovine brain $G_{\beta\gamma}$ (Taussig *et al.*, 1993, 1994. Tang *et al.*, 1992; Gao and Gilman, 1991). The ability of the βγ dimer-sequestering fragment of GRK2 to prevent the stimulation of ACII by opioid receptor activation indicates that the mechanism by which the stimulation occurs involves $G_{\beta\gamma}$ dimers. The importance of the relative abundance of $G_{\beta\gamma}$-stimulated AC isoform or isoforms to the directionality of opioid responsiveness has been demonstrated in several cell lines. For example, dopamine activation of AC types V and VII can be inhibited and stimulated, respectively, by morphine in a concentration-dependent manner (Yoshimura *et al.*, 1996). The stimulatory effects of morphine on AC VII are blocked by pertussis toxin, as well as the α-subunit of transducin, suggesting the importance of $G_{\beta\gamma}$ derived from $G_i$. Similarly, in COS-7 cells, acute opioid treatment inhibits or stimulates transfected AC activity in an isoform-specific fashion (Avidor-Reiss et al., 1997), the pattern of which correlates with that of βγ stimulatory responsiveness.

The phenomenon of rebound supersensitivity after chronic treatment with inhibitory GPCRs is interesting in the context of the effects of chronic $\beta_2$-AR agonist treatment demonstrated in rats. The main mechanisms mediating functional desensitisation in the rat model described in this study was a down-regulation of $G_{s\alpha}$ subunits with no associated change in the level of β subunit protein expression. Cytosolic GRK2 expression is also elevated in this system

contributing to the level of phosphorylation of $\beta_2$ARs, other GRKs and even cytoskeletal elements (Carman *et al.*, 1998; Pitcher *et al.*, 1998; Haga *et al.*, 1998). One could postulate that once chronic salbutamol/salmeterol treatment is terminated, there could be a similar rebound superactivation of AC. This phenomenon has not been observed after chronic stimulatory GPCR agonist treatment thus far and no studies have been performed in animal models such as the one described herein. As $G_{s\alpha}$ is down-regulated by chronic $\beta_2$AR agonist treatment, this frees $\beta\gamma$ subunits to interact with AC. Stimulation of GRK over expression by continual agonist stimulation would also be terminated, thus reducing the expression of GRKs, which act as a sink for $\beta\gamma$ subunits due to their interaction with these G protein subunits (as discussed in section 1.4.2.1). The freeing of $\beta\gamma$ subunits to interact with various AC isoforms could induce a rebound superactivation as seen for opioid agonists. This could be an important consideration in the tolerance response of the drugs used.

In summary, the work from this thesis has demonstrated that chronic treatment of rats with either salbutamol or salmeterol causes *in vivo* functional heterologous desensitisation, associated with a reduction in $\beta_2$AR density, a down regulation in $G_{s\alpha}$ protein expression, and increased GRK2 activity and protein expression. Biochemically, $\beta_2$AR agonists produce a desensitisation that may be multifactorial in mechanism, with most of the steps in the signal transduction cascade affected. The clinical relevance of the data shown in this thesis is unclear, since most mild and moderate asthmatics would not be using doses of salbutamol and salmeterol comparable to those used in this study. However, the use of high doses of $\beta_2$AR agonists are mandatory for acute severe asthma and clinical doses of salbutamol used for acute severe asthma in the U.S. exceed those used in this study. Significantly, the patients receiving these doses for severe asthma are those most in danger of mortality, and have most to gain from therapies combating the adverse effects of chronic $\beta_2$AR agonist treatment.

In future experiments it would be interesting to determine whether the desensitisation demonstrated after chronic infusion of $\beta_2$AR agonist via osmotic mini-pump could be reproduced *in vivo* using inhaled $\beta_2$AR agonists. Treating rats several times a day by inhalation using lower doses would be more

comparable to the doses that mild to moderate asthmatics receive. The effect of glucocorticosteroids in this *in vivo* model also could yield interesting results. Indeed, glucocorticosteroids classically up-regulate $\beta_2$AR (Mak *et al.,* 1994, Aziz *et al.,* 1998) so protection against desensitisation might be predicted. However, the model used in this study showed down-regulation of $\beta_2$AR as only one of several mechanisms responsible for *in vivo* desensitisation and so an assessment of the effects of glucocorticoids in this model would seem merited. A very recent development in research into desensitisation is the observation that the endocytic machinery thought to be solely involved in desensitisation is also involved in the switching of coupling of the $\beta_2$AR to signalling through different G-proteins (Daaka *et al.,* 1997), and in the activation of the mitogen-activated protein (MAP) kinase cascade (van Biesen *et al.,* 1995; Luttrell *et al.,* 1997; Daaka *et al.,* 1998). In the light of these developments, the model employed in the current studies would be a useful tool to observe any switching of signalling between the classical $G_s$-coupled pathway to the $G_{\beta\gamma}$-MAP kinase pathway after chronic $\beta_2$AR agonist treatment.

Other uses for this model could be to:

i) screen novel $\beta_2$AR agonists (with lower efficacy at the $\beta_2$AR) for their ability to effect $\beta_2$AR desensitisation.

ii) screen novel GPCR agonists that act through $G_s$ that could be less prone to cause desensitisation.

iii) devolop the model further to look at inflammatory cell $\beta_2$AR function.

iv) highlight which changes in the AC/cAMP signal transduction cascade are important in regulating desensitisation *in vivo*; i.e. use of knockouts (specific to $G_{s\alpha}$, PKA, GRK).

# Chapter 5

# References

Abbracchio, M. P., Cattabeni, F., Coen, E., Torres-Hernandez, Y., and Omini, C. 1983. β-adrenoceptor desensitization in rat lung: functional and biochemical aspects. *Eur. J. Pharmacol.* **89**:35-42.

Adie, E. J., and Milligan, G. 1994 Agonist regulation of cellular $G_{s\alpha}$-subunit levels in neuroblastoma × glioma hybrid NG108-15 cells transfected to express different levels of the human $\beta_2$ adrenoceptor. *Biochem. J.* **300**:709-715.

Adie, E. J., Mullaney, I., McKenzie, F. R., and Milligan, G. 1992. Concurrent down-regulation of IP prostanoid receptors and the alpha-subunit of the stimulatory guanine-nucleotide-binding protein (Gs) during prolonged exposure of neuroblastoma × glioma cells to prostanoid agonists. Quantification and functional implications. *Biochem. J.* **285**: 529-536.

Ahmed, F., Foster, R. W., and Small, R. C. 1985. Some effects of nifedipine in guinea-pig isolated trachealis. *Br. J. Pharmacol.* **84**:861-869.

Aizawa, H., Inoue, H., Miyazaki, N., Ikeda, T., Shigematsu, N., and Ito, Y. 1991. Effects of procaterol, a $\beta_2$-adrenoceptor stimulant, on neuroeffector transmission in human bronchial tissue. *Respiration* **58**:163-166.

Alvarez, R., Sette, C., Yang, D., Eglen, R. M., Willhelm, R., Shelton, E. R., and Conti, M. 1995. Activation and selective inhibition of a cyclic AMP-specific phosphodiesterase, PDE-4D3. *Mol. Pharmocol.* **48**:616-622.

Ammer, H., and Schulz, R. 1993. Alterations in the expression of G-proteins and regulation of adenylyl cyclase in human neuroblastoma 5H-SY5Y cells chronically exposed to low efficiency mu-opioids. *Biochem. J.* **295**:263-271.

Anderson, G. P. 1995. Adrenaline and noradrenaline. In: Airways smooth muscle: Neurotransmitters, Amines, Lipid Mediators and Signal Transduction. (Raeburn, D. and Giembycz, M. A. Eds). Birkhauser Verlag AG, Basel, pp1-79.

Arriza, J. L., Dawson, T. M., Simerly, R. B., Martin, L. J., Caron, M. G., Snyder, S. H., and Lefkowitz, R. J. 1992. The G-protein-coupled receptor kinases βARK1 and βARK2 are widely distributed at synapses in rat brain. *J. Neurosci.* **12**:4045-4055.

Arshavsky, V. Y., and Bownds, M. D. 1992. Regulation of deactivation of photoreceptor G protein by its target enzyme and cGMP. *Nature*. **357**416-417.

Assem, E. S. K., and Schildt, H. O. 1969. Inhibition by sympathomimetic amines of histamine release induced by antigen in passively sensitised human lung. *Nature* **224**:1028-1029.

Attramadal, H., Arriza, J. L., Aoki, C., Dawson, T. M., Codina, J., Kwatra, M. M., Snyder, S. H., Caron, M. G., and Lefkowitz, R. J. 1992. β-arrestin2, a novel member of the arrestin/β-arrestin gene family. *J. Biol. Chem.* **267**:17882-17890.

Avidor-Reiss, T., Zippel, R., Levy, R., Saya, D., Ezra, V., Barg, J., Matus-Leibevitch, N., and Vogel, Z. 1995. κ-Opioid receptor-transfected cell lines: modulation of adenylyl cyclase activity following acute and chronic opioid treatments. *FEBS Lett.* **361**:70-74.

Avidor-Reiss, T., Nevo, I., Saya, D., and Vogel, Z. 1997. Opiate-induced adenylyl cyclase superactivation is isozyme-specific. *J. Biol. Chem.* **272**:5040-5047.

Avner, B. P., and Noland, B. 1978. In vivo desensitization to beta receptor mediated bronchodilator drugs in the rat: decreased beta receptor affinity. *J. Pharmacol. Exp. Ther.* **207**:23-33.

Aziz, I., McFarlane, L. C., and Lipworth, B. J. 1998. Concomitant inhaled corticosteroid resensitises cardiac $\beta_2$-adrenoceptors in the presence of long acting $\beta_2$-agonist therapy. *Eur. J. Clin. Pharmacol.* **54**:377-381.

Baecker, P. A., Obernolte, R., Bach, C., Yee, C., and Shelton, E. R. 1994. Isolation of a cDNA encoding a human rolipram-sensitive cyclic AMP phosphodiesterase (PDE IVD). *Gene*. **138**:253-256.

Bai, T. R., Lam, R., and Prasad, F. Y. F. 1989. Effects of adrenergic agonists and adenosine on cholinergic neurotransmission in human tracheal smooth muscle. *Pulm. Pharmacol.* **1**:193-199.

Bai, T. R., Mak, J. C., and Barnes, P. J. 1992. A comparison of β-adrenergic receptors and *in vitro* relaxant responses to isoproterenol in asthmatic airway smooth muscle. *Am. J. Respir. Cell Mol. Biol.* **6**:647-651.

Baker, A. J., and Fuller, R. W. 1990. Anti-inflammatory effect of salmeterol on human alveolar macrophages. *Am. Rev. Respir. Dis.* **141**:A394.

Baker, D. G., and Don, U. 1987. Catecholamines abolish vagal but not acetylcholine tone in the cat trachea. *J. Appl. Phsiol.* **63**:2490-2498.

Ball, D. I., Brittain, R. T., Coleman, R. A., Denyer, L. H., Jack, D., Johnson, M., Lunts, L. H., Nials, A. T., Sheldrick, K. E., and Skidmore, I. F. 1991. Salmeterol, a novel, long-acting $\beta_2$-adrenoceptor agonist: characterization of pharmacological activity in vitro and in vivo. *Br. J. Pharmacol.* **104**:665-71.

Barak, L. S., Tiberi, M., Freedman, N. J., Kwatra, M. M., Lefkowitz, R. J., and Caron, M. G. 1994. A highly conserved tyrosine residue in G protein-coupled receptors is required for agonist-mediated $\beta_2$-adrenergic receptor sequestration. *J. Biol. Chem.* **269**:2790-2795.

Barker, J. A., Chediak, A. J., Baier, H. J., and Wanner, A. 1988. Tracheal mucosal blood flow responses to autonomic agonists. *J. Appl. Physiol.* **65**:829-834.

Barnes, P. J. 1985. 1995. β-adrenergic receptors and their regulation. *Am. J. Respir. Crit. Care Med.* **152**:838-860.

Barnes, P.J., Basbaum, C. B., Nadal, J. A., and Roberts, J. M. 1982. Localisation of β-adrenoceptors in mammalian man and rat lung by light microscopic autoradiography. *Nature*. **299**:444-447.

Barnes, P. J., and Chung, K. F. 1992. Questions about inhaled β-agonists in asthma. *Trends Pharmacol. Sci.* **13**:20-23.

Barnes, P. J. 1986. Neural control of human airways in health and disease. *Am. Rev. Resp. Dis.* **134**:1289-1314.

Barnett, D. B., Rugg, E. L., and Nahorski, S. R. 1978. Direct evidence of two types of β-adrenoceptor binding site in lung tissue. *Nature*. **273**:166-168.

Beato, M., Herrlich, P., and Schutz, G. 1995. Steroid hormone receptors: many actors in search of a plot. *Cell* **83**:851-857.

Beavo, J. A., Conti, M., and Heaslip, R. J. (1994). Multiple cyclic nucleotide phosphodiesterases. *Mol. Pharmacol*. **46**: 399-405.

Beebe, S. J., and Corbin, J. D. 1986. Cyclic nucleotide-dependent protein kinases. *The Enzymes* **17**:43-111.

Beer, M., Hacker, S., Poat, J., and Stahl, S. M. 1987. Independent regulation of $\beta_1$- and $\beta_2$-adrenoceptors. *Br. J. Pharmacol*. **92**: 827-834.

Bellofiore, S., Di Maria, G. U., and Martin, J. G. 1987. Changes in upper and lower airway resistance after inhalation of antigen in in sensitised rats. *Am. Rev. Resp. Dis*. **136**:363-368.

Belvisi, M. G., Patel, H. J., Takahashi, T., Barnes, P. J., and Giembycz, M. A. 1996. Paradoxical facilitation of acetylcholine release from parasympathetic nerves innervating guinea-pig trachea by isoprenaline. *Br. J. Pharmacol.* **117**:1413-1420.

Bengtsson, B., and Fagerstrom, P-O. 1982. Extrapulmonary effect of terbutaline during prolonged administration. *Clin. Pharmacol. Ther*. **31**:726-732.

Benovic, J. L., Staniszewski, C., Mayor, F. Jr., Caron, M. G., and Lefkowitz, R, J. 1988. β-Adrenergic receptor kinase. Activity of partial agonists for stimulation of adenylate cyclase correlates with ability to promote receptor phosphorylation. *J. Biol. Chem*. **263**:3893-3897.

Benovic, J., Kuhn, H, Weyland, I., Codina, J., Caron, M., and Lefkowitz, R. J. 1987. Functional desensitisation of the isolated β-adrenergic receptor kinase: potential role of an analog of the retinal protein arrestin (48-kDa protein). *Proc. Natl. Acad. Sci. USA*. **84**:8879-8882.

Benovic, J., Mayor, F. Jr., Staniszewski, C., Lefkowitz, R. J., and Caron, M. G. 1987. Purification and characterisation of the β-adrenergic receptor kinase. *J. Biol. Chem*. **262**:9025-9032.

Benovic, J. L., DeBlasi, A., Stone, W. C., Caron, M. G., and Lefkowitz, R. J. 1989. β-adrenergic receptor kinase: primary structure delineates a multigene family. *Science*. **246**:235-240.

Benovic, J. L., Pike, L. J., Cerione, R. A., Staniszewski, C., Yoshimasa, T., Codina, J., Caron, M. G., and Lefkowitz, R. J. 1985. Phosphorylation of

the mammalian β-adrenergic receptor by cyclic AMP-dependent protein kinase. *J. Biol. Chem.* **260**:7094-7101

Berman, D. M., Kozasa, T., and Gilman, A. G. 1996. The GTPase-activating protein RGS4 stabilizes the transition state for nucleotide hydrolysis. *J. Biol. Chem.* **271**:27209-27212.

Berman, D. M., Wilkie, T. M., and Gilman, A. G. 1996. GAIP and RGS4 are GTPase-activating proteins for the Gi subfamily of G protein alpha subunits. *Cell.* **86**:445-452.

Berstein, G., Blank, J. L., Jhon, D. Y., Exton J. H., Rhee, S. G., and Ross, E. M. 1992. Phospholipase C-β1 is a GTPase-activating protein for Gq/11, its physiologic regulator. *Cell* **70**:411-418.

Bhagat, R., Kalra, S., Swystun, V. A., and Cockcroft, D. W. 1995. Rapid onset of tolerance to the bronchoprotective effect of salmeterol. *Chest.* **108**:1235-1239.

Birnbaumer, L., Abromowitz, J., and Brown, A. M. 1990. Receptor-effector coupling by G proteins. *Biochem. Biophys. Acta* **1031,** 163-224.

Bishopric, N. H., Cohen, H. J., and Lefkowitz, R. J. 1980. Beta adrenergic receptors in lymphocyte subpopulations. *J. Allergy Clin. Immunol.* **65**:29-33.

Blair, A. M. J. N., and Woods, A. 1969. The effects of isoprenaline, atropine and disodium cromoglycate on ciliary motility and mucous flow measured in vivo in cats. *Br. J. Pharmacol.* **35**:379P-380P.

Blake, A. D., Mumford, R. A., Strout, H. V., Slater, E. G., and Strader, C. D. 1987. Synthetic segments of the mammalian βAR are preferentially recognized by cAMP-dependent protein kinase and protein kinase C. *Biochem. Biophys. Res. Commun.* **147**:168-173.

Boekhoff, I., Inglese, J., Schleicher, S., Koch, W. J., Lefkowitz, R. J., and Breer, H. 1994. Olfactory desensitisation requires membrane targeting of receptor kinase mediated by βγ-subunits of heterotrimeric G-proteins. *J. Biol. Chem.* **269**:37-40.

Bokoch, G. M., Katada, T., Northup, J. K., Hewlett, E. L., and Gilman, A. G. 1983. Identification of the predominant substrate for ADP-ribosylation by islet activating protein. *J. Biol. Chem.* **258**:2072-2075.

Bolger, G., Michaeli, T., Martins, T., St. John, T., Steiner, B., Rodgers, L., Riggs, M., Wigler, M., and Ferguson, K. 1993. A family of human phosphodiesterases homologous to the dunce learning and memory gene

product of *Drosophila melanogaster* are potential targets for antidepressant drugs. *Mol. Cell. Biol.* **13**:6558-6571.

Boschetto, P., Roberts, N. M., Rogers, D. F., and Barnes PJ. 1989. The effect of antiasthma drugs on microvascular leak in guinea pic airways. *Am. J. Respir. Dis.* **139**:416-421.

Bourne, H. R., Lichtenstein, L. M., Melmon, K. L., Henney, C. S., Weinstein, Y., and Shearer, G. M. 1974. Modulation of inflammation and immunity by cAMP: receptors for vasoactive hormones and mediators of inflammation regulate many leukocyte functions. *Science Wash. DC.* **184**:19-29.

Bouvier, M., Collins, S., O'Dowd, B. F., Campbell, P. T., DeBlasi, A., Kobilka, B. K., Macgregor, C., Irons, G. P., Caron, M. G., and Lefkowitz, R. J. 1989. Two distinct pathways for cAMP-mediated down-regulation of the $\beta_2$-adrenergic receptor. Phosphorylation of the receptor and regulation of its mRNA level. *J. Biol. Chem.* **264**:16786-16792.

Bouvier, M., Guilbalt, N., and Bonin, H. 1991. Phorbol-ester-induced phosphorylation of the $\beta_2$-adrenergic receptor decreases its coupling to $G_s$. *FEBS Lett.* **279**:288-293.

Bouvier, M., Hausdorff, W. P., De Blasi, A., O'Dowd, B. F., Kobilka, B. K., Caron, M. G., and Lefkowitz, R. J. 1988. Removal of phosphorylation sites from the $\beta_2$-adrenergic receptor delays onset of agonist-promoted desensitization. *Nature.* **333**:370-373.

Bouvier, M., Leeb-Lundberg, L. M. F., Benovic, J. L., Caron, M. G., and Lefkowitz, R. J. 1987. Regulation of arenergic receptor function by phosphoryiation. II. Effects of agonist occupancy on phosphorylation of $\alpha_1$- and $\beta_2$-adrenergic receptors by protein kinase C and the cyclic AMP-dependent protein kinase. *J. Biol. Chem.* **262**:3106-3113.

Bradley, D. E., McNary, W. F., and El-Bermani, A. W. 1970. The distribution of acetylcholinesterase and catecholamine containing nerves in the rat lung. *Anat. Rec.* **167**:205-207.

Bradshaw, J., Brittain, R. T., Coleman, R. A., Jack, D., Kennedy, I., Lunts, L. H. C., and Skidmore, I. F. 1987. The design of salmeterol, a long-acting selective $\beta_2$-adrenoceptor agonist. *Br. J. Pharmacol.* **92**: 920P

Breeze, R. G., and Wheeldon, E. B. 1977. The cells of the pulmonary airways. *Am. Rev.Respir. Dis.* **116**:705-714.

Brittain, R. T. 1990. Approaches to a long-acting selective $\beta_2$-adrenoceptor stimulant. *Lung* **168**:142-153.

Brodde, O-E., Beckeringh, J. J., and Michel, M. C. 1987. Human heart β-adrenoceptors: a fair comparison with lymphocyte-adrenoceptors? *Trends Pharmacol. Sci.* **8**:403-407.

Brooker, G., Harper, J. F., Terasaki, W. L., and Moyolin, R. D. 1979. Radioimmunoassay of cyclic AMP and cyclic GMP. *Adv. Cyclic Nucleotide Res.* **10**: 1-33.

Brown, A. M., and Birnbaumer, L. B. 1988. Direct G protein gating of ion channels. *Am. J. Physiol.* **23**:H401-H410.

Bullowa, J. G. M., and Kaplan, D. M. 1903. On the hypodermatic use of adrenaline chloride in the treatment of asthmatic attacks. *Med News* **83**:787-790.

Bullrich, F., Druck, T., Kunapuli, P., Gomez, J., Gripp, K. W., Schlegelberger, B., Lasota, J., Aronson, M., Cannizzaro, L. A., and Huebner, K. 1995. Chromosomal mapping of the genes GPRK5 and GPRK6 encoding G protein-coupled receptor kinases GRK5 and GRK6. *Cytogenet. Cell Genet.* **70**:250-254.

Busse, W. W., and Sosman, J. M. 1984. Isoproterenol inhibition of isolated neutrophil function. *J. Allergy Clin. Immunol.* **73**:404-410.

Butchers, P. R., Skidmore, I. F., Vardey, C. J., and Wheeldon, A. 1980. Characterisation of the receptor mediating antianaphylactic effects of β-adrenoceptor agonists in human lung tissues *in vitro*. *Br. J. Pharmacol.* **71**:663-667.

Butchers, P. R., Vardey, C. J., and Johnson, M. 1991. Salmeterol: a potent and long acting inhibitor of inflammatory mediator release from himan lung. *Br. J. Pharm.* **104**:672-676.

Buur, T., Clausen, T., Holmberg, E., Hohansson U., and Waldeck B. 1982. Desensitization by terbutaline of β-adrenoceptor in the guinea-pig soleus muscle: biochmeical alterations associated with functional changes. *Br. J. Pharmacol.* **76**:313-317.

Carman, C. V., Som, T., Kim, C. M., and Benovic, J. L. 1998. Binding and phosphorylation of tubulin by G protein-coupled receptor kinases. *J. Biol. Chem.* **273**:20308-20816.

Caron, M. G., and Lefkowitz, R, J. 1976, Solubilization and characterization of the β-adrenergic receptor binding sites of frog erythrocytes. *J. Biol. Chem.* **251**:2374-2384.

Carstairs, J. R., Nimmo, A. J., and Barnes, P. J. 1984. Autoradiographic localisation of β-adrenoceptors in human lung. *Eur. J. Pharmacol.* **103**:189-190.

Carstairs, J. R., Nimmo, A. J., and Barnes, P.J. 1985. Autoradiographic visualization of β-adrenoceptor subtypes in human lung. *Am. Rev. resp. Dis.* **132**:541-547.

Casey, P. J. 1995. Protein lipidation in cell signalling. *Science.* **268**:221-225.

Cerrina, J., Le Roy Ladurie, M., Labat, C., Raffestin, B., Bayol, A., Brink, C. 1986. Comparison of human bronchial muscle responses to histamine *in vivo* with histamine and isoproterenol agonists *in vitro*. *Am. Rev. Respir. Dis.* **134**:57-61

Chapman, K. R., and Rebuck, A. S. 1985. Bronchodilators, hypokaleamia, and fatal asthma. *Lancet* **2**:162.

Chatterjee, T. K., Eapen, A. K., and Fisher, R. A. 1997. A truncated form of RGS3 negatively regulates G protein-coupled receptor stimulation of adenylyl cyclase and phosphoinositide phospholipase C. *J. Biol. Chem.* **272**:15481-15487

Chen, C. K., Wieland, T., and Simon, M. I. 1996. RGS-r, a retinal specific RGS protein, binds an intermediate conformation of transducin an enhances recycling. *Proc. Natl. Acad. Sci. USA* **93**:12285-12889.

Cherrington, A. D., Assimacopoulos, F. D., Harper, S. C., Corbin, J. D., Park, C. R., and Exton, J. H. 1976. Studies on the α-adrenergic activation of hepatic glucose output. II. Investigation of the roles of adenosine cyclic 3', 5'-monophosphate and guanosine cyclic 3', 5'-monophosphate-dependent protein kinase in the actions of phenylephrine in isolated hepatocytes. *J. Biol. Chem.* **251**:5209-5218.

Cherry, J. A., and Davis, R. L. 1995. A mouse homolog of dunce, a gene important for learning and memory in Drosophila, is preferentially expressed in olfactory receptor neurons. *J. Neurobiol.* **28**:102-113.

Cheung, A. H., Huang, R-R. C, Graziano, M. P., and Strader, C. D. 1991. Specific activation of $G_s$ by synthetic peptides corresponding to an intracellular loop of the β-adrenergic receptor. *FEBS lett.* **279**:277-278.

Cheung, D., Timmers, M. C., Zwinderman, A. H., Bel, E. H., Dijkman, J. H., and Sterk, P. J. 1992. Long-term effects of a long-acting $\beta_2$-adrenoceptor agonist, salmeterol, on airway hyperresponsiveness in patients with mild asthma. *N. Engl. J. Med.* **327**:1198-1203.

Chomczynski, P., and Sacchi, N. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**: 156-159.

Chuang, T. T., Lacovelli, L., Sallese, M., and De Blasi, A. 1996. G protein-coupled receptors: heterologous regulation of homologous desensitisation and its implications. *Trends Pharmacol. Sci.* **17**:416-21.

Chuang T. T., LeVine, H. III., and De Blasi, A. 1995. Phosphorylation and activation of β-adrenergic receptor kinase by protein kinase C. *J. Biol. Chem.* **270**:18660-18665.

Chung, F -Z., Lentes, K. U., Gocayne, J., Fitzgerald, M., Robinson, D., Kerlavage, A. R., Fraser, C. M., and Venter, J. C. 1987. Cloning and sequence analysis of the human brain β-adrenergic receptor. *FEBS Lett.* **211**:200-206.

Church, M. K. and Hiroi, J. 1987. Inhibition of IgE-dependent histamine release from human dispersed lung mast cells by anti-allergic drugs and salbutamol. *Br. J. Pharmacol.* **90**:421-429

Clapham. D. E., and Neer, E. J. 1993. New roles for G-protein βγ-dimers in transmembrane signalling. *Nature*. **365**:403-406.

Clapham, D. E. 1996. The G-protein nanomachine. *Nature* **379,** 297-299.

Clark, R. B. 1986. Desensitization of hormonal stimuli coupled to regulation of cyclic AMP levels. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **20**:151-209.

Clark, R. B., Friedman, J., Dixon, R. A. F., and Strader, C. D. 1989. Identification of a specific site required for rapid heterologous desensitization of the β-adrenergic receptor by cAMP-dependent protein kinase. *Mol. Pharmacol.* **36(3)**:343-8.

Clark, R. B., Friedman, J., Johnson, J. A., and Kunkel, M. W. 1987. β-adrenergic receptor Desensitization of wild-type but not cyc lymphoma cells unmasked by submillimolar $Mg^{2+}$. *FASEB J.* **1**:289-97.

Clark, R. B., Kunkel, M. W., Friedman, J., Goka, T. J., and Johnson, J. A. 1988. Activation of cAMP-dependent protein kinase is required for heterologous desensitization of adenylyl cyclase in S49 wild-type lymphoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **85**:1442-6.

Cockcroft, D., McParland, C. P., Britto, S. A., Swystun, V. A., and Rutherford, C. 1993. Regular inhaled salbutamol and airway responsiveness to allergen. *Lancet* **342**:833-837.

Cockcroft, D. W., and Murdock, K. Y. 1987. Comparitive effect of inhaled salbutamol, sodium cromoglycate, and beclomethasone dipropionate on allergen-induced early asthmatic responses, late asthmatic responses, and increased bronchial responsiveness to histamine. *J. Allergy Clin. Immunol.* **79**:734-740.

Colicelli, J., Birchmeier, C., Michaeli, T., O'Neill, K., Riggs M., and Wigler M. 1989. Isolation and characterization of a mammalian gene encoding a high-affinity cAMP phosphodiesterase. *Proc. Natl. Acad. Sci. USA.* **86**:3599-3603.

Collins, S., Altschmied, J., Herbsman, O., Caron, M. G., Mellon, P. L., and Lefkowitz, R. J. 1990. A cAMP response element in the $\beta_2$-adrenergic receptor gene confers transcriptional autoregulation by cAMP. *J. Biol. Chem.* **265**:19330-19335.

Collins, S., Caron, M. G., and Lefkowitz, R J. 1988. $\beta_2$-adrenergic receptors in hamster smooth muscle cells are transcriptionally regulated by glucocorticoids. *J. Biol. Chem.* **263**:9067-9070.

Collins, S., Caron, M. G., and Lefkowitz, R. J. 1992. From ligand binding to gene expression: new insights into the regulation of G protein-coupled receptors. *Trends Biochem. Sci.* **17**:37-40.

Collins, S., Lohse, M. J., O'Dowd, B., Caron, M. G., and Lefkowitz R. J. 1991. Structure and regulation of G protein-coupled receptors: the $\beta_2$-adrenergic receptor as a model. *Vitam. Horm.* **46**:1-39

Conklin, B. R., Farfel, Z., Lustig, K. D., Julius, D., and Bourne, H. R. 1993. Substitution of three amino acids switches receptor specificity of $G_{q\alpha}$ to that of $G_{i\alpha}$. *Nature* **363,** 274-276.

Conolly, M. E., and Greenacre, J. K. 1976. The lymphocyte β-adrenoceptor on normal subjects and patients with asthma:the effect of different forms of treatment on receptor function. *J. Clin. Invest.* **58**:1307-1316.

Conti. M., Nemoz, G., Sette, C., and Vicini, E. 1995. Recent progress in understanding the hormonal regulation of phosphodiesterases. *Endocr. Rev.* **16**:370-389.

Cooper, D. M., Karpen, J. W., Fagan, K. A., and Mons, N. E. 1998. $Ca^{2+}$-sensitive adenylyl cyclases. *Adv. Second Messenger Phosphoprotein Res. Lippincott-Raven Publishers, Philadelphia.* **32**:1-51.

Corea, L., Bentivoglio, M., and Verdecchia, P. 1981. Hypokalaemia due to salbutamol overdosage. *Br. Med. J.* **283**: 500.

Crane, J., Pearce, N., Flatt, A., Burgess, C., Jackson, R., Kwong, T., Ball, M., and Beasley, R. 1989. Prescribed fenoterol and death from asthma in New Zealand, 1981-83:case-control study. *Lancet* **29**:917-22.

Cryer, P. E. 1980. Physiology and pathophysiology of the human sympathoadrenal neuroendocrine system. *N. Engl. J. Med.* **303**:436-444.

Daaka, Y., Luttrell, L. M., and Lefkowitz, R. J. 1997. Switching of the coupling of the $\beta_2$-adrenergic receptor to different G proteins by protein kinase A. *Nature*. **390**:88-91.

Daaka, Y., Luttrell, L. M., Ahn, S., Della Rocca, G. J., Ferguson, S. S., Caron, M. G., and Lefkowitz, R. J. 1998. Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. *J. Biol. Chem.* **273**:685-688.

Danner, S., and Lohse, M. J., 1997, Cell type-specific regulation of $\beta_2$-adrenoceptor mRNA by agonists. *Eur. J. Pharmacol.* **331**: 73-78.

Danser, A. H. J., Van den Ende, R., Lorenz, R. R., Flavahan, N. A., and Vanhoutte, P. M. 1987. Prejunctional $\beta_1$-adrenoceptors inhibit cholinergic neurotransmission in canine bronchi. *J. Appl. Physiol.* **62**:785-790.

Davies, A. O., and Lefkowitz, R. J. 1981. Agonist promoted high affinity state of the β-adrenergic receptor in human neutrophils:modulation by corticosteroids. *J. Clin. Endocrinol. Metab.* **53**:703-708.

Davies, A. O., and Lefkowitz, R. J. 1984. Regulation of β-adrenergic receptors by steroid hormones. *Annu. Rev. Physiol.* **46**:119-130.

Davis, R. L., Takayasu, H., Eberwine, M., and Myres, J. 1989. Cloning and characterization of mammalian homologs of the Drosophila dunce gene. *Proc. Natl. Acad. Sci. USA*. **86**:3604-3608.

De Lanerolle, P., and Paul, R. J., 1991. Myosin phosphorylation/dephosphorylation and regulation of airway smooth muscle contractility. *Am. J. Physiol.* **261**:L1-L14.

Delpierre, S., Orehek, J, Beaupre, A., Velardocchio, M., Fornaris, M., and Grimaud, C. 1983. Comparative reflex action of histamine, acetylcholine and serotonin on dog airways. *Bull. Eur. Physiopathol. Respir.* **19**:489-493.

Dent, G., and Giembycz, M. A. 1995. Selective phosphodiesterase inhibitors in the therapy of asthma. *Clin. Immunother.* **3**: 423-437.

Dent, G., Giembycz, M. A., Rabe, K. F., Evans, P. M., and Barnes, P. J. 1994. Suppression of respiratory burst in human eosinophils by

phosphodiesterase inhibitors: interaction with the β-adrenoceptor agonist albuterol. *J. Pharmacol. Exp. Ther.* **271**:1167-1174.

Dessauer, C. W., and Gilman, A. G. 1996. Purification and characterization of a soluble form of mammalian adenylyl cyclase. *J. Biol. Chem.* **271**:16967-16974.

Diamond, J. 1978. Role of cyclic nucleotides in control of smooth muscle contraction. *Adc. Cyclic. Nucl. Res.* **9**:327-340.

Di Maria, G. U., Bellofiore, S., Sapienz, S., Martin, J. G., and Mistretta, A. 1989. The site of airway responses to methacholine or antigen inhalation challenge. *Eur. Resp. J.* **2**:523-526.

Di Maria, G. U., Martin, J. G., Bellofiore, S., and Mistretta, A. 1988. Relationship between in vivo airway reactivity and in vitro responsiveness of tracheal smooth muscle in inbred rats. Respiration **54**:108-113.

Di Maria, G.U., Wang, C. G., Bates, J. H. T., Guttmann, R., and Martin, J. G. 1987. Partitioning of airway responses to inhaled methacholine in the rat. *J. Appl. Physiol.* **62**:1317-1323.

Dixon, W. E., and Brodie, T. G. 1903. Contributions to the physiology of the lungs. Part 1. The bronchial muscles, their innervation, and the action of drugs upon them. *J. Physiol.* **29**:97-173.

Dixon, R. A. F., Kobilka, B. K., Strader, C. D., and Benovic, J. L. 1986. Cloning of the gene and cDNA for mammalian β-adrenergic receptor and homology with rhodopsin. *Nature* **321**:75-79.

Dixon, R. A. F., Sigal, I. S., Candelor, M. R., Register, R. B., Scattergood, W., Rands, E., and Strader, C. D. 1987b. Structural features required for ligand binding to the β-adrenergic receptor. *EMBO J.* **6,** 3269-3275.

Dixon, R. A. F., Sigal, I. S., Rands, E., Register, R. B., Candelore, M. R., Blake, A. D., and Strader, C. D. 1987a. Ligand binding to the β-adrenergic receptor involves its rhodopsin-like core. *Nature* **326:**73-77.

Dixon, R. A. F., Sigal, I. S., and Strader, C. D. 1988. Structure-function analysis of the beta-adrenergic receptor. *Cold Spring Harb Symp Quant Biol.* **53**:487-97.

Dohlman, H. G., Bouvier, M., Benovic, J. L., Caron, M. G., and Lefkowitz, R. J. 1987. The multiple membrane spanning topography of the $\beta_2$-adrenergic receptor. *J. Biol. Chem.* **262**:14282-14288.

Dohlman, H. G., Caron, M. G., De Blasil, A., Frielle, T., and Lefkowitz, R. J. 1990. Role of extracellular disulfide-bonded cysteines in the ligand

binding function of the $\beta_2$-adrenergic receptor. *Biochemistry* **29**:2335-2342.

Donnelly, L. E., Boyd, R.S., Williams, R. J., Kelly, E., and Macdermot, J. 1995. Inhibition of ADP-ribosyltransferase increases synthesis of $G_s\alpha$ in neuroblastoma x glioma hybrid cells and reverses iloprost-dependent heterologous loss of fluoride-sensitive adenylate cyclase. *Biochem. Pharmacol.* **49**:767-776.

Drmota, T., Novotny, J., Kim, G. D., Eidne, K. A., Milligan, G., and Svobada, P. 1998. Agonist-induced internalization of the G prtoein $G_{11\alpha}$ and thyrotropin-releasing hormone receptors proceed on different time scales. *J. Biol. Chem.* **273**:21699-21707.

Dulis, B. H., and Wilson, T. B. 1980. The β-adrenergic receptor of live human polymorphonuclear leukocytes. *J. Biol. Chem.* **225**:1043-1048.

Dupree, P., Parton, R. G., Raposo, G., Kurzchalia, T. V., and Simons, K. 1993. Caveolae and sorting in the trans-Golgi network of epithelial cells. *EMBO J.* **12**:1597-1605

Eason, M. G., Kurose, H., Holt, B. D., Raymond, J. R., and Liggett, S. B. 1992. Simultaneous coupling of $\alpha_2$-adrenergic receptors to two G proteins with opposing effects. Subtype-selective coupling of α2C10, α2C4 and α2C2 adrenergic receptors to Gi and Gs *J. Biol. Chem.* **267**, 15795-15801.

El-Bermani, A. W. 1978. Pulmonary noradrenic innervation of rat and monkey: a comparitive study. *Thorax* **33**:167-174.

Ellis, K. E., Mistry, R., Boyle, J. P., and Challiss, R. A. J. 1995. Correlation of cyclic AMP accumulation and reiaxant actions of saimeterol and salbutamol in bovine tracheal smooth muscle. *Br. J. Pharmacol.* **116**: 2510-2516

Emorine, L. J., Marullo, S., Briend-Sutren, M -M., Patey, G., Tate, K., Delavier-Klutchko, C., and Strosberg, A. D. 1989. Molecular characterization of the human $\beta_3$-adrenergic receptor. *Science* **245**:1118-1121.

Encio, P. J., and Detgra-Wadleigh, S. D. 1991. The genomic structure of the human glucocorticoid receptor. *J. Biol. Chem.* **266**:7182-7188.

Engel, G., Hoyer, D., Berthold, R., and Wagner, H. 1981. (±)-[$^{125}$Iodo]-cyanopindalol, a new ligand for β-adrenoceptors. Identification and quantitation of subclass of β-adrenoceptors in guinea pig. *Naunyn-Schmeidebergs Arch. Pharmacol.* **317**:277-285.

Engels, F., Carstairs, J. R., Barnes, P. J., and Nijkamp, F. P. 1989. Autoradiographic localization of changes in pulmonary β-adrenoceptors in an animal model of atopy. *Eur. J. Pharmacol.* **164**:139-146.

Engels, P., Sullivan, M., Muller, T., and Lubbert, H. 1995. Molecular cloning and functional expression in yeast of a human cAMP-specific phosphodiesterase subtype (PDEIV-C). *FEBS Lett.* **358**:3085-310.

Erjefält, I., and Persson, C. G. A. 1991. Pharmacologic control of plasma exudation into tracheo-bronchial airways. *Am. Rev. Respir. Dis.* **143**:1008-1014.

Farley, J. M., and Miles, P. R. 1978. The sources of calcium for acetylcholine-induced contractions of dog tracheal smooth muscle. *J. Pharmac. exp. Ther.* **207**:340-346.

Faurobert, E., and Hurley, J. B. 1997. The core domain of a new retina specific RGS protein stimulates the GTPase activity of transducin in vitro. *Proc. Natl. Acad. Sci. USA.* **94**:2945-2950.

Federman, A. D., Conklin, B. R., Schrader, K. A., Reed, R. R., and Bourne, H. R. 1992. Hormonal stimulation of adenylyl cyclase through Gi-protein βγ subunits. *Nature* **356,** 159-161.

Felbel, J., Trockur, B., Ecker, T., Landgraf, W., and Hoffman, F. 1988. Regulation of cytosolic calcium by cAMP and cGMP in freshly isolated smooth muscle cells from bovine trachea. *J. Biol. Chem.* **263**: 16764-16771.

Feldman, R. D. 1987. β-adrenergic receptor-mediated suppression of interleukin 2 receptors in human lymphocytes. *J. Immunol.* **139**:3355-3359.

Ferguson, S. S., Downey, W. E., Colapietro, A. M., Barka, L. S., Menard, L., and Caron, M. G. 1996. Role of β-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. *Science.* **271**:363-366.

Ferguson, S. S., Manard, L., Barak, L. S, Kock, W. J., Colapietro, A. M., and Caron, M. G. 1995. Role of phosphorylation in agonist-promoted $\beta_2$-adrenergic receptor sequestration. Rescue of a sequestration-defective mutant receptor by βARK1. *J. Biol. Chem.* **270**:24782-24789.

Fernandes, L. B., Fryer, A. D., and Hirschman, C. A. 1992. $M_2$ muscarinic receptors inhibit isoproterenol-induced relaxation of canine airway smooth muscle. *J. Pharmacol. Exp. Ther.* **262**:119-126.

Fisher, D. A., Smith, J. F., Pillar, J. S., St Denis, S. H., and Cheng, J. B. 1998. Isolation and characterization of PDE9A, a novel human cGMP-specific phosphodiesterase. *J. Biol. Chem.* **273**:15559-15564.

Fitzgerald, G. A., Barnes, P. J., Hamilton, C. A., and Dollery, C. T. 1980. Circulating adrenaline and blood pressure: the metabolic effects and kinetics of infused adrenaline in man. *Eur. J. Clin. Invest*. **10**:401-406.

Florio, V. A., and Sternweis, P. C. 1989. Mechanisms of muscarinic receptor action on $G_o$ in reconstituted phospholipid vesicles. *J. Biol. Chem.* **260,** 3909-3915.

Fredericks, Z. L., Pitcher, J. A., and Lefkowitz, R. J. 1996. Identification of the G protein-coupled receptor kinase phosphorylation sites in the human $\beta_2$-adrenergic receptor. *J. Biol. Chem*. **271**:13796-13803.

Freedman, N. J., and Lefkowitz, R. J. 1996. Desensitisation of G protein coupled receptors. *Recent Horm. Res*. **51**:319-353.

Frielle, T., Collins, S., Daniel, K. W., Capron M. G., Lefkowitz, R. J., and Kobilka, B. K. 1987. Cloning of the cDNA for the $\beta_1$-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **84**:7920-7924.

Frigas, E., and Gleich, J. 1986. The eosinophil and the pathophysiology of asthma. *J. Allergy Clin. Immunol.* **77**:527-537.

Fujishige, K., Kotera, J., Michibata, H., Yuasa, K., Takebayashi, S., Okumura, K., and Omori, K. 1999. Cloning and characterization of a novel human phosphodiesterase that hydrolyzes both cAMP and cGMP (PDE10A). *J. Biol. Chem*. **274**:18438-18445.

Fuller, R. W., O'Malley, G., Baker, A. J., and MacDermot, J. 1988. Human alveolar macrophage activation: inhibition by forskolin but not β-adrenoceptor stimulation or phosphodiesterase inhibition. *Pulm. Pharmacol*. **1**:101-106.

Galant, S.P., and Allred, S. 1981. Binding and functional characteristics of beta-adrenergic receptors in the intact neutrophils. *J. Lab. Clin. Med.* **98**:227-237.

Galant, S. P., Durisetti, L., Underwood, S., Allred, S., and Insel, P. A. 1980. β-adrenergic receptors of polymorphonuclear particulates in bronchial asthma. *J. Clin. Invest*. **65**:577-585.

Gao, B. N., and Gilman, A. G. 1991. Cloning and expression of a widely distributed (type IV) adenylyl cyclase. *Proc. Natl. Acad. Sci. USA*. **88**:10178-10182.

Gardiner, P. V., Ward, C., Booth, H., Allison, A., Hendrick, D. J., and Walters, E. H. 1994. Effect of eight weeks of treatment with salmeterol on

bronchoalveolar lavage inflammatory indices in asthmatics. *Am. J. Respir. Crit. Care Med.* **150**:1006-1011

Garritsen, A., and Simonds, W. F. 1994. Multiple domains of G protein beta confer subunit specificity in beta gamma interaction. *J. Biol. Chem.* **269**:24418-24423.

Garritsen, A., van Galen, P. J. M., and Simonds, W. F. 1993. The N-terminal coiled-coil domain of β is essential for γ association: a model for G-protein βγ subunit interaction. *Proc. Natl. Acad. Sci USA.* **90**:7706-7710.

Gatto, C., Green, T. P., Johnson, M. G., Marchessault, R. P., Seybold, V., and Johnson, D. E. 1987. Localisation of quantitiative changes in pulmonary β-receptors in ovalbumin-sensitized guinea-pigs. *Am. Rev. Repir. Dis.* **136**:150-154.

Gauthier, C., Tavernier, G., Charpentier, F., Languin, D., and Le Marec, H. 1996. Functional $\beta_3$-adrenoceptor in the human heart. *J. Clin. Invest.* **98**:556-562.

Gibson, G. J., Greenacre, J. R., König, P., Conolly, M. E., and Pride, N. B. 1978. Use of exercise challenge to investigate possible tolerance to β-adrenoceptor stimulation in asthma. *Br. J. Dis. Chest* **72**:199-206.

Giembycz, M. A. and Raeburn, D. 1991. Putative substrates for cyclic nucleotide-dependent protein kinases and the control of airway smooth muscle tone. *J. Autonomic Pharmacol.* **11**:365-398.

Giembycz, M. A. 1996. Phosphodiesterase 4 and tolerance to $\beta_2$-adrenoceptor agonists in asthma. *Trends. Pharmacol. Sci.* **17**:331-336

Giembycz, M. A. 1992. Could isoenzyme-selective phosphodiesterase inhibitors render bronchodilator therapy redundant in the treatment of bronchial asthma? *Biochem. Pharmacol.* **43**: 2041-2051.

Giembycz, M. A., and Diamond, J. 1990a. Characterisation of cyclic AMP-dependent protein kinases in guinea-pig lung employing the novel synthetic substrate, Kemptide: In vitro sensitivity of the soluble enzyme to isoprenaline, forskolin, methacholine and leukotriene $D_4$. *Biochem. Pharmacol.* **39**:1297-1312.

Giembycz, M. A., and Diamond, J. 1990b. Evaluation of Kemptide, a serine containing heptapeptide, for the estimation of cyclic AMP-dependant protein kinase activity in respiratory tissues. *Biochem. Pharmacol.* **39**: 271-283.

Gilman, A. G. 1987. G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56**:615-649.

Gleich, G. J., and Adolphson, C. R. 1986. The eosinophil leukocyte:structure and function. *Adv. Immunol.* **39**:177-253.

Goldie, R. G., Papadimitriou, J. M., Paterson, J. W, Rigby, P. J., Self, H. M, and Spina, D. 1986. Influence of epithelium on responsiveness of guinrea-pig isolated trachea to contractile and relaxant agonists. *Br. J. Pharmacol.* **87**:5-14.

Goldie, R. G., Spina, D., Henry, P. J., Lulich, K. M., and Paterson, J. W. 1986. *In vitro* responsiveness of human asthmatic bronchus to carbachol, histamine, β-adrenoceptor agonists and theophylline. *Br. J. Clin. Pharmacol.* **22**:669-676.

Goodman, O. B., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. 1997. β-arrestin acts as a clathrin adaptor in endocytosis of the $\beta_2$-adrenergic receptor. *Nature.* **383**:447-450.

Grainger, J., Woodman, K., Pearce, N., Crane, J., Burgess, C., Keane, A., and Besley, R. 1991. Prescribed fenoterol and death from asthma in New Zealand, 1981-87: A further case-controlled study. *Thorax* **46**:105-111.

Gratziou, C., Roberts, J. A., Bradding, P., Holgate, S. T., and Howarth, P. H. 1992. The influence of the long acting β-agonist, salmeterol xinafoate on T-lymphocyte lavage populations and activation status in asthma. *Am. Rev. Respir. Dis.* **145**:A67.

Graziano, M. P., Freissmuth, M., and Gilman, A. G. 1989. Expresson of $G_{s\alpha}$ in *Escherichia coli*: purification and properites of two forms of the protein. *J. Biol. Chem.* **264**:409-418.

Green, A., Johnson, J. L., and Milligan, G. 1990. Down-regulation of Gi subtypes by prolonged incubation of adipocytes with an A1 adenosine receptor agonist. *J. Biol. Chem.* **265,** 5206-5210.

Green, S. A., Spasoff, A. P., Coleman, R. A., Johnson, M., and Liggett, S. B. 1996. Sustained activation of a G protein-coupled receptor via "anchored" agonist binding. Molecular localization of the salmeterol exosite within the $\beta_2$-adrenergic receptor. *J. Biol. Chem.* **271**:24029-2403.

Greening, A. P., Ind, P. W., Northfield, M., and Shaw, G. 1994. Added salmeterol versus higher-dose corticosteroid in asthma patients with symptons on existing inhaled corticosteroid. *Lancet* **344**:219-224.

Gronomeyer, H. 1992 Control of transcription activation by steroid hormone receptors. *FASEB J.* **6**:2524-2529.

Grove, A., and Lipworth, B. J. 1995. Bronchodilator subsensitivity to salbutamol after twice-daily salmeterol in asthmatic patients. *Lancet*. **346**:201-206.

Guderman, T., Kalkbrenner, F., and Schultz, G. 1996. Diversity and selectivity of receptor-G protein interaction. *Annu. Rev. Pharmacol. Toxicol*. **36**: 429-459.

Guillot, C., Fornaris, M., Badger, M., and Orehek, J. 1984. Spontaneous and provoked resistance to isoproterenol in isolated human bronchi. *J. Allergy Clin. Immunol*. **74**:713-718.

Gundogdu, A. S., Brown, P. M., Juul, S., Sachs, L., and Sonkson, P. H. 1979. Comparison of hormonal and metabolic effects of salbutamol infusion in normal subjects and insulin-requiring diabetics. *Lancet* **2**:1317-1321.

Gunst, S. S., and Stropp, J. Q. 1988. Effect of Na-K adenosinetriphosphatase activity on relaxation of canine tracheal smooth muscle. *J Appl. Physiol*. **64**:635-641.

Gurevich, V. V., Dion, S. B., Onorato, J. J., Ptasienski J., Kim, C. M., Sterne-Marr, R., Hosey, M. M., and Benovic, J. L. 1995. Arrestin interactions with G protein-coupled receptors. *J. Biol. Chem*. **270**:720-731.

Haalboom, J. R. E., Deenstra, M., and Struyrenberg, A. 1985. Hypokalemia induced by inhalation of fenoterol. *Lancet* **1**:1125-1127.

Hadcock, J. R., and Malbon, C. C. 1988. Regulation of β-adrenergic receptors by permissive hormones: glucocorticoids increase steady state levels of receptor mRNA. *Proc. Natl. Acad. Sci. USA*. **85**:8415-8419.

Hadcock, J. R., and Malbon, C. C. 1993. Agonist regulation of gene expression of adrenergic receptors and G proteins. *J. Neurochem*. **60**:1-9.

Hadcock, J. R., Port, J. D., and Malbon, C. C. 1991. Cross-regulation between G-protein-mediated pathways. Activation of the inhibitory pathway of adenylyl cyclase increases the expression of $\beta_2$-adrenergic receptors. *J. Biol. Chem*. **266**:11915-11922.

Hadcock, J. R., Ros, M., and Malbon, C. C. 1989. Agonist regulation of β-adrenergic receptor mRNA:analysis in S49 mouse lymphoma mutants. *J. Biol. Chem*. **264**:13956-13961.

Hadcock, J. R., Wang, H., and Malbon, C. C. 1989. Agonist-induced destabilization of β-adrenergic receptor mRNA. Attenuation of glucocorticoid-induced up-regulation of β-adrenergic receptors. *J. Biol. Chem*. **264**:19928-19933.

Haga, K., and Haga, T. 1992. Activation by G protein beta gamma subunits of agonist- or light-dependent phosphorylation of muscarinic acetylcholine receptors and rhodopsin. *J. Biol. Chem.* **267**:2222-2227.

Haga, T., Haga, K., and Kameyama, K. 1994. G protein-coupled receptor kinases. *J. Neurochem.* **63**:400-412.

Haga, K., Ogawa, H., Haga, T., and Murofushi, H. 1998. GTP-binding protein-coupled receptor kinase 2 (GRK2) binds and phosphorylates tubulin. *Eur. J. Biochem.* **255**:363-368.

Hall, I. P., Donaldson, J., and Hill, S. J. 1989. Inhibition of histamine stimulated inositol phospholipid hydrolysis by agents which increase cyclic AMP levels in bovine tracheal smooth muscle. *Br. J. Pharmacol.* **97**:603-613

Haraguchi, K., and Rodbell, M. 1990. Isoproterenol stimulates shift of G proteins from plasma membrane to pinocytotic vesicles in rat adipocytes: a possible means of signal dissemination. *Proc. Natl. Acad. Sci. USA* **87**:1208-1212.

Harvey, J. E., Baldwin, C. J., Wood, P. J., Alberti, K. G. M. M., and Tattersfield A. E. 1981. Airway and metabolic responsiveness to intravenous salbutamol in asthma: effect of regular inhaled salbutamol. *Clin Sci.* **60**:579-585.

Hauck, R. W., Harth, M., Schulz, C., Präuer, H., Böhm, M., and Schömig, A. 1997. Effects of $\beta_2$-agonist- and dexamethasone-treatment on relaxation and regulation of β-adrenoceptors in human bronchi and lung tissue. *Br. J. Pharmacol.* **121**:1523-1530.

Hausdorff, W. P., Bouvier, M., O'Dowd, B. F., Irons, G. P., Caron, M. G., and Lefkowitz, R. J. 1989. Phosphorylation sites on two domains of the $\beta_2$-adrenergic receptor are involved in distinct pathways of receptor desensitization. *J. Biol. Chem.* **264**:12657-12665.

Hausdorff, W. P., Campbell, P. T., Ostrowski, J., Yu, S. S., Caron, M. G., and Lefkowitz, R. J. 1991. A small region of the β-adrenergic receptor is selectively involved in its rapid regulation. *Proc. Natl. Acad. Sci. USA.* **88**:2979-2983.

Hayashi, M., Matsushima, K., Ohashi, H., Tsunoda, H., Murase, S., Kawarada, Y., and Tanaka, T. 1998. Molecular cloning and characterization of human PDE8B, a novel thyroid-specific isozyme of 3',5'-cyclic nucleotide phosphodiesterase. *Biophys. Biochem. Res. Commun.* **250**:751-756.

Heck, S., Kullmann, M., Gast, A., Ponta, H., Rahmsdorf, H. J., Herrlich, P., and Cato, A. C. 1994. A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1. *EMBO J.* **13**:4087-4095

Hellstrand, K., Hermodsson, S., and Stranegård, Ö. 1985. Evidence for a β-adrenoceptor-mediated regulation of human natural killer cells. *J. Immunol.* **134**:4095-4099.

Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Beckman, E., and Downing, K. H. 1990. Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy. *J. Mol. Biol.* **213,** 889-929.

Henricks, P. A. J., Van Esch, B., Van Oosterhout, A. J. M., and Nijkamp, F. P. 1988. Specific and non-specific effects of β-adrenoceptor agonists on guinea-pig alveolar macrophage function. *Eur. J. Pharmacol.* **152**:321-330.

Hepler, J. R. 1992. G proteins. *Trends Biochem. Sci.* **17**:383-387.

Hepler, J. R., Berman, D. M., Gilman, A. G., and Kozasa, T. 1997. RGS4 and GAIP are GTPase-activating proteins for $G_{q\alpha}$ and block activation of phospholipase Cβ by gamma-thio-GTP-$G_{q\alpha}$. *Proc. Natl. Acad. Sci. USA.* **94**:428-432.

Hesse, H.R., Kasparek, R., Mizera, W., Unterholzner, C., and Konierzko, N. 1981. Influence of reproterol on ciliary beat frequency of human bronchial epithelium in vitro. *Arzneim. Forsch.* **31**:716-718.

Hilbert, M. F., Trumpp-Kallmeyer, S., Hoflack, J., and Bruinvels, A. 1992. This is not a G protein-coupled receptor. *Trend. Pharmacol. Sci.* **14**, 7-12.

Hildebrandt, J. D., Codina, J., Rosenthal, W., Birnbaumer, L., Nee,r E. J., Yamazaki, A., and Bitensky, M. W. 1985. Characterization by two-dimensional peptide mapping of the γ subunits of Ns and Ni, the regulatory proteins of adenylyl cyclase, and of transducin, the guanine nucleotide-binding protein of rod outer segments of the eye. *J. Biol. Chem.* **260**:14867-14872.

Hildebrandt, J. D., Codina, J., Risinger, R., and Birnbaumer, L. 1984. Identification of a γ subunit associated with the adenylyl cyclase regulatory proteins Ns and Ni. *J. Biol. Chem.* **259**:2039-2042.

Hjemdahl, P., Larsson, K., Johansson, M. C., Zetterlund, A. and Eklund, A. 1990. β-adrenoceptors in human alveolar macrophages isolated by elutriation. *Br. J. Clin. Pharmacol.* **30**:673-682.

Holgate, S. T., Robinson, C., and Church, M. K. 1988. Mediators of immediate hypersensitivity. In:Allergy: Principles and Practise, edited by E. J. Middleton, C. E. Reed, E. F. Ellis, N. F. J. Adkinson and J. W. Yunginger. St Louis, MO:*Mosby*. p135-163.

Homburger, V., Lucas, M., Cantau, R., Barabe, J., Penit, J., and Bockaert, J. 1980. Further evidence that desensitisation of β-adrenergic-sensitive adenylate cyclase proceeds in two steps: modification of the coupling and loss of β-adrenergic receptors. *J. Biol Chem.* **255**:10436-10444.

Huang, J. C., Garcia, J. L., Reuben, J. P., and Kaczorowski, G. J. 1993. Inhibition of beta-adrenoceptor agonist relaxation of airway smooth muscle by $Ca^{2+}$-activated $K^+$ channel blockers. *Eur. J. Pharmacol.* **235**:37-43.

Hui, K. K., Conolly, M. E., and Tashkin, D. P. 1982. Reversal of human lymphocyte β-adrenoceptor desensitisation by glucocorticoids. *Clin. Pharmocol. Ther.* 32:556-565.

Hui, K. P., Ventresca, P., Brown, A. C., Barnes, P. J., and Chung, K. F. 1992. Modulation on neurally mediated airway microvascular leak in guinea-pig airways by $\beta_2$ adrenoceptor agonists. *Agents Actions.* **32**:29-33.

Hulme, E. C., Birdsall, N. J. M., and Buckley, N. J. 1990. Muscarinic receptor subtypes. *Ann. Rev. Pharmacol. Toxicol.* **30,** 633-650.

Hunt, T. W., Fields, T. A., Casey, P. J., and Peralta, E. G. 1996. RGS10 is a selective activator of $G_{\alpha i}$ GTPase activity. *Nature.* **383**:175-177.

Hybbinnette, J., and Mercke, U. 1982. Effects of the parasympathomimetic drug methacholine and its antagonist atropine on mucociliary transport. *Acta Oto-Laryngol.* **93**:465-473.

Iaccorino, G., Tomhave, E. D., Lefkowitz, R. J., and Koch, W. J. 1998. Reciprocal in vivo regulation of myocardial G protein-coupled receptor kinase expression by β-adrenergic receptor stimulation and blockade. *Circulation* **98**:1783-1789.

Inglese, J., Freedman, N. J., Koch, W. J., and Lefkowitz, R. J. 1993. Structure and mechanism of the G protein-coupled receptor kinases. *J. Biol. Chem.* **268**:23735-23738.

Ito, Y. 1988. Pre- and post-junctional actions of procaterol, a β-adrenoceptor stimulant, on dog tracheal tissue. *Br. J. Pharmacol.* **95**:268-274.

Iyengar, R., and Birnbaumer, L. 1993. Molecular and functional diversity of mammalian $G_s$-stimulated adenylyl cyclases. *FASEB J.* 7: 768-775.

Jackson, D. A., and Nathanson, N. M. 1995. Subtype-specific regulation of muscarinic receptor expression and function by heterologous receptor activation. *J. Biol. Chem.* **270,** 22374-22377.

January, B., Seibold, A., Allal, C., Whaley, B. S., Knoll, B. J., Moore, R. H., Dickey, B. F., Barber, R., and Clark, R. B. 1998. Salmeterol-induced

desensitization, internalization and phosphorylation of the human $\beta_2$-adrenoceptor. *Brit. J. Pharmacol.* **123**:701-711.

January, B., Seibold, A., Whaley, B., Hipkin, R. W., Lin, D., Schonbrunn, A., Barber, R., and Clark, R. B. 1997. $\beta_2$-adrenergic receptor desensitization, internalization, and phosphorylation in response to full and partial agonists. *J. Biol. Chem.* **272**:23871-23879.

Janssen, L. J., and Daniel, E. E. 1991. Characterization of the prejunctional β-adrenoceptors in canine bronchial smooth muscle. *J. Pharmacol. Exp. Ther.* **254**:741-749.

Jiang, H., Colbran, J. L., Francis, S. H., and Corbin, J. D. 1992. Direct evidence for cross-activation of cGMP-dependent protein kinase by cAMP in pig coronary arteries. *J. Biol. Chem.* **267**:1015-1019.

Jiang, H., Shabb, J. B., and Corbin, J. D. 1992. Cross-activation: overriding cAMP/cGMP selectivities of protein kinases in tissues. *Biochem. Cell Biol.* **70**:1283-1289.

Johnson, J. A., Clark, R. B., Friedman, J., Dixon, R. A. F., and Strader C. D. 1990. Identification of a specific domain in the beta-adrenergic receptor required for phorbol ester-induced inhibition of catecholamine-stimulated adenylyl cyclase. *Mol. Pharmacol.* **38**:289-293.

Johnson, M. 1990. The pharmacology of salmeterol. *Lung (suppl).* **168**:115-9.

Johnson, M. 1994. The preclinical effects of salmeterol: non-bronchodilator effects. *Eur. Respir. Rev.* **1;4**:257-260.

Jones, T. R., Charette, L., Garcia, M. L., and Kaczorowski, G. J. 1990. Selective inhibition of relaxation of guinea-pig trachea by charybdotoxin, a potent $Ca^{2+}$-activated $K^+$ channel inhibitor. *J. Pharmacol. Exp. Ther.* **255**:697-706.

Jones, K. A., Lorenz, R. R., Warner, D. O., Katusic, Z. S., and Sieck, G. C. 1994. Changes in cytosolic cGMP and calcium in airways smooth muscle relaxed by 3-morpholineosydnonimine. *Am. J. Physiol.* **266**:L9-16.

Kahn, R. 1907. Zur Physiologie der Trachea. *Arch Physiol.* 398-426.

Kallos, P., and Kallos, L. 1984. Experimental asthma in guinea-pigs revisited. *Int. Arch Allergy Appl. Immunol.* **73**:77-85.

Kamikawa, Y., and Shimo, Y. 1990. Inhibitory effects of catecholamines on cholinergically mediated contractions of guinea-pig isolated bronchial muscle. *J. Pharm. Pharmacol.* **42**:131-134.

Kammer, G. M. 1988. The adenylate cyclase-cAMP-protein kinase A pathway and regulation of immune response. *Immunol. Today.* **9**:222-228.

Kariman, K. 1980. β-adrenergic receptor binding in lymphocytes from patients with asthma. *Lung.* **158**:41-51.

Karnick, S. S., Sakmar, T. P., Chen, H.-B., and Khorana, H. G. 1988. Cysteine residues 110 and 187 are essential for the formation of correct structure in bovine rhodopsin. *Proc. Natl. Acad. Sci.USA* **85**: 8459-8463.

Katada, T., Bockoch, G. M., Northup, J. K., Ui, M., and Gilman, A. G. 1984[a]. The inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. Properties and function of the purified protein. *J. Biol. Chem.* **259**:3568-3577.

Katada, T., Bokoch, G. M., Smigel, M. D., Ui, M., and Gilman, A. G. 1984[b]. The inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. Subunit dissociation and the inhibition of adenylate cyclase in S49 lymphoma cyc- and wild type membranes. *J. Biol. Chem.* **259**:3586-3595.

Katada, T., Northup, J. K., Bokoch, G. M., Ui, M., and Gilman, A. G. 1984c. The inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. Subunit dissociation and guanine nucleotide-dependent hormonal inhibition. *J. Biol. Chem.* **259**:3578-3585

Katz, A., Simon, M. I. 1995. A segment of the C-terminal half of the G-protein beta 1 subunit specifies its interaction with the gamma 1 subunit. *Proc. Natl. Acad. Sci. USA.* **92**:1998-2002.

Kaumann, A. J. 1997. Four β-adrenoceptor subtypes in the mammalian heart. *Trends Pharmacol. Sci.* **18**:70-78.

Kaumann, A. J., and Molenaar, P. 1997. Modulation of human cardiac function through 4 β-adrenoceptor populations. *Naunyn Schmiedebergs Arch Pharmacol.* **355**:667-81.

Kelly, E., Keen, M., Nobbs, P., and MacDermot, J. 1990. Segregation of discrete $G_{s\alpha}$-mediated responses that accompany homolgous or heterologous desensitization in two related somatic hybrids. *Br. J. Pharmacol.* **99**:309-316.

Kelsen SG, Anekwe OO, Aksoy MO, Reddy PJ, Dhanesekaran N, Penn R and Benovic JL. 1997. Chronic effects of catecholamines on the $\beta_2$-adrenoceptor system in cultured human airway epithelial cells. *Am. J. Physiol.* **272**:L916-L924.

Kenny, A., and Hooper, N. 1991. Peptidases involved in the metabolism of bioactive peptides. In: *Degradation of Bioactive substances: Physiology*

*and Pathophysiology*, edited by JH Henriksen. Boca Raton, FL:CRC p47-49.

Khan, M. M., Melmon, K. L., Fathman, C. G., Hertelwulf, B., and Strober, S. 1985. The effects of autacoids on cloned murine lymphoid cells:modulation of IL 2 secretion and the activity of natural suppressor cells. *J. Immunol.* **134**:4100-4106.

Khorana, H. G. 1992. Rhodopsin, photoreceptor of the rod cell. An emerging pattern for structure and function. *J. Biol. Chem.* **267**:1-4.

Kim, G-D., and Milligan, G. 1994. Receptor availability defines the extent of agonist-mediated G-protein down-regulation in neuroblastoma x glioma hybrid cells transfected to express the $\beta_2$-adrenoceptor. *FEBS Letters.* **355**: 166-170.

Kirkpatrick, C. T., Jenkinson, H. A., and Cameron, A. R. 1975. Interaction between drugs and potassium-rich solutions in producing contraction in bovine tracheal smooth muscle: studies in normal and calcium-depleted tissues. *Clin. Exp. Pharmac. Physiol.* **2**: 559-570.

Klein, W. L., Sullivan, J., Skorupa, A., and Aguilar, J. S. 1989. Plasticity of neuronal receptors. *FASEB J.* **3**:2132-2140.

Knox, A. J., and Brown, J.K. 1991. Differential regulation of $Na^+/K^+$ ATPase by the cyclic nucleotide dependent protein kinases in airway smooth muscle. *Clin. Res.* **39**:331A.

Knox, A. J., and Tattersfield, A. E. 1995. Airway smooth muscle relaxation. *Thorax.* **50**:894-901.

Kobilka, B. K., Kobilka, T. S., Daniel, K., Regan, J. W., Caron, M. G., and Lefkowitz, R. J. 1988. Chimeric $\alpha_2$-/$\beta_2$-adrenergic receptors: delineation of domains involved in effector coupling and ligand binding specificity. *Science* **240,** 1310-1316.

Koch, W. J., Inglese, J., Stone, W. C., and Lefkowitz, R. J. 1993. The binding site for the βγ subunits of heterotrimeric G proteins on the β-adrenergic receptor kinase. *J. Biol. Chem.* **268**:8256-8260.

Koch, W. J., Rockman, H. A., Samama, P., Hamilton, R., Bond, R. A., Milano, C. A., and Lefkowitz, R. J. 1995. Cardiac function in mice overexpressing the β-adrenergic receptor kinase or a βARK inhibitor. *Science.* **268**:1350-1353.

Koenig, J. A., and Edwardson J. M. 1997. Endocytosis and recycling of G protein-coupled receptors. *Trends Pharmacol. Sci.* **18**:277-287.

Kochetkova, M., Burns, F. M. F., and Souness, J. E. 1995. Isoprenaline induction of cAMP-phosphodiesterase in guinea-pig macrophages occurs in the presence, but not in the absence, of the phosphodiesterase type IV inhibitor rolipram. *Biochem. Pharmacol.* **50**:2033-2038.

Kong, G., Penn, R., and Benovic, J. L. 1994. A β-adrenergic receptor kinase dominant negative mutant attenuates desensitization of the $\beta_2$-adrenergic receptor. *J. Biol. Chem.* **269**:13084-13087.

Konietzko, N., and Kasparek, R. 1982. Effect of adrenergic, cholinergic, and histaminergic stimulation on ciliary beat frequency of human nasal mucosa in vitro. *Am. Rev. Respir. Dis.* **127**. Suppl. 294: 243-249.

Kono, T., Robinson, F. W., Sarver, J. A., Vega, F. V., Pointer, R. H. 1977. Actions of insulin in fat cells. Effects of low temperature, uncouplers of oxidative phosphorylation, and respiratory inhibitors. *J. Biol. Chem.* **252**:2226-2233.

Kozasa, T., Itoh, H., Tsukamoto, T., and Kaziro, Y. 1988. Isolation and characterization of the human $G_{s\alpha}$ gene. *Proc. Natl. Acad. Sci. USA* **85**:2081-2085.

Krueger, K. M., Daaka, Y., Pitcher, J. A, Lefkowitz, R. J. 1997. The role of sequestration in G protein-coupled receptor resensitisation. *J. Biol. Chem.* **272**:5-8.

Krupnick, J. G. and Benovic, J. L. 1998. The role of receptor kinases and arrestins in G protein-coupled receptor regulation. Annu. Rev. Pharmacol. Toxicol. 38:289-319.

Kudlacz, E. M., Navarro, H. A., Slotkin, T. A. 1990. Regulation of β-adrenergic receptor-mediated processes in fetal rat lung: selective desensitization caused by chronic terbutaline exposure. *J. Dev. Physiol.* **14**:103-108.

Kume, H., Graziano, M. P., and Kotlikoff, M. I. 1992. Stimulatory and inhibitory regulation of calcium-activated potassium channels by guanine nucleotide-binding proteins. *Proc. Natl. Acad. Sci. USA*. **89**:11051-11055.

Kume, H., Hall, I. P., Washabau, R. J., Takagi, K., and Kotlikoff, M. I. 1994. β-adrenergic agonists regulate KCa channels in airway smooth muscle by cAMP-dependent and -independent mechanisms. *J. Clin. Invest.* **93**:371-379.

Kume, H., and Kotlikoff, M. I. 1993. Dual pathways of β-adrenergic action of $K/Ca^{2+}$ channels of tracheal smooth muscles. *Am. Rev. Respir. Dis.* **147**:A208.

Kume, H., and Takagi, K. 1997. Inhibitory effects of $G_s$ on desensitization of β-adrenergic receptors in tracheal smooth muscle. *Am. J. Physiol.* **273**:L556-64.

Kume, H., Takai, A., Tokuno, H., and Tomita, T. 1989. Regulation of $Ca^{2+}$-dependent $K^+$ channel activity in tracheal myocytes by phosphorylation. *Nature*. **341**:152-154.

Kurtenbach, E., Curtis, C. A. M., Pedder, E. K., Aitken, A., Harris, A. C. M., and Hulme, E. C. 1990. Muscarinic acetylcholine receptors. Peptide sequencing identifies residues involved in antagonist binding and disulfide bond formation. *J. Biol. Chem.* **265**:13702-13708.

Kvapil, P., Novotny, J., Svoboda, P., and Ransnas, L. A. The short and long forms of the alpha subunit of the stimulatory guanine-nucleotide-binding protein are unequally redistributed during (-)-isoproterenol-mediated desensitization of intact S49 lymphoma cells. *Eur. J. Biochem.* **226**:193-199.

Lands, AM, Luduena, FP, and Buzzo, HJ, 1967. Differentiation of receptors responsive to isoproterenol. *Life Sci.* **6**:2241-2249.

Larsson, S., and Svedmyr, N. 1977. Bronchodilating effect and side effects of $\beta_2$-adrenergic stimulants by different modes of administration (tablets, metered aerosol, and combinations thereof). *Am. Rev. Resp. Dis.* **116**:861-869.

Law, P. Y., McGinn, T. M., Wick, M. J., Erikson, L. J., Evans, C., and Loh, H. H. 1994. Analysis of δ-opioid receptor activities stably expressed in CHO cell lines: function of receptor density? *J. Pharmacol. Exp. Ther.* **271**:1686-1694.

Lazarus, S. C., Basbaum, C. B., and Gold, W. M. 1984. Localisation of cAMP in dog and cat trachea: effects of β-adrenergic agonists. *Am. J. Physiol.* **247**:C327-C334.

Lee, T. C., Lenihan, D. J., Malone, B., Roddy, L. L., and Wasserman, S. I. 1984. Increased biosynthesis of platelet-activating factor in activated human eosinophils. *J. Biol. Chem.* **259**:5526-5530.

Lee, N. H., and Fraser, C. M. 1993. Cross-talk between m1 muscarinic acetylcholine and $\beta_2$-adrenergic receptors. *J. Biol. Chem.* **268,** 7949-7957.

Lefkowitz, R. J. 1993. G protein-coupled receptor kinases. *Cell.* **74**:409-412.

Lefkowitz, R. J., Kobilka, B. K., and Caron, M. G. 1989. The new biology of drug receptors. *Biochem. Pharmacol.* **38**:2941-2948.

Leitch, A. G., Clancy, L. J., Costello, J. F., and Flenley, D. C. 1976. Effect of intravenous infusion of salbutamol on ventilatory response to carbon dioxide and hypoxia and a heart rate and plasma potassium in normal men. *Bri. Med. J.* **1**:365-367.

Li, P. L., and Campbell, W. B. 1997. Epoxyeicosatrienoic acids activate $K^+$ channels in coronary smooth muscle through a guanine nucleotide binding protein. *Circ. Res.* **80**(6):877-884.

Liggett, S. B., Caron, M. G., Lefkowitz, R. J., and Hnatowich, M. 1991. Coupling of a mutated form of the human $\beta_2$-adrenergic receptor to $G_i$ and $G_s$. *J. Biol. Chem.* **266**:4816-4821.

Liggett, S. B., Freedman, N. J., Schwinn, D. A., and Lefkowitz, R. J. 1993. Structural basis for receptor subtype-specific regulation revealed by a chimeric $\beta_3/\beta_2$-adrenergic receptor. *Proc. Natl. Acad. Sci. USA.* **90**:3665-3669.

Liggett, S. B. 1989. Identification and characterization of a homogeneous population of $\beta_2$-adrenergic receptors on human alveolar macrophages. *Am. Rev. Respir. Dis.* **139**:552-555.

Liles, W. C., and Nathanson, N. M. 1986. Regulation of neuronal muscarinic acetylcholine receptor number by protein glycosylation. *J. Neurochem.* **46**:85-95.

Lipworth, B., Tan, S., Devlin, M., Aiken, T., Baker, R., and Hendrick, D. 1998. Effects of treatment with formoterol on bronchoprotection against methacholine. *Am. J. Med.* **104**:431-438.

Livi, G. P., Kmetz, P., McHale, M., Cieslinski, L. B., Sathe, G. M., Taylor, D. P., Davis, R. L., Torphy, T. J, and Balcarek, J. M. 1990. Cloning and expression of cDNA for a human low-Km, rolipram-sensitive cyclic AMP phosphodiesterase. *Mol. Cell. Biol.* **10**:2678-2686.

Lohse, M. J., Andexinger, S., Pitcher, J., Trukawinxki, S., Codina, J., Faure, J. P., Caron, M. G., and Lefkowitz, R. J. 1992. Receptor-specific desensitization with purified proteins. Kinase dependence and receptor specificity of β-arrestin and arrestin in the $\beta_2$-adrenergic receptor and rhodopsin systems. *J. Biol. Chem.* **267**:8558-8564.

Lohse, M. J., Benovic, J. L., Caron, M. G., and Lefkowitz, R. J. 1990. Multiple pathways of rapid $\beta_2$-adrenergic receptor desensitization. Delineation with specific inhibitors. *J. Biol. Chem.* **265**:3202-3209.

Lohse, M. J., Benovic, J. L., Codina, J., Caron, M. G., and Lefkowitz, R. J. 1990. β-arrestin: a protein that regulates β-adrenergic receptor function. *Science.* **248**:1547-1550.

Lohse, M. J., Krasel, C. K., Winstel, R., and Mayor, F. 1996. G-protein-coupled receptor kinases. *Kidney Int.* **49**:1047-1052.

Lohse, M. J. 1993. Molecular mechanisms of membrane receptor desensitization. *Biochim. Biophys. Acta.* **1179**:171-188.

Longabough, J. P., Didsbury, J., Spiegel, A., and Stiles, G. L. 1989. Modification of the rat adipocyte A1 adenosine receptor-adenylate cyclase system during chronic exposure to an A1 adenosine receptor agonist: alterations in the quantity of $G_{s\alpha}$ and $G_{i\alpha}$ are not associated with changes in their mRNAs. *Mol. Pharmacol.* **36**: 681-688.

Loughney, K., Snyder, P. B., Uher, L., Rosman, G. J., Ferguson, K., and Florio, V. A. 1999. Isolation and characterization of PDE10A, a novel human 3', 5'-cyclic nucleotide phosphodiesterase. *Gene* **234**:109-117.

Lupas, A. N., Lupas, J. M., and Stock, J. B. 1992. Do G protein subunits associate via a three-stranded coiled coil? *FEBS Lett.* **314**:105-108.

Luttrell, L. M., van Biesen, T., Hawes, B. E., Koch, W. J., Krueger, K. M., Touhara, K., and Lefkowitz, R. J. 1997. G-protein-coupled receptors and their regulation: activation of the MAP kinase signaling pathway by G-protein-coupled receptors. *Adv. Second Messenger Phosphoprotein Res.* **31**:263-277.

Mack, J. A., Neilson, C.P., Stevens, D. L., and Vestal, R. E. 1986. β-adrenoceptor-mediated modulation of calcium ionophore activated polymorphonuclear leukocytes. *Br. J. Pharmacol.* **88**:417-423.

Mackenzie, F. R., and Milligan, G. 1990. Prostaglandin $E_1$-mediated, cyclic AMP-independent, down-regulation of $G_{s\alpha}$ in neuroblastoma X glioma hybrid cells. *J. Biol. Chem.* **265**: 17084-17093.

Madison, J. M., and Brown, J. K. 1988. Differential effects of forskolin, isoprenaline, and dibutyryl-cyclic adenosine monophosphate on phospho inositide hydrolysis in canine tracheal smooth muscle. *J. Clin. Invest.* **82**:1462-5.

Mak, J. C. W., Grandordy, B., and Barnes, P. J. 1994. High affinity [$^3$H]formoterol binding sites in lung:characterisation and autoradiographic mapping. *Eur. J. Pharmacol.* **269**:35-41.

Mak, J. C., Nishikawa, M., Shirasaki, H., Miyayasu, K., and Barnes, P. J. 1995. Protective effects of a glucocorticoid on downregulation of pulmonary $\beta_2$-adrenergic recptors *in vivo*. *J. Clin. Invest.* **96**:99-106.

Malbon, C. C., and Hadcock, J. R. 1988. Evidence that glucocorticoid response elements in the 5' non-coding region of the hamster $\beta_2$-adrenergic receptor gene are obligate for glucocorticoid regulation of hamster mRNA levels. *Biochem. Biophys. Res. Commun.* **154**:676-681.

Manchee, G. R., Barrow, A., Kulkarni, S., Palmer, E., Oxford, J., Colthup, P. V., Maconochie, J. G., and Tarbit, M. H. 1993. Disposition of salmeterol xinafoate in laboratory animals and in humans. *Drug Metab. Dispos.* **21**: 1022-1028.

Manning, D. R., and Gilman, A. G. 1983. The regulatory components of adenylate cyclase and transducin. A family of structurally homologous guanine nucleotide-binding proteins. *J. Biol. Chem.* **258**:7059-7063.

Manning, C. D., McLaughlin, M. M., Livi, G. P., Cieslinski, L. B., Torphy, T. J., and Barnette, M. S. 1996. Prolonged β-adrenoceptor stimulation up-regulates cAMP phosphodiesterase activity in human monocytes by increasing mRNA and protein for phosphodiesterases 4A and 4B. *J. Pharmacol. Exp. Ther.* **276**:810-818.

Marquetant, R., Brehm, B., and Strasser, R. H. 1992. Chronic β-blockade transregulates inhibitory $A_1$-adenosine and muscarinic $M_2$ receptors of the adenylyl cyclase system. *J. Mol. Cell Cardiol.* **24,** 535-548.

Masayuma, K., and Ishikana, T. 1985. Direct interaction of guinea-pig eosinophils and adrenergic agents. *Int. Arch. Allergy Appl. Immunol.* **78**:243-248

Matthews, J. M., Falckh, P. H. J., Molenaar, P., and Summers, R. J. 1996. Chronic (-) isoprenaline infusion down-regulates $\beta_1$- and $\beta_2$-adrenoceptors but does not transregulate muscarinic cholinoceptors in rat heart. *Naunyn Schmeideberg's Arch. Pharmacol.* **353**: 213-225.

Matthews, J. M., Falckh, P. J. H., and Hoffman, B. B. 1987. Adenylate cyclase supersensitivity: a general means of cellular adaption to inhibitory agonists? *Trends Pharmacol. Sci.* **8**: 308-311.

Mayor, F., Jr., Benovic J. L., Caron, M. G., and Lefkowitz. R. J. 1987. Somatostatin induces translocation of the β-adrenergic receptor kinase and desensitizes somatostatin receptors in S49 lymphoma cells. *J. Biol. Chem.* **262**:6468-6471.

McFadden, ER. 1981. Comparison of two dosage regimens of albuterol in acute asthma. J. Allergy Clin. Immunol. **68**: 91-97.

McGraw, D. W., and Liggett, S. B. 1997. Heterogeneity in β-adrenergic receptor kinase expression in the lung accounts for cell-specific desensitisation of the $\beta_2$adrenergic receptor. *J. Biol. Chem.* **272**:7338-7344.

McKenzie, F. R. and Milligan, G. 1990. Prostaglandin $E_1$-mediated, cyclic AMP-independant, down-regulation of Gsα in neuroblastoma × glioma hybrid cells. *J. Biol. Chem.* **265**:17084-17093.

McLaughlin, M. M., Cieslinski, L. B., Burman, M., Torphy, T. J., and Livi, G. P. 1993. A low-Km, rolipram-sensitive, cAMP-specific phosphodiesterase from human brain. Cloning and expression of cDNA, biochemical characterization of recombinant protein, and tissue distribution of mRNA. *J. Biol. Chem.* **268**:6470-6476.

Meurs, H., Koëter, G. H., De Vries, K., and Kauffman, H. F. 1982. The β-adrenergic system and allergic bronchial asthma: changes in lymphocyte β-adrenergic receptor number and adenylate cyclase activity after an allergen-induced attack. *J. Allergy Clin. Immunol.* **70**:272-280.

Michel, M. C., Wang, X. L., Schlicker, E., Göthert, M., Beckering, J. J., and Brodde, O. E. 1987. Increased $\beta_2$-adrenoceptor density in heart, kidney and lung of spontaneous hypertensive rats. *J. Auton. Pharmacol.* **7**:41-51.

Milligan, G., Kim, G. D., Mullaney, I., and Adie, E. J. 1995[a]. Regulation of cellular Gsα levels and basal adenylyl cyclase activity by expression of $\beta_2$-adrenoceptor in neuroblastoma cell lines. *Mol. Cell. Biochem.* **149**:213-216.

Milligan, G., and Unson, C. G. 1989. Persistant activation of the α subunit of $G_s$ promotes its removal from the plasma membrane. *Biochem J.* **260**:837-841.

Milligan, G. 1993. Agonist regulation of cellular G protein levels and distribution:mechanisms and functional implications. *Trends Pharmacol Sci.* **14**(11):413-418.

Miner, J. N., and Yamamoto, K. R. 1991. Regulatory crosstalk at composite response elements. *Trends Biochem. Sci.* **16**:423-426.

Miura, M., Belvisi, M. G., Stretton, D., Yacoub, M. H., and Barnes, P. J. 1992. Role of potassium channels in bronchodilator responses in human airways. *Am. Rev. Respir. Dis.* **146**:132-136.

Mohammed, S. P., Weyman-Jones, C., Taylor, C. V., and Young, A. 1996. Duration of action of $\beta_2$-agonists when given intravenously. *Am. J. Resp. Crit. Care Med.* **153**:A383.

Molimard, M., Naline, E., Zhang, Y., Le Gros, V., Begaud, B., and Advenier, C. 1998. Long- and short-acting $\beta_2$-adrenoceptor agonists: interactions in human contracted bronchi. *Eur. Respir J.* **11**:583-588.

Monaco, L., Vicini, E., and Conti, M. 1994. Structure of two rat genes coding for closely related rolipram-sensitive cAMP phosphodiesterases. Multiple mRNA variants originate from alternative splicing and multiple start sites. *J. Biol. Chem.* **269**:347-357.

Moore, R. H., Sadovnikoff, N., Hoffenberg, S., Liu, S., Woodford, P., Angelides, K., Trial, J., Carsrud, N. D. V., Dickey, B. F., and Knoll, B. J. 1995. Ligand-stimulated $\beta_2$-adrenergic receptor internalization via the constitutive endocytic pathway into rab5-containing endosomes. *J. Cell Sci.* **108**:2983-91.

Motomura, S., Deighton, N. M., Zerowski, H. -R., Oetsch, N., Michel, M. C., and Brodde, O.-E. 1990. Chronic $\beta_1$-adrenoceptor antagonist treatment sensitizes $\beta_2$-adrenoceptors but desensitizes $M_2$-muscarinic receptors in the human right atrium. *Br. J. Pharmacol.* **101,** 363-369.

Motulsky, H. J., and Insel, P. A. 1982. Adrenergic receptors in men. Direct identification, physiological regulation and clinical alteration. *N. Engl. J. Med.* **307**:18-29.

Mueller, E., and Van Breemen, C. 1979. Role of intracellular $Ca^{2+}$ sequestration in β-adrenergic relaxation of airway smooth muscle. *Nature.* **281**:682-683.

Muller, M., and Renkawitz, R. 1991. The glucocorticoid receptor. *Biochim. Biophys. Acta.* **1088**:171-182.

Münch, G., Dees, C., Hekman, M., and Palm, D. 1991. Multisite contacts involved in coupling of the β-adrenergic receptor with the stimulatory guanine-nucleotide-binding regulatory protein. Structural and functional studies by β-receptor-site-specific synthetic peptides. *Eur. J. Biochem.* **198**:357-364.

Munoz, N. M., Vita, A. J., Neely, S. P., McAllister, K., Spaethe, S. M., White, S. M., and Leff, A. R. 1994. β-adrenergic modulation of formyl-methionone-leucine-phenylalanine stimulates secretion of eosinophil peroxidase and leukotriene $C_4$. *J. Pharmacol. Exp. Ther.* **268**:1339-1343.

Murakami, A., Yajima, T., Sakuma, H., McLaren, M., and Inana, G. 1993. X-arrestin: a new retinal arrestin mapping to the X chromosome. *FEBS Lett.* **334**:203-209.

Murzin, A. G. 1992. Structural principles for the propeller assembly of β-sheets: the preference for seven-fold symmetry. *Proteins.* **14**:191-201.

Nanoff, C., Freissmuth, M., Tuisl, E., and Schütz, W. 1989. A different desensitisation pattern of cardiac β-adrenoceptor subtypes by prolonged *in vivo* infusion of isoprenaline. *J. Cardiovasc. Pharmacol.* **13**:198-203.

Neer, E. J. 1995. Heterotrimeric G proteins: organizers of transmembrane signals. *Cell* **80**:249-57.

Neer, E. J. Schmidt CJ, Nambrudripad R, and Smith TF. 1994. The ancient regulatory-protein family of WD-repeat proteins. *Nature.* **371**:297-300.

Nekrasova, E. R., Berman, D. M., Rustandi, R. R., Hamm, H. E., Gilman, A. G., and Arshavsky, V. Y. 1997. Activation of transducin guanosine triphosphatase by two proteins of the RGS family. *Biochemistry* **36**:7638-7643.

Nerme, V., Abrahamsson, T., Vauqulin, G. 1990. Chronic isoproterenol administration causes altered β-adrenoceptor-Gs-coupling in guinea pig lung. *J. Pharmacol. Exp, Ther.* **252**:1341-1346.

Newnham, D. M., Grove, A., McDevitt, D. G., and Lipworth, B. J. 1995. Subsensitivity of bronchodilator and systemic $\beta_2$-adrenoceptor responses after regular twice-daily treatment with eformoterol dry powder in asthmatic patients. *Thorax.* **50**:497-504.

Newnham, D. M., McDevitt, D. G., Lipworth, B. J. 1994. Bronchodilator subsensitivity after chronic dosing with eformoterol in patients with asthma. *Am. J. Med.* **97**:29-37.

Nials, A. T., Bail, D. I., Butchers, P. R., Coleman, R. A., Humbles, A. A., Johnson, M., Vardey, C. J. 1994. Formoterol on airway smooth muscle and human lung mast cells: a comparison with salbutamol and salmeterol. *Eur. J. Pharmacol.* **251**: 127-135.

Nials, A.T., Coleman, R. A., Johnson, M., Magunssen, H., Rabe, K. F., and Vardey, C. J. 1993. Effects of β-adrenoceptor agonists in human bronchial smooth muscle. *Br. J. Pharmacol.* **110**:1112-1116.

Nichol, G., Nix, A., Barnes, P. J., and Chung, K. F. 1990. Prostaglandin $F_{2\alpha}$ enhancement of capsaicin induced cough in man:modulation by $\beta_2$-adrenergic and anticholinergic drugs. *Thorax* **45**:694-698.

Nishikawa, M., Mak, J. C., Shirasaki, H., and Barnes, P. J. 1993. Differential down-regulation of pulmonary $\beta_1$- and $\beta_2$-adrenoceptor messenger RNA with prolonged *in vivo* infusion of isoprenaline. *Eur. J. Pharmacol.* **247**(2):131-138.

Nishikawa, M., Mak, J. C., Shirasaki, H., Harding, S. E., and Barnes, P. J. 1994. Long-term exposure to norepiniphrine results in down-regulation and reduced mRNA expression of pulmonary β-adrenergic receptors in guinea pigs. *Am. J. Respir. Cell Mol. Biol.* **10**:91-99.

Nishikawa, M., Mak, J. C. W., and Barnes, P. J. 1996. Effect of short- and long-acting $\beta_2$-adrenoceptor agonists on pulmonary $\beta_2$-adrenoceptor expression in human lung. *Eur. J. Pharmacol.* **318**:123-129.

Nogrady, S. G., Hartley, J. P. R., and Seaton, A. 1977. Metabolic effects of intravenous salbutamol in the course of acute severe asthma. *Thorax.* **32**:559-562.

Northup, J. K., Sternwies, P. C., Smigel, M. D., Schleifer, L. S., Ross, E. M., and Gilman, A. G. 1980. Purification of the regulatory component of adenylate cyclase. *Proc. Natl. Acad. Sci. USA* **77**:6516-6520.

Obernolte, R., Bhakta, S., Alvaraez, R., Bach, C., Zuppan, P., Mulkins, M., Jarnagin, K., and Shelton, E. R. 1993. The cDNA of a human lymphocyte cyclic-AMP phosphodiesterase (PDE IV) reveals a multigene family. *Gene*. **129**:239-247.

O'Connor, B. J., Aikman, S. L., and Barnes, P. J. 1992. Tolerance to the non-bronchodilator effects of inhaled $\beta_2$-agonists. *N. Engl. J. Med.* **327**:1204-1208.

O'Connor, B. J., Fuller, R. W., and Barnes, P. J. 1994. Non-bronchodilator effects of inhaled $\beta_2$-agonists. *Am. J. Respir. Crit. Care. Med.* **150**:381-387.

O'Connor, B. J., Ridge, S. M., Barnes, P. J., and Fuller, R. W. 1991. Comparative effect of terbutaline on mast cell and neurally meidated bronchoconstriction in asthma. *Thorax* **46**:745P.

O'Donnell, T. V., Sears, M. R., and Rea, H. H. 1989. Fenoterol and fatal asthma. *Lancet* **2**:45.

O'Dowd, B. F., Hnatowich, M., Caron, M. G., Lefkowitz, R. J., and Bouvier, M. J. 1989. Palmitoylation of the human $\beta_2$-adrenergic receptor. Mutation of Cys 341 in the carboxyl tail leads to an uncoupled nonpalmitoylated form of the receptor. *J. Biol. Chem.* **264,** 7564-7569.

O'Dowd, B. F., Hnatowich, M., Regan, J. W., Leader, W. M., Caron, M. G., and Lefkowitz, R. J. 1988. Site-directed mutagenesis of the cytoplasmic domains of the human $\beta_2$-adrenergic receptor. *J. Biol. Chem.* **263**: 15985-15992.

Ohguro, H., Van Hooser, J. P., Milam, A. H., and Palczewski, K. 1995. Rhodopsin phosphorylation and dephosphorylation *in vivo*. *J. Biol. Chem.* **270**:14259-14262.

Ovchinnikov, Y. A., Abdulaev, N. G., and Bogachuk, A. S. 1988. Two adjacent cysteine residues in the C-terminal cytoplasmic fragment of bovine rhodopsin are palmitoylated. *FEBS Lett.* **230**:1-5.

Palczewski, K. 1994. Structure and functions of arrestins. *Protein Sci.* **3**:1355-1361.

Palczewski, K., and Benovic, J. L. 1991. G-protein-coupled receptor kinases. *Trends Biochem. Sci.* **16**:387-391.

Palczewski, K., Buczylko, J., Kaplan, M. W., Polans, A. S., and Crabb, J. W. 1991. Mechanism of rhodopsin kinase activation. *J. Biol. Chem.* **266**:12949-12955.

Palczewski, K., McDowell, J. H., Jakes, S., Ingebritsen, T. S. and Hargrave, P. A. 1989. Regulation of rhodopsin dephosphorylation by arrestin. *J. Biol. Chem.* **264**:15770-15773.

Palczewski, K., Ohguro, H,, Premont, R. T., and Inglese, J. 1995. Rhodopsin kinase autophosphorylation. Characterization of site-specific mutations. *J. Biol. Chem.* **270**:15294-12598.

Palmqvist, M., Balder, B., Löwhagen, O., Melander, B., Svedmyr, N., and Wahlander, L. 1992. Late asthmatic reaction decreased after pretreatment with salbutamol and formoterol, a new long-acting $\beta_2$-agonist. *J. Allergy Clin. Immunol.* **89**:844-849.

Paraschos, A., and Karliner, J. S. 1994. Receptor crosstalk: Effects of prolonged carbachol exposure on $\beta_1$-adrenoceptors and adenylyl cyclase activity in neonatal rat ventricular myocytes. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **350**: 267-276.

Parsons, C. G., Martin, D., Franklin, R, J., Wood, J, L., and Headley, P. M. 1986. Do κ-opioids mimic ε-agonists as amino acid antagonists? *Neuropharmacology.* **25**:217-20.

Parruti, G., Peracchia, F., Sallese, M., Ambrosini, G., Msini, M., Rotilio, D., and De Blasi, A. 1993. Molecular analysis of human β-arrestin1: cloning, tissue distribution, and regulation of expression. Identification of two isoforms generated by alternative splicing. *J. Biol. Chem.* **268**:9753-9761.

Paterson, J. W., Evans, R. J., and Prime, F. J. 1971. Selectivity of bronchodilator action of salbutamol in asthmatic patients. *Br. J. Dis. Chest.* **65**:21-38.

Paterson, J. W., Lulich, K. M., and Goldie, R. G. 1983. A comment on $\beta_2$-agonists and their use in asthma. *Trends Pharmacol. Sci.* **4**:67-69.

Paterson, J. W., Woolcock, A. J., and Shenfeld, G. M. 1979. Bronchodilator drugs. *Am. Rev. resp. Dis.* **120**:1149-1188.

Pederson, S. E., and Ross, E. M. 1985. Functional activation of β-adrenergic receptors by thiols in the presence and absence of agonists. *J. Biol. Chem.* **264**:14150-14157.

Penn, R. B., Pannettieri, R. A. Jr., and Benovic, J. L. 1998. Mechanisms of acute desensitization of the $\beta_2$AR-adenylyl cyclase pathway in human airway smooth muscle. *Am. J. Resp. Cell. Mol. Biol.* **19**: 338-348.

Perkins, J. P., Hausdorff, W. P., and Lefkowitz, R. J. 1991. Molecular biology of adrenergic receptors. In The β-Adrenergic Receptors (Perkins, J. P., ed) pp73-124, *The Humana Press Inc.*,

Persson, C. G. A., Ekman, M., and Erjefalt, I. 1978. Terbutaline prevents permeability effects of histamine in the lung. *Acta Pharmacol. Toxicol.* **42**:395-397.

Persson, C. G. A., Erjefalt, I., Grega, G. J., and Svensjo, E. 1982. The role of β-receptor agonists in the inhibition of pulmonary edema. *Ann. NY Acad. Sci.* **384**:544-556.

Peters, S. P., Schulman, E. S., Schleimer, R. P., MacGlashan, D. W., Newball, H. H., and Lichtenstein, L. M. 1982. Dispersed human lung mast cells:pharmacologic aspects and comparison with human lung tissue fragments. *Am. Rev. Respir. Dis.* **126**:1034-1039.

Pippig, S., Andexinger, S., Daniel, K., Puzicha, M., Caron, M. G., Lefkowitz, R. J., and Lohse, M. J. 1993. Overexpression of β-arrestin and β-adrenergic receptor kinase augment desensitization of $\beta_2$-adrenergic receptors. *J. Biol. Chem.* **268**:3201-3208.

Pippig, S., Andexinger, S., and Lohse, M. J. 1995. Sequestration and recycling of $\beta_2$-adrenergic receptors permit receptor resensitization. *Mol. Pharmacol.* **47**:666-676.

Pitcher, J. A., Csoitos, C., DePaoli-Roach, A. A., and Lefkowitz, R. J. 1995. The G-protein-coupled receptor phosphatase: a protein phosphatase type 2A with a distinct subcellular distribution and substrate specificity. *Proc. Natl. Acad. Sci. USA.* **92**:8343-8347.

Pitcher, J. A., Hall, R. A., Daaka, Y., Zhang, J., Ferguson, S. S., Hester, S., Muller, S., Caron, M. G., Lefkowitz, R. J., and Barak, L. S. 1998. The G protein-coupled receptor kinase 2 is a microtubule-associated protein kinase that phosphorylates tubulin. *J. Biol. Chem.* **273**:12316-12324.

Pitcher, J. A., Inglese, J., Higgins, J. B., Arriza, J. L., Casey, P. H., Kim, C., Benovic, J. L., Kwatra, M. M., Caron, M. G., and Lefkowitz, R. J. 1992. Role of βγ subunits of G proteins in targeting the β-adrenergic receptor kinase to membrane-bound receptors. *Science*. **257**:1264-1267.

Pitcher, J., Lohse, M. J., Codina, J., Caron, M. G., and Lefkowitz, R. J. 1992. Desensitization of the isolated $\beta_2$-adrenergic receptor by β-adrenergic receptor kinase, cAMP-dependent protein kinase, and protein kinase C occurs via distinct molecular mechanisms. *Biochemistry* **31**:3193-3197.

Pitcher, J. A., Touhara, K., Payne, E. S., and Lefkowitz, R. J. 1995. Pleckstrin homology domain-mediated membrane association and activation of the β-adrenergic receptor kinase requires coordinate interaction with $G_{\beta\gamma}$ subunits and lipid. *J. Biol. Chem.* **270**:11707-11710.

Plaut, M., Pierce, J. H., Watson, C. J., Hanley-Hyde, J., Nordan, R. P., and Paul, W. E. 1989. Mast cell lines produce lymphokines in response to cross-linkage of FcRI or to calcium ionophores. *Nature* **339**:64-67.

Port, J.D., Huang, L-Y., and Malbon, C. C. 1992. β-adrenergic receptor agonists that down-regulate receptor mRNA up-regulate a M(r) 35,000 protein(s) that selectively binds to β-adrenergic receptor mRNAs. *J. Biol. Chem.* **267**:24103-24108.

Premont, R. T., Inglese, J., and Lefkowitz, R. J. 1995. Protein kinases that phosphorylate activated G protein-coupled receptors. *FASEB J.* **9**:175-182.

Premont, R. T., Koch, W. J., Inglese, J., and Lefkowitz, R. J. 1994. Identification, purification, and characterisation of GRK5, a member of the family of G protein-coupled receptor kinases. *J. Biol. Chem.* **269**:6832-6841.

Premont, R. T., Matsuoka, I., Mattei, M. G., Pouille, Y., Defer, N., and Hanoune, J. 1996. Identification and characterisation of a widely expressed form of adenylyl cyclase. *J. Biol. Chem.* **271**:13900-13907.

Quing, F., Hayes, M., Rhodes, C. G., Ind, P. W., Jones, T., Hughes, J. M. B. 1994. The effects of chronic salbutamol therapy on β-adrenergic receptors: peripheral mononuclear leucocytes compared to lung tissue. *Thorax* **49**:1046-1047.

Rabe, K., Dent, G., Giembycz MA., Perkins, R. S., Evans, P., and Barnes, P. Effects of long-acting beta adrenoceptor agonist formoterol vs albuterol on $LTB_4$-induced $Ca^{2+}$ mobilisation, $H_2O_2$ and $TXB_2$ release in guinea-pig eosinophils. *Am. Rev. Respir. Dis.* 1990; 141>A29.

Rabe, K. F., Giembycz, M. A., Dent, G., Perkins, R. S., Evans, P., and Barnes, P. J. 1993. Salmeterol is a competitive antagonist at β-adrenoceptors

mediating inhibition of respiratory burst in in guinea-pig eosinophils. *Eur. J. Pharmacol.* **231**:305-308.

Ramage, L., Lipworth, B. J., Ingram, C. G., Cree, I. A., and Dhillon, D. P. 1992. Reduced protection against exercise-induced bronchoconstriction after chronic dosing with salmeterol. *Respir. Med.* **88**:363-368.

Ransnas, L. A., Svoboda, P., Jasper, J. R., and Insel, P. A. 1989. Stimulation of β-receptors of S49 lymphoma cells redistributes the α subunit of the stimulatory G protein between cytosol and membranes. *Proc. Natl. Acad. Sci.* **86,** 7900-7903.

Ray, K., Kunsch, C., Bonner, L. M., and Robishaw, J. D. 1995. Isolation of cDNA clones encoding eight different human G protein γ-subunits, including three novel forms designated the γ4, γ10, and γ11 subunits. *J. Biol. Chem.* **270**:21765-71.

Rees, D. C., Deantonio, L., and Eisenberg, D. 1989. Hydrophobic organisation of membrane proteins. *Science* **245,** 510-513.

Rhoden, K. J., Meldrum, L. A., and Barnes, P. J. 1988. Inhibition of cholinergic neurotransmission in human airways by $\beta_2$-adrenoceptors. *J. Appl. Physiol.* **65**:700-705.

Rhodes, D. G., Newton, R., Butler, R., and Herbette, L. 1992. Equilibrium and kinetic studies of the interactions of salmeterol with membrane bilayers. *Mol. Pharmacol.* **42**:596-602.

Richardson, J. B. 1979. Nerve supply to the lungs. *Am. Rev. Respir. Dis.* **119**:785-790.

Rivkin, I., Rosenblatt, J., and Becker, E. L. 1975. The role of cyclic AMP in the chemotactic responsiveness and spontaneous motility of rabbit peritoneal neutrophils. The inhibition of neutrophil movement and the elevation of cyclic AMP levels by catecholamines, prostaglandins, theophylline and cholera toxin. *J. Immunol.* **115**:1126-1134.

Roberts, J. A., Bradding, P., Walls, A. F., Holgate, S. T., and Howarth, P. H.. 1992a. The influence of salmeterol xinafoate on mucosal inflammation in asthma. *Am. Rev. Respir. Dis.* **145**:A418.

Roberts, J. A., Bradding, P., Walls, A. F., Holgate, S. T., and Howarth, P. H. 1992b. The effect of salmeterol xinafoate therapy on lavage findings in asthma. *Am. Rev. Respir. Dis.* **145**:A418.

Rocha e Silva, M. 1969. Concerning the histamine receptor (H1). *J. Pharm. Pharmacol.* **21**(11):778-780.

Roffel, A. F., Meurs, M., Elzinga, C. R. S., and Zaagsma, J. 1993. Muscarinic $M_2$ receptors do not participate in the functional antagonism between methacholine and isoprenaline in guinea pig tracheal smooth muscle. *Eur. J. Pharmacol.* **249**:235-238.

Rolf Smith, S., Ryder, C., Kendall, M. J., and Holder, R. 1984. Cardiovascular and biochemical responses to nebulised salbutamol in normal subjects. *Br. J. Clin. Pharmacol.* **18**:641-644.

Ross, E. M. 1995. G protein GTPase-activating proteins: regulation of speed, amplitude, and signaling selectivity. *Recent Prog. Horm. Res.* **50**:207-219.

Roth, N., Campbell, P. T., Caron, M. G., Lefkowitz, R. J., and Lohse, M. J. 1991. Comparative rates of desensitization of β-adrenergic receptors by the beta-adrenergic receptor kinase and the cyclic AMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA*. **88**:6201-6204.

Roux, F. J., Grandordy, B., and Douglas, J. S. 1996. Functional and binding characteristics of long-acting $\beta_2$-agonists in lung and heart. *Am. J. Respir. Crit. Care Med.* **153**:1489-1495.

Rugg, E. L., Barnett, D. B., and Nahorski, S. R. 1978. Coexistence of $beta_1$ and $\beta_2$-adrenoceptors in mamalian lung: Evidence from direct binding studies. *Mol. Pharmacol.* **14**:996-1005.

Ruiz-Gómez, A., and Mayor, F. 1997. GRK2 colocalizes with β-adrenergic receptors during agonist-induced receptor internalization. *J. Biol. Chem.* **272**:9601-9604.

Sabol, S. L., and Nirenberg, M. 1979. Regulation of adenylate cyclase of neuroblastoma × glioma hybrid cells by α-adrenergic receptors. II. Long lived increase of adenylate cyclase activity mediated by α-receptors. *J. Biol. Chem.* **254**:1921-6.

Sanders, V. M., and Munson, A. E. 1984. β-adrenoceptor mediation of the enhancing effect of norepinephrine on the murine primary antibody response *in vitro*. *J. Pharmacol. Exp. Ther.* **230**:183-192.

Sanjar, S., McCabe, P. J., and Humbles, A. H. 1991. Inhibition by salmeterol of antigen-induced eosinophil accumulation in guinea-pig lung. *Eur. Respir. J.* **4**:200s.

Santa Cruz, R., Landa, J., Hirsch, J., and Sackner, M. A. 1974. Tracheal mucous velocity in normal man and patients with obstructive lung disease:effects of terbutaline. *Am. Rev. Respir Dis.* **109**:458-463.

Savarese, T. M., Wang, C-D., and Fraser, C. M. 1992. Site-directed mutagenesis of the rat m1 muscarinic acetylcholine receptor. Role for conserved cysteines in receptor function. *J. Biol. Chem.* **267,** 11439-11448.

Sears, M. R., Taylor, D. R., Print, C. G., Lake, D. C., Li, Q. Q., Flannery, E. M., Yates, D. M., Lucas, M. K., and Herbison, G. P. 1990. Regular inhaled β-agonist treatment in bronchial asthma. *Lancet* **336**:1391-6.

Sette, C., Vicini, E., and Conti, M. 1994. Modulation of cellular responses by hormones: role of cAMP specific, rolipram-sensitive phosphodiesterases. *Mol. Cell. Endocrinol.* **100**:75-79.

Sette, C., Iona, S., and Conti, M. 1994a. The short-term activation of a rolipram-sensitive, cAMP-specific phosphodiesterase by thyroid-stimulating hormone in thyroid FRTL-5 cells is mediated by a cAMP-dependent phosphorylation. *J. Biol. Chem.* **269**:9245-9252.

Sette, C., Vicini, E., and Conti, M. 1994b. The rat PDE3/IVd phosphodiesterase gene codes for multiple proteins differentially activated by cAMP-dependent protein kinase. *J. Biol, Chem.* **269**:18271-18279.

Seybold, J., Newton, R., Wright, L., Finney, P. A., Suttorp, N., Barnes, P. J., Adcock, I. M., Giembycz, M. A. 1998. Induction of phosphodiesterases 3B, 4A4, 4D1, 4D2, and 4D3 in Jurkat T-cells and in human peripheral blood T-lymphocytes by 8-bromo-cAMP and $G_s$-coupled receptor agonists. *J. Biol. Chem.* **272**: 20575-20588.

Shahid, M., Cottney, J. E., Walker, G. B., McInnewar, I., Bruin, J. C., Spiers, I., Logan, R. T., and Nicholson, C. D. 1991. The presence of five cyclic nucleotide phosphodiesterase isoenzyme activities in bovine tracheal smooth muscle and the functional effects of selective inhibitors. *Br. J. Pharmacol.* 102:314P

Sharma, S. K., Klee, W. A., and Nirenberg, M. 1975. Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. *Proc. Natl. Acad. Sci. USA.* **72**:3092-3092.

Shih, M., and Malbon, C. C. 1994. Oligodeoxynucleotides antisense to mRNA encoding protein kinase A, protein kinase C and β-adrenergic receptor kinase reveal distinctive cell-type specific roles in agonist-induced desensitization. *Proc. Natl. Acad. Sci. USA* **91**:12193-12197.

Shinohara, T., Dietzschold, B., Craft, C. M., Wistow, G., Early, J. J., Donoso, L. A., Horwitz, J., and Tao, R. 1987. Primary and secondary structure of bovine retinal S antigen (48-kDa). *Proc. Natl. Acad. Sci. USA.* **84**:6975-6979.

Sibley, D. R., Strausser, R. H., Benovic, J. A., Daniel, K., and Lefkowitz, R. J. 1986. Phosphorylation/dephosphorylation of the β-adrenergic receptor regulates its functional coupling to adenylate cyclase and subcellular distribution. *Proc. Natl. Acad. Sci. USA* **83**:9408-9412.

Silver, P., and Stull, J. T. 1982. Phosphorylation of myosin light chain and phosphorylase in tracheal smooth muscle in response to KCl and carbachol. *Mol. Pharmacol.* **25**:267-74.

Simon, M. I., Strathmann, M. P., Gautam, N. 1991. Diversity of G proteins in signal transduction. *Science* **252**:802-8.

Skoogh, B-E., and Svedmyr, N. 1989. $\beta_2$-adrenoceptor stimulation inhibits ganglionic transmission in ferret trachea. *Pulm. Pharmacol.* **1**:167-172.

Small, R. C., Foster, R. W., and Boyle, J. P. 1988. $K^+$-channel opening as a mechanism for relaxing airways smooth muscle. *Agents Actions Suppl.* **23**:89-94.

Soderling, S. H., Bayuga, S. J., and Beavo, J. A. 1998a. Cioning and characterization of a cAMP-specific cyclic nucleotide phosphodiesterase. *Proc. Natl. Acad. Sci. USA* **95**:8991-8996.

Soderling, S. H., Bayuga, S. J., and Beavo, J. A. 1998b. Identification and characterization of a novel family of cyclic nucleotide phosphodiesterases. *J. Biol. Chem.* **273**:15553-15558.

Soderling, S. H., Bayuga, S. J., Beavo, J. A. 1999. Isolation and characterization of a dual-substrate phosphodiesterase gene family: PDE10A. *Proc. Natl. Acad. Sci. USA.* **96**:7071-7076.

Söhleman, P., Hekman, M., Puzicha, M., and Lohse, M. J. 1995. Binding of purified recombinant β-arrestin to guanine-nucleotide-binding-protein-coupled receptors. *Eur. J. Biochem.* **232**:464-472.

Sparrow, M. P., Pfitzer, G., Gagelmann, M., and Rüegg, J. C. 1984. Effect of calmodulin, $Ca^{2+}$, and cAMP protein kinase on skinned tracheal smooth muscle. *Am. J. Physiol.* **246**:C308-C314.

Spina, D., Rigby, P. J., Paterson, J. W., and Goldie, R. G. 1989. Autoradiographic localization of β-adrenoceptors in asthmatic human lung. *Am. Rev. Respir. Dis.* **140**:1410-1415.

Spitzer, W.O., Suissa, S., Ernst, P., Horwitz, R. I., Habbick, B., Cockcroft, D., Boivin, J., McNutt, M., Buist, A. S., and Rebuck, A. S. 1992. The use of β-agonists and the risk of death and near-death from asthma. *N. Engl. J. Med.* **326**:501-506.

Spring, D. J., and Neer, E. J. 1994. A 14-amino acid region of the G protein γ subunit is sufficient to confer selectivity of γ binding to the β subunit. *J. Biol. Chem.* **269**:22882-86.

Stern-Marr R, and Benovic J. 1995. Regulation of G protein-coupled receptors by receptor kinases and arrestins. *Vitam. Horm.* **51**:193-234.

Stern-Marr, R., Gurevich, V. V., Goldsmith, P., Bodine, R. C., Sanders, C., Donoso, L. A., and Benovic, J. L. 1993. Polypeptide variants of β-arrestin and arrestin3. *J. Biol. Chem.* **268**:15640-15648.

Sternweis, P. C. 1994. The active role of βγ in signal transduction. *Curr. Opin. Cell. Biol.* **6**:198-203.

Sternwies, P. C., Northup, J. K., Smigel, M. D., and Gilman, A. G. 1981. The regulatory component of adenylate cyclase. Purification and properties. *J. Biol. Chem.* **256**:11517-11526.

Stolley, P. D. 1972. Asthma mortality:Why the United States was spared an epidemic of deaths due to asthma. *Am Rev Respir Dis.* **109**:883-8893.

Strader, C. D., Candelore, M. R., Hill, W. S., Sigal, I. S., and Dixon, R. A. F. 1989. Identification of two serine residues involved in agonist activation of the β-adrenergic receptor. *J. Biol. Chem.* **264**:13572-78.

Strader. C. D., Fong, T. M., Tota, M. R., and Underwood, D. 1994. Structure and function of G protein-coupled receptors. *Annu Rev Biochem.* **63**:101-132.

Strader. C. D., Sigal, I. S., Blake, A. D., Cheung, A. H., Register. R. B., Rands, E., Zemik, B. A., Candelore, M R., and Dixon, R. A. F. 1987. The carboxyl terminus of the hamster β-adrenergic receptor expressed in mouse L cells is not required for receptor sequestration. *Cell* **49**:855-863.

Strader, C. D., Sigal, I. S., Candelore, M. R., Rands, E., Hill, W. S., and Dixon, R. A. F. 1988. Conserved aspartic acid residues 79 and 113 of the β-adrenergic receptor have different roles in receptor function. *J. Biol. Chem.* **263**:10267-10271.

Strader, C. D., Sigal, I. S., and Dixon, R. A. F. 1989. Structural basis of β-adrenergic receptor function. *FASEB J.* **3**:1825-1832.

Strader, C. D., Sigal, I. S., Register, R. B., Candelore, M. R., Rands, E., and Dixon, R. A. F. 1987. Identification of residues required for ligand binding to the β-adrenergic receptor. *Proc. Natl. Acad. Sci.USA* **84**:4384-4388.

Stransky, A., Szereda-Przestaszewsky, M., and Widdicombe, J. G. 1973. The effects of lung reflexes on laryngeal resistance and motor-neuron discharge. *J. Physiol.* **231**:417-438.

Strasser. R. H., Benovic, J. L., Caron, M. G., and Lefkowitz, R. J. 1986. β-Agonist- and prostaglandin $E_1$-induced translocation of the β-adrenergic receptor receptor kinase: evidence that the kinase may act on multiple adenylate cyclase-coupled receptors. *Proc. Natl. Acad. Sci. USA* **83**:6362-6366.

Strasser. R. H., Stiles, G. L., and Lefkowitz, R. J. 1984. Translocation and uncoupling of the β-adrenergic receptor in rat lung after catecholamine promoted desensitisation in vivo. *Endocrinology*. **115**:1392-1400.

Strosberg, A. D. 1993. Structure, function and regulation of adrenergic receptors. *Protein Sci.* **2**:1198-1209.

Strulovici, B., Cerione, R. A., Kilpatrick, B. F., Caron, M. G., and Lefkowitz, R. J. 1984. Direct demonstration of impaired functionality of a purified desensitised β-adrenergic receptor in a reconstituted system. *Science*. **225**:837-840.

Su, Y.-F., Harden, T. K., and Perkins, J. P. 1979. Isoproterenol-induced desensitisation of adenylate cyclase in human astrocytoma cells. *J. Biol. Chem.* **254**:38-41.

Sulakvelidze, I., and McDonald, D. M. 1994. The anti-edema action of formoterol in rat trachea does not depend on capsaicin-sensitive sensory nerves. *Am. J. Respir. Crit. Care. Med.* **149**:232-238.

Sullivan, M., Egerton, M., Shakur, Y., Marquardsen, A., and Houslay, M. D. 1994. Molecular cloning and expression, in both COS-1 cells and *S. cerevisiae*, of a human cytosolic type-IVA, cyclic AMP specific phosphodiesterase (hPDE-IVA-h6.1). *Cell Signal.* **6**:793-812.

Summer, R. 1985. Status asthmaticus. *Chest* **87**:87S-94S.

Sunahara, R. K., Dessauer, C. W., and Gilman, A. G. 1996. Complexity and diversity of mammalian adenylyl cyclases. *Annu. Rev. Pharmacol. Toxicol.* **36**:461-80.

Svedmyr, N. 1981. $\beta_2$-adrenoceptor stimulants and theopylline in asthma therapy. *Eur. J. Respir. Dis.* **62** (Suppl. 116):1-48.

Svedmyr, N., and Thiringer, G. 1971. The effects of slabutamol and isoprenaline on β-receptors in patients with chronic lung disease. *Postgrad. Medl. J.* **47**(Suppl.):44-46.

Swinnen, J. V., Joseph, D. R., and Conti, M. 1989a. Molecular cloning of rat homologues of the *Drosophila melanogaster* dunce cAMP phosphodiesterase: evidence for a family of genes. *Proc. Natl. Acad. Sci. USA*. **86**:5325-5329.

Swinnen, J. V., Joseph DR, and Conti M. 1989b. The mRNA encoding a high-affinity cAMP phosphodiesterase is regulated by hormones and cAMP. *Proc. Natl. Acad. Sci. USA*. **86**:8197-8201.

Szefler, S. J., Edwards, C. K., Haslett, C., Zahniser, N. R., Miller, J. A., and Henson, P. M. 1987. Effects of cell isolation procedures and radioligand selection on the characterization of human leukocyte β-adrenergic receptors. *Biochem. Pharmacol*. **36**:1589-1597.

Tai, E., and Read, J. 1967. Response of blood gas tensions to aminophylline and isoprenaline in patients with asthma. *Thorax* **22**: 543-549.

Tang, W. J., and Gilman, A. G. 1992. Adenylyl cyclases. *Cell*. **70**:869-872.

Tang, W-J., and Gilman, A. G. 1995. Construction of a soluble adenylyl cyclase activated by $G_{s\alpha}$ and forskolin. *Science* **268:**1769-1772.

Tang, W-J., Iñiguez-Lluhi, J. A., Mumby, S. M., and Gilman, A. G. 1992. Regulation of mammalian adenylyl cyclases by G-protein α and βγ subunits. *Cold Spring Harbor Lab. Symp. Quant. Biol*. **57**:135-144.

Tang, W. J., Yan, S-Z., and Drum, C. 1997. Class III adenylyl cyclases - regulation and underlying mechanisms. *Adv. Second Messenger Phosphoprotein Res*. **32**:127-151.

Taussig, R., and Gilman, A. G. 1995. Mammalian membrane-bound adenylyl cyclases. *J. Biol. Chem*. **270**:1-4.

Taussig, R., Quarmby, L. M., and Gilman, A. G. 1993. Regulation of purified type I and type II adenylate cyclases by G protein βγ subunits. *J. Biol. Chem*. **268**:9-12.

Taylor, S. E. 1987. Potentiation of isoproterenol-induced relaxation of isolated trachea by aminophylline: modulation by desensitization. *Proc. Soc. Exp. Biol. Med*. **185**:385-391.

Taylor, D. R., Sears, M. E., Herbison, G. P., Flannery, E. M., Print, C. G., Lake, D. C., Yates, D. M., Lucas, M. K., and Li, Q. 1993. Regular inhaled β agonist in asthma; effects on exacerbations and lung function. *Thorax* **48**:134-138.

Tenor, H., Hatzelmann, A., Wendel, A., and Schudt, C. 1995. Identification of phosphodiesterase IV activity and its cyclic adenosine monophosphate-

dependent up-regulation in a human keratinocyte cell line (HaCaT). *J. Invest. Dermatol.* **105**:70-74.

Tesmer, J. J. G., Berman, D. M., Gilman, A G., and Sprang, S. R. 1997. Structure of RGS4 bound to AlF4--activated $G_{i\alpha 1}$: stabilization of the transition state for GTP hydrolysis. *Cell* **89**:251-261.

Tholanikunnel, B. G., Granneman, J. G., and Malbon, C. C. 1995. The Mr 35,000 β-adrenergic receptor mRNA-binding protein binds transcripts of G-protein-linked receptors which undergo agonist-induced destabilization. *J. Biol. Chem.* **270**:12787-12793.

Thomas, J. M., and Hoffman, B. B. 1987. Adenylate cyclase supersensitivity: a general means of cellular adaptation to inhibitory agonists? *Trends Pharmacol. Pharmacol.* **18**:308-311.

Thomas, J. M., and Hoffman, B. B. 1989. Chronic somatostatin treatment induces enhanced forskolin-stimulated cAMP accumulation in wild-type S49 mouse lymphoma cells but not in protein kinase-deficient mutants. *Mol. Pharmacol.* **34**:116-124.

Thomas, J. M., and Hoffman, B. B. 1992. Adaptive increase in adenylyl cyclase activity in NG108-15 and S49 cells induced by chronic treatment with inhibitory drugs is not due to a decrease in cAMP concentrations. *Cell Signal.* **4**:417-428.

Thomas, J. M., and Hoffman, B. B. 1996. Isoform-specific sensitization of adenylyl cyclase activity by prior activation of inhibitory receptors: role of beta gamma subunits in transducing enhanced activity of the type VI isoform. *Mol. Pharmacol.* **49**:907-914.

Tokuyama, K., Kuo, H., Rohde, J. A. L., Barnes, P. J., and Rogers, D. F. 1990. Neural control of goblet cell secretion in guinea pig airways. *Am J. Physiol.* **259**:L108-L115.

Tokoyama, K., Lötvall, O., Löfdahl, C-G., Barnes, P. J., and Chung, K. F. 1991. Inhaled formoterol inhibits histamine-induced airflow obstruction and airway microvascular leakage. *Eur. J. Pharmacol.* **193**:35-40.

Torphy, T. J. 1994. β-adrenoceptors, cAMP and airway smooth muscle relaxation: challenges to the dogma. *Trends Pharmacol. Sci.* **15**:370-374.

Torphy, T, J., Barnette, M, S., Hay, D. W., and Underwood, D. C. 1994. Phosphodiesterase IV inhibitors as therapy for eosinophil-induced lung injury in asthma. *Environ. Health. Perspect.* **102**: 79-84.

Torphy, T. J., Rinard, G. A., Rietola, M. G., and Mayer, S. E. 1983. Functional antagonism in canine tracheal smooth muscle: inhibition by methacholine

of the mechanical and biochemical responses to isoprotenerol. *J. Pharmacol. Exp. Ther.* **227**:694-699.

Torphy, T. J., Undem, B. J. 1991. Phosphodiesterase inhibitors: new opportunities for the treatment of asthma. *Thorax*. **46**(7):512-523.

Torphy, T. J., Zhou, H. L., Burman, M., and Huang, L, B. 1991. Role of cyclic nucleotide phosphodiesterase isozymes in intact canine trachealis. *Mol. Pharmacol.*. **39**: 376-384.

Torphy, T. J., Zhou, H. L., and Cieslinksi, L. B. 1992. Stimulation of β-adrenoceptors in a human monocyte cell line (U937) up-regulates cyclic AMP-specific phosphodiesterase activity. *J. Pharmacol. Exp. Ther.* **263**:1195-1205.

Torphy, T. J., Zhou, H., Foley, J. E., Sarau, H. M., Manning, C. D., and Barnette, M. S. 1995. Salbutamol up-regulates PDE4 activity and induces a heterologous desensitisation of U937 cells to prostaglandin $E_2$. *J. Biol. Chem*. **270**:23598-23604.

Touhara, K., Inglese, J., Pitcher, J. A., Shaw, G., and Lefkowitz, R. J. 1994. Binding of G protein βγ-subunits to pleckstrin homology domains. *J. Biol. Chem*. **269**:10217-10220.

Traber, J., Gullis, R., and Hamprecht, B. 1975. Influence of opiates on the levels of adenosine 3':5'-cyclic monophosphate in neuroblastoma × glioma hybrid cells. *Life Sci*. **16**:1863-1868.

Trowbridge, I. S., Collawn, J. F., and Hopkins, C. R. 1993. Signal-dependent membrane protein trafficking in the endocytic pathway. *Annu. Rev. Cell. Biol.* **9**:129-161.

Truss, M., and Beato, M. 1993 Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. *Endocr. Rev*. **14**:459-479.

Twentyman, O. P., Fennerty, J. P., and Holgate, S. T. 1991. The inhibitory effect of nebulised albuterol on the early and late asthmatic reactions and increase in airway responsiveness provoked by inhaled allergen in asthma. *Am. Rev. Respir. Dis*. **144**:782-787.

Ullman, A., and Svedmyr, N. 1988. Salmeterol, a new long acting inhaled $\beta_2$-adrenoceptor agonist: comparison with salbutamol in adult asthmatic patients. *Thorax* **43**:674-678.

Valiquette, M., Bonin, H., Hnatowich, M., Caron, M. G., Lefkowitz, R. J., and Bouvier, M. 1990. Involvement of tyrosine residues located in the carboyl tail of the human $\beta_2$-adrenergic receptor in agonist-induced down-regulation of the receptor. *Proc. Natl. Acad. Sci. USA*. **87**:5089-5093.

Van As, A. 1974. The role of selective $\beta_2$-adrenergic stimulants in the control of ciliary activitiy. *Respiration* **31**:146-151.

Van Biesen, T., Hawes, B. E., Luttrell, D. K., Krueger, K. M., Touhara, K., Porfiri, E., Sakaue, M., Luttrell, L. M., and Lefkowitz, R. J. 1995. Receptor-tyrosine-kinase and G βγ-mediated MAP kinase activation by a common signalling pathway. *Nature* **376**:781-784.

Van Liefde, I., Van Ermen, A., Van Witzenburg, A., Fraeyman, N., and Vauquelin, G. 1994. Species and strain-related differences in the expression and functionality of β-adrenoceptor subtypes in adipose tissue. *Arch. int. Pharmacodyn.* **327**: 69-86.

Van Schayck, C. P., Dompeling, E. van Herwaarden, C L., Folgering, H., Verbeek, A. L., van der Hoogen, H. J., and van Weel, C. 1991. Bronchodilator treatment in moderate asthma or chronic bronchitis: continuous or on demand? A randomised controlled study. *Br. Med. J.* **303**:1426-1431.

Vanscheeuwijck, P., Velde E. V., and Fraeyman, N. 1989. Effect of aging on properties and function of β-adrenoceptors in rat lung. *Eur. J. Pharmacol.* **172**:373-380.

Vassilev, P. P., Danchev, N., and Staneva-Stoytcheva, D. 1998. In vivo induced desensitization of β-adrenoceptors in rat lung. Effect of nitrendipine. *Methods Find Exp. Clin. Pharmacol.* **20**:833-840.

Verghese, M. W., McConnell, R. T., Lenhard, J. M., Hamacher, L., and Jin, S. L. 1995. Regulation of distinct cyclic AMP-specific phosphodiesterase (phosphodiesterase type 4) isozymes in human monocytic cells. *Mol. Pharmacol.* **47**:1164-1171.

Verleden, G. M., Belvisi, M. G., Rabe, K. F., Miura, M., and Barnes, P. J. $\beta_2$-adrenoceptors inhibit NANC neural bronchoconstrictor responses *in vitro*. *J. Appl. Physiol.* **74**:1195-1199.

Vick, R. L., Todd, E. P., and Luedke, D. W. 1972. Epinephrine-induced hypokalaemia: relation to liver and skeletal muscle. *J. Pharmacol. Exp. Ther.* **181**:139-146.

Von Zastrow, M., and Kobilka, B. K. 1992. Ligand-regulated internalization and recycling of human $\beta_2$-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. *J. Biol. Chem.* **267**:3530-3538.

Wahedna, I., Wong, C. S., Wisniewswki, A. F. Z., and Pavord, I. D. 1993. Asthma control during and after cessation of regular $\beta_2$-agonist treatment. *Am. Rev. Respir. Dis.* **148**:707-712.

Wang, Z., Bilsky, E. J., Porreca, F., and Sadée, W. 1994. Constitutive μ opioid receptor activation as a regulatory mechanism underlying narcotic tolerance and dependence. *Life Sci.* **54**:339-350.

Watson, A. J., Katz, A., and Simon, M. I. 1994. A fifth member of the mammalian G-protein β-subunit family. Expression in brain and activation of the $\beta_2$ isotype of phospholipase C. *J. Biol. Chem.* **269**:22150-22156.

Watson, N., Linder, M. E., Druey, K. M., Kehrl, J. H., and Blumer, K. J. 1996. RGS family members: GTPase-activating proteins for heterotrimeric G-protein α-subunits. *Nature.* **383**:172-175.

Wedegaertner, P. B., Bourne, H. R.; and von Zastrow, M. 1996. Activation-induced subcellular redistribution of $G_{s\alpha}$ in the cell. *Mol. Cell Biol.* **7**:1225-1233.

Weller, P. F., Lee, C. W., Foster, D. W., Corey, E. J., Austen, K. F., and Lewis, R. A. 1983. Generation and metabolism of 5-lipoxygenase pathway leukotrienes by human eosinophils:predominant production of leukotriene $C_4$. *Proc. Natl. Acad. Sci. USA.* **80**:7626-7630.

Weston, A. H. 1989. Smooth muscle $K^+$ channel openers; their pharmacology and clinical potential. *Pflügers Arch.* **414** (suppl. 1):S99-S105.

Whelan, C. H., and Johnson, M. 1992. Inhibition by salmeterol of increased vascular permeability and granulocyte accumulation in guinea-pig lung and skin. *Br. J. Pharmacol.* **105**:831-8.

Whisnant, R. E., Gilman, A. G., and Dessauer, C. W. 1996. Interaction of the two cytosolic domains of mammalian adenylyl cyclase. *Proc. Natl. Acad. Sci. USA.* **93**:6621-6625.

Widdicombe, J. G., and Pack, R. J. 1982. The Clara cell. *Eur. J. Respir. Dis.* **63**:202-220.

Wilden, U., Hall, S. W., and Kühn, H. 1986. Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. *Proc. Natl. Acad. Sci. USA* **83,** 1174-1178.

Williams, S. J., Panish, R. W., and Seaton, A. 1975. Comparison of intravenous aminophylline and salbutamol in severe asthma. *Br. Med. J.* **1**:685. 1975.

Williams, L. T., Snyderman, R., and Lefkowitz, R. J. 1976. Identification of β-adrenergic receptors in human lymphocytes by (-)[$^3$H]alprenolol binding. *J. Clin. Invest.* **57**:149-155.

Williams, S. J., Winner, S. J., and Clark, T. J. H. 1981. Comparison of inhaled and intravenous terbutaline in acute severe asthma. *Thorax*. **36:**629-631.

Winstel, R., Freund, S. Krasel, C., Hoppe, E., and Lohse, M. J. 1996. Protein kinase cross-talk: Membrane targeting of the β-adrenergic receptor kinase by protein kinase C. *Pro.c Natl. Acad. Sci. USA*. **93**:2105-9.

Wong, Y. H., Conklin, B. R., and Bourne, H. R. 1992. Gz-mediated hormonal inhibition of cyclic AMP accumulation. *Science* **255**:339-342.

Wong, Y. H., Federman, A., Pace, A. M., Zachary, I., Evans, T., Pouyssegur, J., and Bourne, H. R. 1991. Mutant α subunits of $G_{i2}$ inhibit cyclic AMP accumulation. *Nature* **351,** 63-65.

Woolcock, A., Lundback, B., Ringdal, N., and Jacques, L. A. 1996. Comparison of addition of salmeterol to inhaled steroids with doubling the dose of inhaled steroids. *Am. J. Respir. Crit. Care Med.* **153**:1481-1488.

Xiao, R-P., Ji, X., and Lakatta, E. G. 1995. Functional coupling of the $\beta_2$-adrenoceptor to a pertussis toxin-sensitive G protein in cardiac myocytes. *Mol. Pharmacol*. **47**:322-29.

Yamaki, T., Takeuchi, Y., Komori, N., Kobayshi, H., Sakai, Y., and Hotta, Y., Matsumoto, H. 1990. A 49-kilodalton phosphoprotein in the *Drosophila* photoreceptor is an arrestin homologue. *Science*. **248**:483-486.

Yan, S-Z., Hahn, D., Huang, Z. H., and Tang, W. J. 1996. Two cytoplasmic domains of mammalian adenylyl cyclase form a $G_{s\alpha}$- and forskolin-activated enzyme *in vitro*. *J. Biol. Chem*. **271**:10941-10945.

Yatani, A., Matteram, R., Codina, J., Graf, R., Okabe, K., Padrell, E., Iyengar, R., Brown, A. M., and Birnbaumer, L. 1988. The G protein-gated atrial $K^+$ channel is stimulated by three distinct $G_{i\alpha}$-subunits. *Nature* **336**:680-682.

Yoshimura, M., Ikeda, H., and Tabakoff, B. 1996. Mu-Opioid receptors inhibit dopamine-stimulated activity of type V adenylyl cyclase but enhance dopamine-stimulated activity of type VII adenylyl cyclase. *Mol Pharmacol.* **50**:43-51.

Yu, S. S., Lefkowitz, R. J., and Hausdorff, W. P. 1993. β-adrenergic receptor sequestration. A potential mechanism of receptor resensitization. *J. Biol. Chem.* **268**:337-341.

Yukawa, T., Ukena, D., Chanez, P., Dent, G., Chung, K. F., and Barnes, P. J. 1990. β-adrenergic receptors on eosinophils: binding and functional studies. *Am. Rev. Respir. Dis.* **141**:1446-1452.

Zeiders, J. L., Seidler, F. J., Iaccarino, G., Koch, W. J., and Slotkin, T. A. 1999. Ontogeny of cardiac β-adrenoceptor desensitization mechanisms: agonist treatment enhances receptor/G-protein transduction rather than eliciting uncoupling. *J. Mol. Cell. Cardiol.* **31**:413-423.

Zhang, J., Ferguson, S. G., Barak, L. S., Menard, L., and Caron, M. G. 1996. Dynamin and β-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. *J. Biol. Chem.* **271**:18302-18305.

# Appendix 1

*Abbreviations for Amino Acids.*

| Amino Acid | Three Letter Abbreviation |
|---|---|
| Alanine | Ala |
| Arginine | Arg |
| Asparagine | Asn |
| Aspartic Acid | Asp |
| Asparagine or Aspartic Acid | Asx |
| Cysteine | Cys |
| Glutamine | Gln |
| Glutamic Acid | Glu |
| Glutamine or Glutamic Acid | Glx |
| Glycine | Gly |
| Histadine | His |
| Isoleucine | Iso |
| Leucine | Leu |
| Lysine | Lys |
| Methionine | Met |
| Phenylalanine | Phe |
| Proline | Pro |
| Serine | Ser |
| Threonine | Thr |
| Tryptophan | Trp |
| Tyrosine | Tyr |
| Valine | Val |

## Appendix 2: References Erratum


Beavo, J. A. 1995. Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiol. Rev.* **75**:725-748.

Benovic, J. L., Mayor, F. Jr., Somers, R. L., Caron, M. G., and Lefkowitz, R. J. 1986. Light-dependent phosphorylation of rhodopsin by β-adrenergic receptor kinase. *Nature* **321**:869-872.

Böhm, M., Flesch, M., Schnabel, P. 1997. β-adrenergic signal transduction in the failing and hypertrophied myocardium. *J. Mol. Med.* **75**:842-848.

Dougall, I. G., Harper, D., Jackson, D. M., and Leff, P. 1991. Estimation of the efficacy and affinity of the $\beta_2$-adrenoceptor agonist salmeterol in guinea-pig trachea. *Br. J. Pharmacol.* **104**:1057-1061.

Hall, J. A., Kaumann, A. J., and Brown, M. J. 1990. Selective $\beta_1$-adrenoceptor blockade enhances positive inotropic responses to endogenous catecholamines mediated through $\beta_2$-adrenoceptors in human atrial myocardium. *Circ. Res.* **66**:1610-1623.

Hamid, Q. A., Mak, J. C., Sheppard, M. N., Corrin, B., Venter, J. C., and Barnes, P. J. 1991. Localization of $\beta_2$-adrenoceptor messenger RNA in human and rat lung using in situ hybridization: correlation with receptor autoradiography. *Eur. J. Pharmacol.* **206**:133-138.

Hausdorff. W. P., Caron, M. G., and Lefkowitz, R. J. 1990. Turning off the signal: desensitization of β-adrenergic receptor function. *FASEB J.* **4**:2881-2889.

Jones, T. R., Charette, L., Garcia, M. L., and Kaczorowski, G. J. 1993. Interaction of iberiotoxin with β-adrenoceptor agonists and sodium nitroprusside on guinea pig trachea. *J. Appl. Physiol.* **74**:1879-1884.

Kaumann, A. J., and Lemoine, H. 1987. $\beta_2$-adrenoceptor-mediated positive inotropic effect of adrenaline in human ventricular myocardium. Quantitative discrepancies with binding and adenylate cyclase stimulation. *Naunyn Schmiedebergs Arch. Pharmacol.* **335**:403-11.

Konzett, H., and Rossler, R. 1940 Versuchsanordnung zu untersuchungen an der bronchialmuskulatur. *Arch. Exp. Path. Pharmak.* **195**: 71-74.

Kwatra, M. M., Leung, E., Maan, A. C., McMahon, K. K., Ptasiensk,i J., Green, R. D., and Hosey, M. M. 1987. Correlation of agonist-induced phosphorylation of chick heart muscarinic receptors with receptor desensitization. *J. Biol. Chem.* **262**:16314-16321.

Lemoine, H., and Kaumann, A. J. 1991. Regional differences of $\beta_1$- and $\beta_2$-adrenoceptor-mediated functions in feline heart. A $\beta_2$-adrenoceptor-mediated positive inotropic effect possibly unrelated to cyclic AMP. *Naunyn Schmiedebergs Arch. Pharmacol.* **344**:56-69.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the Folin phenol agent. *J. Biol. Chem.* **193**: 265-270.

Martling, C-R, Saria, A., Brodin, E., Rosell, S., and Folkers, K. 1984. Capsaicin pretreatment inhibits vagal cholinergic control of pulmonary mechanics in the guinea-pig. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **325**:343-348.

Nerme, V., Abrahamsson, T., and Vauqulin, G. 1990. Chronic isoproterenol administration causes altered β-adrenoceptor-Gs-coupling in guinea pig lung. *J. Pharmacol. Exp. Ther.* **252**:1341-1346.

Palczewski, K., and Benovic, J. L. 1990. G-protein-coupled receptor kinases. *Trends Biochem. Sci.* **16**:387-391.

Palczewski, K., Carruth, M. E., Adamus, G., McDowell, J. H., and Hargrave, P. A. 1990. Molecular, enzymatic and functional properties of rhodopsin kinase from rat pineal gland. *Vision Res.* **30**:1129-11.37.

Rabe, K., Dent, G., Giembycz, M. A., Perkins, R. S., Evans, P., and Barnes, P. 1990. Effects of the long-acting β-adrenoceptor agonist formoterol vs albuterol on $LTB_4$-induced $Ca^{2+}$ mobilisation, $H_2O_2$ and $TXB_2$ release in guinea-pig eosinophils. *Am. Rev. Respir. Dis.* **141**:A29.

Raeburn, D., Underwood, S. L., and Villamil, M. E. 1992. Techniques for drug delivery to the airways, and the assessment of lung function in animal models. *J. Pharmacol. Toxicol. Methods.* **27**:143-159.

Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51**:600-672.

Taussig, R., Tang, W. J., Hepler, J. R., and Gilman, A. G. 1994. Distinct patterns of bidirectional regulation of mammalian adenylyl cyclases. *J. Biol. Chem.* **269**:6093-6100.

Thomas, J. M., and Hoffman, B. B. 1989. Chromic somatostatin treatment induces enhanced forskolin-stimulated cAMP accumulation in wild-type S49 mouse lymphoma cells but not in protein kinase-deficient mutants. *Mol. Pharmacol.* **34**:116-124.

Travis, M. D., Whalen, E. J., and Lewis, S. J. 1997. Heterologous desensitization of β-adrenoceptor signal transduction in vivo. *Eur. J. Pharmacol.* **5**:R1-3.

Verleden, G. M., Belvisi, M. G., Rabe, K. F., Miura, M., Barnes, P. J. 1993. $\beta_2$-adrenoceptor agonists inhibit NANC neural bronchoconstrictor responses in vitro. *J. Appl. Physiol.* **74**:1195-1199.



BIBL MONDON UNIV