

**CHARACTERIZATION OF THE ~~HUMAN~~ LYMPHOBLAST
BETA-ADRENERGIC RECEPTOR IN BIPOLAR DEPRESSION**

by

GILLIAN KAY

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**Department of Pharmacology & Toxicology
St. Mary's Hospital Medical School
London, W2 1PG**

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ABSTRACT

Bipolar depression is characterised by periods of depression interspersed with episodes of mania. Of all the affective disorders it shows the strongest hereditary tendency. No consistent biochemical defect has yet been associated with bipolar depression. The aim of this study was to characterise the *beta*-adrenergic receptor (β -AR) on virally transformed human lymphoblasts from bipolar depressed and control subjects. Transformation of lymphocytes using the Epstein-Barr virus allowed cells to be grown in culture for long time periods under controlled conditions.

The β -AR was characterised initially in controls in terms of receptor binding, using [^{125} I]-iodocyanopindolol ([^{125} I]-CYP), and stimulation of the associated enzyme, adenylyl cyclase. Scatchard analysis indicated a single binding site which displacement studies showed to be a β_2 type adrenoceptor. Comparison to lymphocyte [^{125} I]-CYP binding showed no differences in binding affinity but significantly lower binding capacity. Adenylyl cyclase stimulation was concentration and time dependent. β -AR regulation was investigated by coincubation with isoprenaline (10^{-10} - 10^{-5} M) for 4 hours, 24 hours or 7 days. Down-regulation of lymphoblast β -ARs in response to isoprenaline was concentration and time dependent and accompanied by decreased receptor function as defined by adenylyl cyclase activity.

Cell lines from 12 subjects with bipolar depression and 12 sex and age matched controls were established and basal binding characteristics and adenylyl cyclase activity compared. There were no significant differences in binding capacity or in β -AR-coupled cAMP accumulation. Down-regulation in response to 24 hour coincubation with 1nM isoprenaline was also examined. Down-regulation was significantly less in bipolar cell lines.

7 day coincubation with lithium chloride had a differential effect on β -AR regulation in bipolar and control cell lines. Down-regulation was increased in bipolar cell lines and slightly decreased in controls. 7 and 14 day coincubation with desipramine had a similar differential effect on β -AR regulation.

It is proposed that bipolar depression is accompanied by a dysfunction in β -AR regulation that is normalized by lithium and desipramine.

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to the memory of my dear mother
Esther Kay
whose values will always guide me and
whose love will always be with me.

CHAPTER ONE

INTRODUCTION

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1.1 The β -Adrenergic Receptor

1.1.1 Structure and function

Adrenergic receptors have been delineated by pharmacological approaches into two distinct classes, α - and β -adrenergic receptors, based on the relative potencies of agonists and antagonists (Alquist, 1948). The β -adrenergic receptor (β -AR) has been further subdivided into two subclasses termed β_1 and β_2 (Lands *et al.*, 1967).

β -ARs are coupled to the membrane-bound enzyme complex adenylyl cyclase, which catalyzes the formation of the second messenger 3',5'-cyclic adenosine monophosphate (cAMP) from the substrate adenosine triphosphate (ATP) (Sutherland *et al.*, 1968). The β -AR is functionally linked to adenylyl cyclase by a distinct guanine nucleotide regulatory protein, Gs (Gilman, 1984). Both the β_1 - and the β_2 -AR stimulate adenylyl cyclase.

When successful direct radioligand-binding techniques for studying the β -AR were established in 1974, the subclassification of these receptors into β_1 and β_2 subtypes was confirmed (see below). The β_1 -AR appears to predominate in mammalian heart and adipose tissue, whereas the β_2 -AR predominates in the lung, liver and smooth muscle. In the rat brain, the β_1 -AR is at its highest concentration in the pineal gland, the cerebral cortex and the striatum, while the β_2 -AR is present at low concentrations in several areas (Lefkowitz *et al.*, 1983; Frielle *et al.*, 1988a). In the human brain, 80-90 % of the β -ARs are the β_1 -type in the caudate and putamen, 60-80 % are β_1 in the frontal, occipital and temporal cortical areas, whilst the amygdala, thalamus and hippocampus have an equal proportion of both β_1 - and β_2 -ARs (De Paermentier *et al.*, 1989 & 1990).

Purification of receptor-binding macromolecules and photoaffinity labelling of these receptor structures have contributed much to our knowledge of the β -ARs' structure and molecular properties, the elucidation of which is important in order to understand the biochemical mechanisms of hormone and drug action. Using photoaffinity labelling, β -AR-binding peptides were labelled in erythrocytes from frogs, turkeys, ducks and rats (Lavin *et al.*, 1982; Rashidbaigi & Ruoho,

1982), in membranes from several mammalian tissues and species (Lavin *et al.*, 1982; Benovic *et al.*, 1983) and in S49 lymphoma cells (Rashidbaigi *et al.*, 1982). β -ARs from several sources have been purified to apparent homogeneity by coupling repeated affinity chromatography cycles with ion exchange chromatography and several high performance liquid chromatography steps (HPLC) (Shorr *et al.*, 1981; 1982a,b). These purified receptors were shown to bind to radioligands with β -adrenergic specificity, stereoselectivity and affinity and the results from photoaffinity labelling in the purified membranes were in accord with those from crude membranes (Shorr *et al.*, 1981; 1982b). A further technique, that of radiation inactivation, estimates the functional target size of a protein under denaturing conditions. These techniques led to the conclusion that in mammalian systems, and in the frog, both β_1 - and β_2 -AR binding sites reside on peptides of M_r 58-62 000, whilst the avian erythrocyte β_1 -like-AR binding site resides on a peptide with a maximum M_r of 40-50 000 (Shorr *et al.*, 1982b; Lefkowitz *et al.*, 1983). The human placenta, which contains both β_1 - and β_2 -ARs, was used as a source of β -ARs for purification by Bahouth and Malbon (1987). The purified receptors were shown to have a molecular weight of 67 000 and agonist and antagonist competition profiles demonstrated the presence of both β_1 - and β_2 -ARs. The authors suggested that the pharmacologically distinct β_1 - and β_2 -ARs are structurally similar peptides.

The techniques of molecular biology have greatly added to our knowledge of adrenergic receptor structure. Gene cloning and sequencing have provided data showing that a high degree of structural homology exists between a number of neurotransmitter receptors and opsins. Each of the receptors cloned to date appears to be a member of one of a limited number of super-families which have sequence and presumably structural homologies (Birdsall, 1989). There are three main super-families: neurotransmitter-gated ion channels (Stevens, 1987); ligand activated tyrosine kinase receptors; and receptors capable of activating guanine nucleotide regulatory proteins (G-proteins) and so modulating adenylyl cyclase activity, phosphatidylinositol biphosphate hydrolysis or the activity of certain brain channels (Hulme & Birdsall, 1986). Secondary-structure similarities have been found by analysis of the protein sequences deduced from receptor gene sequences and by hydropathy calculations which predict potential membrane-spanning

regions. By comparison of the secondary structural features, relationships between different receptors becomes more apparent. The primary structures of several members of the G-protein-coupled family of receptors have been determined in recent years. These include all the adrenergic receptor subtypes (α_1 , α_2 , β_1 and β_2), several serotonergic, dopaminergic and muscarinic cholinergic receptors, and rhodopsin. These receptors all share a conserved structure and presumed membrane topography (O'Dowd *et al.*, 1989).

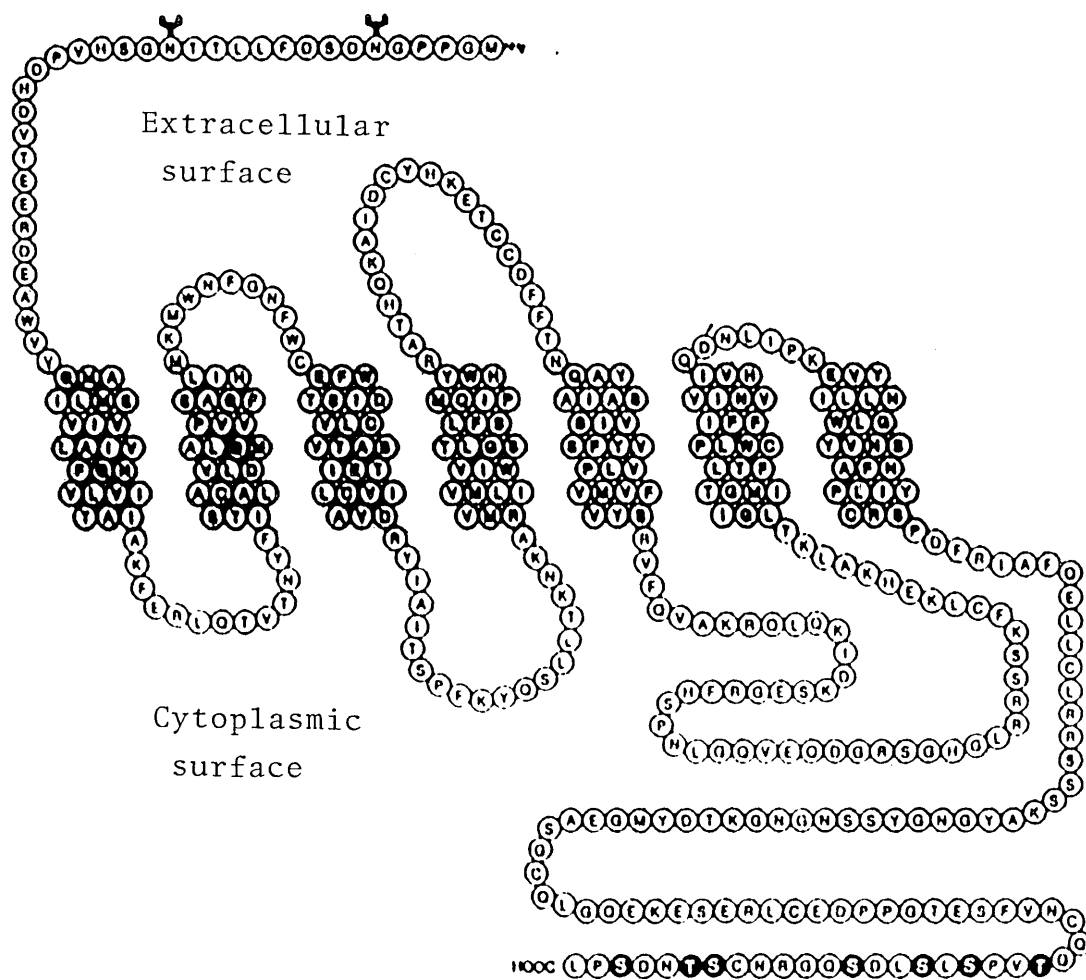
Between 1981 and 1984, the avian erythrocyte β_1 -like, the amphibian erythrocyte β_2 and the mammalian lung β_2 receptors were purified to homogeneity and shown to retain binding activity in detergent-solubilized form (Shorr *et al.*, 1981 & 1982b; Homcy *et al.*, 1983; Benovic *et al.*, 1984). Using the purified hamster lung β_2 -AR, the gene and cDNA were cloned for the mammalian β_2 -AR by Dixon's group in 1986. The primary amino acid sequence of portions of the receptor protein was determined by fragmenting the purified receptor with cyanogen bromide, resolving the resulting peptides by reverse phase HPLC and then subjecting several of these peptides to gas phase sequencing. Based on sequences of several such peptides, synthetic oligonucleotides were constructed and used to screen genomic libraries. Appropriate restriction fragments from genomic clones were then used to screen cDNA libraries. The entire amino acid sequence of the β_2 -AR was then elucidated based on the sequences of the cDNA and genomic clones.

Hydropathy profiles of the amino acid sequence predicted for the β_2 -AR indicated that the β -AR sequence would encode a largely hydrophobic polypeptide, with the N-terminal region of the receptor being predominantly hydrophobic and the C-terminal end being hydrophilic. It was observed that there was significant amino acid homology with bovine rhodopsin and that the hydropathy profile was very similar to that of the rhodopsins in general. Bacteriorhodopsin was already known to contain seven membrane-spanning helices, and they proposed that the β -AR also has multiple membrane-spanning regions. It was also noted that the sequence homology between β -AR and rhodopsin parallels similarities in their function as both polypeptides are involved in signal transduction mechanisms involving the guanine nucleotide regulatory

proteins, Gs and transducin, respectively (Gilman, 1984). Furthermore, rhodopsin kinase multiply phosphorylates rhodopsin at its C-terminus, which contains seven serine and threonine residues, and the C-terminus of the postulated sequence for β -AR also contained several serine and threonine residues that could serve as phosphorylation sites (Dixon *et al.*, 1986). This led to the proposal that β -AR agonists and antagonists interact with the β -AR in a similar manner to that by which retinal interacts with opsin; that is, they intercalate among the transmembrane helices and so determine whether the receptor is in its active or inactive conformation (Dixon *et al.*, 1986). The similarity between the β -AR and rhodopsin was extended further when it was shown that β -AR kinase (β ARK), which phosphorylates only the agonist-occupied β -AR (Benovic *et al.*, 1986a), was capable of phosphorylating rhodopsin in a light-dependent manner similar to that in which rhodopsin kinase phosphorylates rhodopsin (Benovic *et al.*, 1986b). A proposed structure of the β -AR was therefore suggested (Fig. 1.1), based on the similarities in the sequence, function and regulation of the β -AR and rhodopsin (Benovic *et al.*, 1986b). The seven membrane-spanning regions are connected by a series of extracellular and cytoplasmic loops. There are two sites of N-linked glycosylation near the amino terminus and two cAMP-dependent kinase phosphorylation sites in the presumed cytoplasmic domains in addition to the serine- and threonine-rich C-terminus (Lefkowitz *et al.*, 1986).

The cloning and determination of the complete nucleotide sequence of the turkey β -AR (Yarden *et al.*, 1986) and the human β_2 -AR rapidly followed that of the hamster β_2 -AR (Kobilka *et al.*, 1987). As for the hamster receptor, the deduced amino acid sequence of the human β_2 -AR is that of a protein containing seven clusters of hydrophobic amino acids. This protein is 87% identical overall with the hamster β_2 -AR, with the most highly conserved regions being the putative membrane-spanning regions (95% homology) and the cytoplasmic loops (93% homology). This suggested that these regions of the molecule contain important functional domains. The most divergent regions are the N- and C-termini. The two sites of N-linked glycosylation, the two sites of cAMP-dependent phosphorylation and the serine- and threonine-rich C-terminus present in the hamster β_2 -AR, are all conserved. This group also localized the gene for the β_2 -AR to q31-q32 on chromosome 5.

Fig. 1.1 The proposed structure of the β -AR, showing the glycosylation and phosphorylation sites (after Benovic *et al.*, 1986b)



Dixon *et al.* (1987) have shown that most of the hydrophilic residues are not directly involved in the binding of agonists and antagonists and suggested that ligands interact with a site located close to the membrane or within the hydrophobic core of the protein in a similar manner to which retinal binds to a site within the hydrophobic core of rhodopsin. This suggestion was supported and extended by the work of Wong *et al.* (1988) who determined the locations at which two similar photoaffinity ligands covalently attached to the turkey erythrocyte β -AR. Their results indicated that β -AR ligands bind within a bundle of membrane spanning domains and that the seventh membrane span borders the binding site as it does in rhodopsin. They proposed that a catecholamine agonist would promote activation of the β -AR by distorting its structure in the same manner as *trans*-retinal distorts the structure of rhodopsin resulting in increased affinity for Gs.

When the human β_2 -AR cDNA was used to probe human genomic DNA, the G-21 clone was isolated due to its cross-hybridization with β_2 -AR (Kobilka *et al.*, 1987). This was determined to encode a protein with striking sequence and structural similarities with the G protein-coupled family of receptors, which was later identified as the 5-HT_{1A} receptor (Fargin *et al.*, 1988). The G-21 DNA was used to screen a human placenta cDNA library and a cDNA clone encoding the human β_1 -AR was isolated (Frielle *et al.*, 1987). This clone is very similar to the turkey β -AR, which suggests that the avian receptor is closely related to the human β_1 -AR despite differences in pharmacology. Comparison of the deduced structures of the two β -AR subtypes showed that there is the greatest similarity in the membrane-spanning regions. As the hydrophobic core is thought to contain the ligand binding site, differences in sequence in these regions may explain the differences in the pharmacology of the two subtypes.

As the β_1 - and β_2 -ARs are structurally, functionally and biochemically similar, the question arose whether the two receptor subtypes are the products of different genes or whether they represent post-translationally modified products of a common gene. However, examination of the β -ARs' nucleotide sequences ruled out post-translational modification as they contain no introns and Frielle *et al.* (1988a) have proposed that they are, in fact, products of distinct genes.

Like the β_2 -AR, the β_1 -AR appears to couple to Gs, and it was suggested that the cytoplasmic receptor domains are involved with this coupling. There is significant similarity between the first and second cytoplasmic loops of the two human receptors and the avian β -AR, but the third loop and the C-terminus are quite divergent in all three proteins (Frielle *et al.*, 1987). Dixon *et al.* (1987) demonstrated that a mutant of the hamster β_2 -AR, that had amino acids 239-372 deleted, had high agonist affinity, but did not couple to adenylyl cyclase. This group later showed that deletion of either amino acids 222-229 or 258-270 produced mutant receptors that did not couple to adenylyl cyclase, and whose interactions with Gs were also altered (Strader *et al.*, 1987). These two regions of the receptor protein span the entire third cytoplasmic loop, with amino acids 222-229 being at the N-terminal portion of the loop and amino acids 258-270 at the C-terminal end, with both peptide segments forming the junctions with the adjacent hydrophobic domains (Strader *et al.*, 1987).

α_2 - and β -ARs have different ligand binding properties and opposite effects on the adenylyl cyclase system (α_2 -ARs are inhibitory and β -ARs stimulatory). The construction and expression of chimeric $\alpha\beta$ -AR genes and then the investigation of the ligand binding and adenylyl cyclase activating properties of the resulting chimeric receptors, allows the structural domains determining the specificity of ligand binding and effector coupling to be deduced. Kobilka *et al.* (1988) used chimeric human $\alpha_2\beta_2$ -ARs to deduce that the specificity for coupling to Gs lies within a region extending from the N-terminus of the fifth hydrophobic domain to the C-terminus of the sixth, a region that includes the third cytoplasmic loop.

The importance of the C-terminal portion of the third cytoplasmic loop for productive receptor-Gs coupling was confirmed by O'Dowd *et al.* (1988). This group examined the ability both of human β_2 -AR mutants, with site-directed mutations and deletions, and of chimeric human $\alpha_2\beta_2$ -ARs to mediate isoprenaline stimulation of adenylyl cyclase via Gs. They showed that the N-terminal segment of the cytoplasmic tail was also involved in productive coupling to Gs. In addition, they implicated the second cytoplasmic loop and a conserved cysteine residue in the cytoplasmic tail for supportive roles in maintaining the proper orientation of

the Gs binding site. Kobilka *et al.* (1988) also deduced that the major determinants of α_2 - and β_2 -AR agonist and antagonist ligand binding specificity are within the seventh membrane spanning domain, and that several of the first five membrane spanning domains may contribute to agonist binding specificity.

Chimeric human $\beta_1\beta_2$ -ARs have been used to elucidate the structural basis for the pharmacological distinctions between these two receptor subtypes (Frielle *et al.*, 1988). Agonist and antagonist binding studies and adenylyl cyclase assays with these chimeric $\beta_1\beta_2$ -ARs indicated that the fourth membrane-spanning region is largely responsible for determining β_1 as opposed to β_2 agonist binding properties, whilst the sixth and seventh membrane-spanning regions have an important role in determining β_1 as opposed to β_2 antagonist binding properties. A number of other membrane-spanning regions were also shown to contribute, to a lesser extent, to agonist and antagonist binding specificity. Thus subtype specificity is determined by most of the transmembrane regions of the receptors, lending support to the hypothesis that the binding of ligands to β -ARs occurs in a pocket formed by the clustering of these membrane-spanning α -helices. However, antagonists interact with structural determinants distinct from those with which agonists interact (Frielle *et al.*, 1988).

1.1.2 Receptor occupation and response

The hypothesis that tissue response is dependent on the proportion of receptors occupied by the stimulating agent - the "occupation theory" of drug action - was first proposed by Clark (1926; 1933). Several modifications to this theory have since been made. Ariens (1954) proposed a proportionality constant " α " (intrinsic activity) between response and receptor occupation. Stephenson (1956) differentiated between the response of the tissue and the stimulus caused by the agonist compound, proposing that the latter was directly related to the degree of receptor occupation. He termed the proportionality constant between receptor occupation and the stimulus, efficacy.

The simplest model of a ligand-receptor interaction assumes that each molecule of ligand (D) binds independently and reversibly to a single receptor molecule (R) according to the law of mass action, forming a complex (DR). This gives the following equation:

$$[DR] = \frac{[D] [R_t]}{[D] + K_d} \quad (1)$$

where R_t = total receptor population

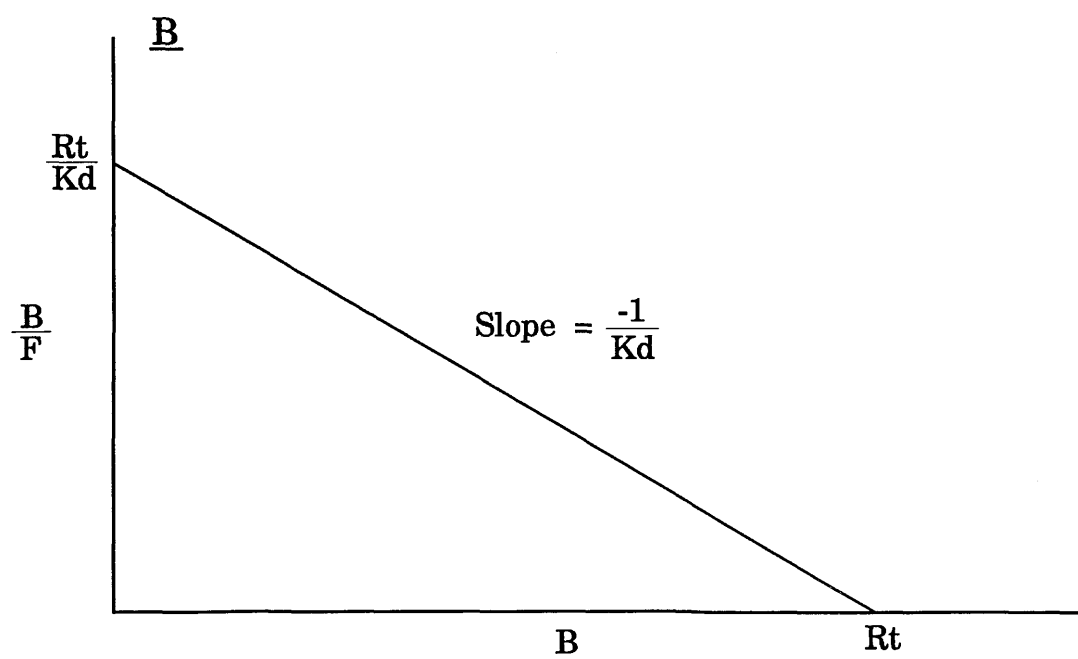
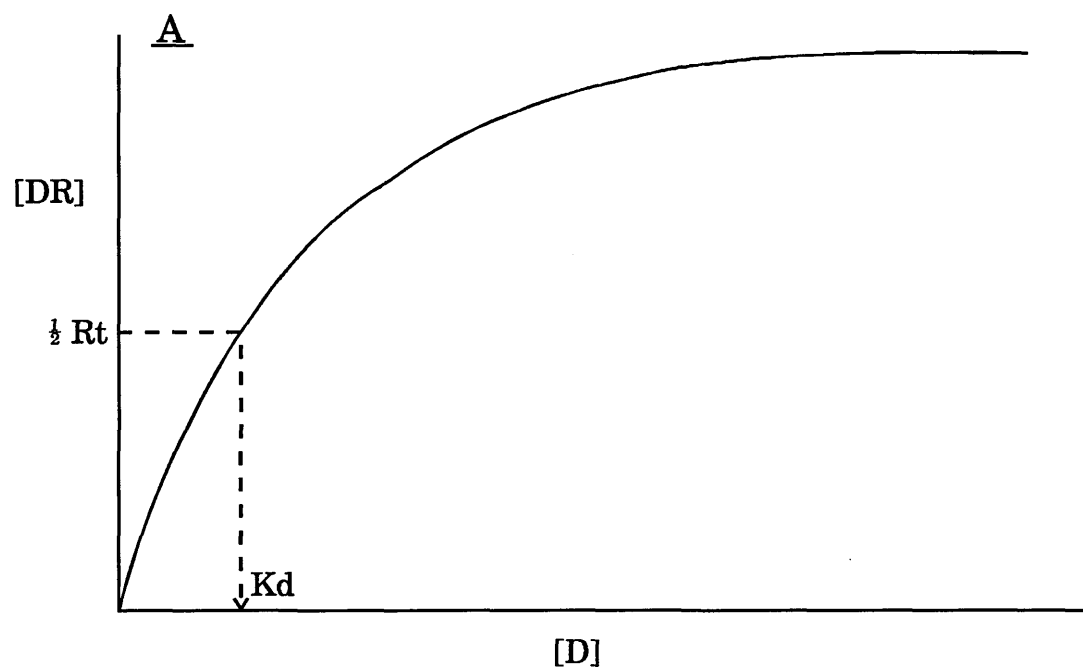
K_d = dissociation equilibrium constant.

Hence the concentration of bound ligand-receptor complex increases in a hyperbolic manner with increasing free ligand concentration towards a maximum value R_t . From (1) it can be seen that when the concentration of DR is equal to $\frac{1}{2}R_t$, then the concentration of D is equivalent to the K_d value. Thus the free ligand concentration at which half the total number of receptors are bound is equivalent to the dissociation equilibrium constant. This value is characteristic of the interaction between ligand and receptor and describes the affinity of the receptor for that ligand. The theoretical binding curve is shown in Fig. 1.2A. If at equilibrium the amount of ligand bound is negligible compared to the total ligand concentration, then the concentration of ligand can be measured as the total ligand present rather than the amount free in solution. This holds true in the case of [^{125} I]-iodocyanopindolol ([^{125} I]-CYP) binding to β -ARs on the lymphoblast as the receptor density is quite low.

Scatchard (1949) produced a linearized version of the binding equation with B replacing [DR] to describe the concentration of bound ligand and F in the place of [D] for the concentration of free ligand:

$$\frac{B}{F} = \frac{R_t - B}{K_d} \quad (2)$$

Fig. 1.2 A. Theoretical binding curve
 B. Scatchard plot



A comparison of B/F against B results in a straight line in the case of independent monomeric binding with a slope of $-1/K_d$ and intercepts of R_t and R_t/K_d (Fig. 1.2B). This Scatchard plot is most applicable to the curvilinear region of the hyperbolic binding curve at free ligand concentrations between $0.1-10 \times K_d$.

Non-linear Scatchard plots result when the binding of the ligand occurs to more than one binding site or when cooperativity of binding is present. The Scatchard plot resulting from binding to multiple sites is a composite of individual curves, so that binding at two sites produces a hyperbola whose asymptotes are the component binding curves. Cooperative binding of ligand to receptor complexes results in a sigmoidal or a flattened hyperbolic curve for positive and negative cooperativity, respectively.

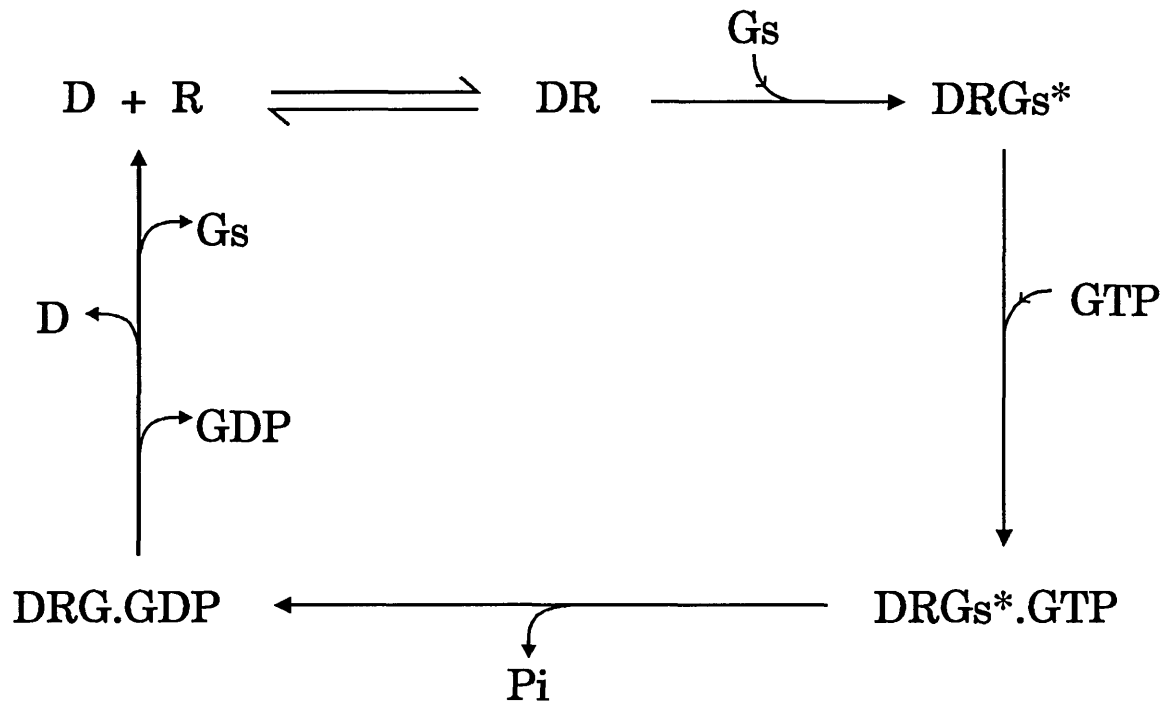
The Hill plot is used to investigate cooperativity and is a linear plot of $\log [DR]/[R_t]-[DR]$ against $\log [D]$, where the slope of the line is the Hill coefficient. In cases of positive cooperativity this value is greater than 1 and conversely, in cases of negative cooperativity, it is less than 1. Values of the Hill coefficient greater than unity also result when binding of more than one ligand to each receptor is occurring. A Hill coefficient of 1 usually indicates the absence of cooperativity and a simple 1:1 relationship between the ligand and the receptor.

The binding of agonists has been shown to be sensitive to the presence of certain cations and purine nucleotides which do not affect the binding of antagonists (Lefkowitz *et al.*, 1976; Peroutka *et al.*, 1979). In general these receptor modulators reduce agonist binding affinity. This indicates that the mechanism of receptor binding and activation is probably more complex than the "occupation theory" would suggest. It would also seem that antagonist binding will give a more accurate and consistent measure of binding affinities.

Rodbell (1980) suggested a cyclic agonist-receptor interaction, with the extent of the final response being dependent on the rate of turnover of the activated ligand-receptor complex. According to the mechanism shown in Fig. 1.3, the binding of antagonist would occur by a simple reversible process as described by equation (1). Agonist binding, however, would be followed by activation and

formation of the excited complex DRG_s^* . Endogenous GTP would then bind to this complex, forming $DRG_s^*.GTP$ which would in turn activate an intracellular component by phosphorylation and hence initiate the response. GTP is then dephosphorylated to GDP, causing the dissociation of the remaining $DRG.GDP$ complex to its components D, R, G_s , and GDP.

Fig. 1.3 Mechanism of agonist-receptor interaction and the role of G_s .



1.1.3 Ligand-binding studies

The development of direct radioligand binding techniques has been very important both for progress in the molecular biology of β -ARs and for the study of β -AR density and regulation in membrane preparations. Early attempts to identify β -ARs by direct binding methods with [^{3} H]catecholamines were associated with discrepancies between binding data and data from physiological response measurements. Cuatrecasas *et al.* (1974) showed that binding of [^{3} H]-noradrenaline (NA) to intact cells and microsomes was not in fact a measure of adrenergic receptors, but of a membrane catecholamine binding protein related to catechol-O-methyl transferase. Therefore criteria for β -AR identification which were more refined and more stringent than those previously used were proposed (Cuatrecasas *et al.*, 1974; Lefkowitz, 1974). These criteria are:

- a) β -AR binding should be rapid and reversible;
- b) Receptor binding should be saturable indicating a finite number of binding sites;
- c) β -AR agonists and antagonists should compete for binding sites in a manner that closely parallels the known specificity of physiological β -AR mediated responses; structurally related compounds which are devoid of physiological β -AR mediated effects should not compete for the binding sites;
- d) (-)Stereoisomers of adrenergic agonists and antagonists should be considerably more potent than the (+)stereoisomers in competing for the binding sites, thus reflecting the stereospecificity observed in physiological β -AR responses.

Lymphocytes were shown to contain a β -AR coupled adenylyl cyclase system by Bourne & Melmon (1971). Having identified binding sites in frog erythrocytes and canine myocardium which satisfied all of the above criteria, in 1976 Williams *et al.* identified the β -AR in the lymphocyte using ($\pm$)[^{3} H]-dihydroalprenolol ([^{3} H]-DHA), the first time the β -AR had been identified in a human tissue using direct binding techniques. Binding studies on human lymphocytes have been performed using [^{3} H]-DHA and an iodinated

ligand (-)[¹²⁵I]-iodohydroxybenzylpindolol ([¹²⁵I]-HYP) (Aarons *et al.*, 1980), but Brodde *et al.* (1981) suggested that neither of these ligands were ideal for β -AR binding studies. [³H]-DHA has an affinity to β -ARs in the nanomolar range and has a low specific activity of 20-60 Ci/mmol, both of which facts necessitate the use of large amount of tissue. Another drawback of [³H]-DHA is that it has been shown to label a second binding site. Binding to this site can be displaced by 5-HT_{1A} receptor-selective compounds (Riva & Creese, 1989). [¹²⁵I]-HYP is a racemate with a higher affinity, in the 0.1-0.01 nM range, and a higher specific activity of 2175 Ci/mmol. However, this radioligand has high nonspecific binding which can only be partially suppressed by high concentrations of phentolamine or 5-HT.

A highly specific β -AR radioligand, ($\pm$)[¹²⁵I]-iodocyanopindolol ([¹²⁵I]-CYP), which binds with high affinity and specificity to β -ARs, was used to identify and characterize lymphocyte β -ARs by Brodde *et al.* in 1981. The [¹²⁵I]-CYP binding in the lymphocyte was rapid, saturable and of high affinity (K_d value in the 0.01 nM range). The binding was stereospecific and had relatively low nonspecific binding. Binding was not affected by phentolamine or 5-HT. Linear Hofstee plots were obtained indicating the presence of a homogenous population of β -ARs, which were identified to be of the β_2 subtype. This suggests that [¹²⁵I]-CYP is the ligand of choice for characterization of the lymphocyte β -AR, a view that is supported by Werstiuk *et al.* (1990) in a review of studies of the lymphocyte β -AR in depression.

β -AR agonists bind to two distinct states of the β -AR, a high affinity state, which is sensitive to guanyl nucleotides, and a low affinity state, which is insensitive to guanyl nucleotides (Kent *et al.*, 1980). The lymphocyte β_2 -AR is no exception to this rule. In the absence of GTP, the displacement curve for selective β_2 -AR agonists is shallow, resulting in a nonlinear Hofstee plot, indicating the presence of two affinity states of the receptor. The addition of GTP to the assay shifts the inhibition-concentration curve to the right and steepens it, so the Hofstee plot becomes linear, and all the receptors are in the low affinity state (Brodde *et al.*, 1981). The ratio of the affinity of a ligand to the receptors in the low affinity state to that of those in the high affinity state, the K_L/K_H ratio, is a

measure of the agonist affinity state of the β -ARs, with a higher ratio indicating higher β -AR sensitivity to NA.

1.1.4 β -Adrenoceptor-coupled activation of adenylyl cyclase

Adenylyl cyclase is activated by β -ARs through the stimulatory G protein, Gs (Gilman, 1987). Gs is required for adenylyl cyclase activation by receptors under physiological conditions (Ross *et al.*, 1978). Hormone-sensitive adenylyl cyclase activity has been reconstituted in phospholipid vesicles from three purified proteins - the β -AR, Gs, and adenylyl cyclase itself (May *et al.*, 1985). Thus, these three proteins suffice to constitute a primary pathway for hormonal stimulation of cAMP synthesis.

Gs is made up of three subunits α , β , and γ , that are associated with the cell membrane. The β and γ subunits form a single functional unit and are functionally interchangeable with $\beta\gamma$ subunits from other G proteins, including Gi which inhibits adenylyl cyclase (Levitzki, 1988).

The demonstration of β -AR-dependent GTPase activity in the turkey erythrocyte provided conclusive evidence for the role of GTP in the regulation of hormone-dependent adenylyl cyclase (Cassel & Selinger, 1976). Hormone-stimulated GTPase has since been demonstrated for many other stimulatory and inhibitory adenylyl cyclase systems (Levitzki, 1986; Gilman, 1987). The agonist-bound β -AR interacts with Gs and it has been suggested that this induces a conformational change in Gs to an "open" state which facilitates GDP to GTP exchange (Cassel & Selinger, 1976; Braun *et al.*, 1982). The process of GDP/GTP exchange is much slower than the hydrolysis of GTP to GDP at the Gs α -subunit (Arad & Levitzki, 1979). Thus the rate limiting step in the on-off cycle is the GDP to GTP exchange reaction. The interaction of the agonist-bound receptor with Gs facilitates the exchange of GDP for GTP and this stimulates GTPase activity and increases the flux of GTP through the system.

The hormonal signal is amplified in two ways. Firstly, each agonist-bound β -AR activates several Gs molecules (Tolkovsky & Levitzki, 1978; Hekman *et al.*, 1984). Secondly, further signal amplification occurs because the rate constant for GTP hydrolysis ($\sim 13 \text{ min}^{-1}$ at 37°C) is 100-times smaller than the turnover number of adenylyl cyclase ($\sim 1100 \text{ min}^{-1}$), so several hundred molecules of cAMP can be synthesised during the lifetime of a single Gs.GTP complex (Gilman, 1987). The combined amplification at the receptor-Gs interaction is therefore in the order of 1000 (Levitzki, 1988). This mechanism where one receptor molecule catalyses the activation of numerous G proteins is known as "collision coupling" since the agonist-bound receptor interacts transiently with the cyclase system (Tolkovsky & Levitzki, 1978; Levitzki, 1986).

Gilman (1984) suggested that the $\beta\gamma$ subunit dissociates from Gs upon its activation by GTP binding. The active $\text{Gs}\alpha.\text{GTP}$ subunit then seeks the catalytic unit of adenylyl cyclase (C) to generate the active complex $\text{Gs}\alpha.\text{GTP.C}$. According to this model, $\beta\gamma$ and the catalyst C compete for $\text{Gs}\alpha.\text{GTP}$. Since $\beta\gamma$ from Gs and Gi are functionally interchangeable and probably identical (Gilman, 1987), the elevation of their concentration can result from the activation of Gi by inhibitory receptors, thus causing inhibition of the cyclase. As the formation of a Gi.C complex has yet to be demonstrated, this model provides a mechanism of cyclase inhibition via Gi.

Gs, or at least $\text{Gs}\alpha$, may in fact be permanently associated with C, an idea which is supported by kinetic findings and biochemical data (Gilman, 1987), although this hypothesis implies that the amplification step described above could not occur. If $\text{Gs}\alpha$ is permanently associated with C, then it is still possible that $\beta\gamma$ dissociates from Gs.C to yield the active species $\text{Gs}\alpha.\text{GTP.C}$, and to play an attenuating role when associated (Gilman, 1987). However, recent results from Levitzki's group (Marbach *et al.*, 1990) have demonstrated that the β subunit co-purifies with activated adenylyl cyclase, suggesting that subunit dissociation does not occur during Gs.C activation. It may be that the $\beta\gamma$ subunits do not provide a regulatory role, but simply serve to anchor the α subunit to the membrane bilayer (Sternweis, 1986).

1.1.5 β -Adrenoceptor regulation

A very general mechanism of cellular adaptation is an attenuation of responsiveness to stimuli with time, variously termed tachyphylaxis, desensitization, refractoriness or tolerance. Agonist-promoted desensitization appears to be a ubiquitous homeostatic mechanism by which target cells can modulate responsiveness to agents acting at cell-surface receptors. There are many clinical examples of desensitization to catecholamines, such as the rapid loss of the bronchodilating effects of β -AR agonists administered to patients with asthma and the attenuated inotropic response of β -AR agonists administered to patients with congestive heart failure (Lefkowitz *et al.*, 1984). β -AR stimulation by agonists, such as isoprenaline, causes a rapid increase in cAMP levels, which then decrease, often to near basal levels, with continued presence of the agonist (Shear *et al.*, 1975).

Hormone-induced desensitization has been categorized into two types (Perkins *et al.*, 1978; Sibley & Lefkowitz, 1985): homologous, or agonist-specific, desensitization; and heterologous, or agonist-nonspecific, desensitization. Desensitization is termed homologous when the subsequent response to the desensitizing hormone only is attenuated whilst responsiveness to other types of agonists is unimpaired. Conversely, in heterologous desensitization, incubation with one agonist results in an attenuated response to different agonists operating through distinct receptors. Response to stimulants that act at points distal to the receptor, such as guanine nucleotides and the fluoride ion, may also be decreased (Sibley & Lefkowitz, 1985).

It is likely that both types of desensitization occur simultaneously in many situations although different mechanisms are involved (Lefkowitz *et al.*, 1990). It has been proposed that rapid heterologous desensitization is mediated by phosphorylation of receptors by the cAMP-dependent protein kinase, protein kinase A (PKA), whereas rapid homologous desensitization is mediated primarily by receptor phosphorylation by a cAMP-independent protein kinase, β -adrenoceptor kinase (β ARK) (Lefkowitz *et al.*, 1990). β ARK is found in most mammalian tissues and has the ability to phosphorylate the β -AR and several

other closely related receptors only when they are occupied by agonists (Benovic *et al.*, 1986a; Sibley *et al.*, 1987). β ARK appears to be analogous to rhodopsin kinase, a retinal enzyme that phosphorylates and inactivates only the light-bleached form of rhodopsin (Benovic *et al.*, 1989).

Almost 20 years of work by many laboratories has indicated that a number of different mechanisms, operating at the levels of the receptor, G proteins, adenylyl cyclase and beyond, contribute to β -AR desensitization (Lefkowitz *et al.*, 1990). Events associated with the loss of adenylyl cyclase response are the uncoupling of the G-proteins from the receptors; internalization of the β -ARs from the cell surface (sequestration); β -AR phosphorylation; and the loss of β -ARs from the cell (down-regulation) (Fishman & Perkins, 1988). The order and interdependency of these events and their relative importance in the process of desensitization of adenylyl cyclase response have been the subject of much research. Receptor uncoupling is an impaired ability of the receptor to interact with Gs. This may be due to intrinsic changes in the receptors that diminish the effectiveness with which they are able to activate G proteins (biochemical uncoupling) or to the sequestration of the receptors from the plasma membrane, away from Gs (physical uncoupling) (Fishman & Perkins, 1988). Phosphorylation of the receptors by protein kinases reduces the efficiency of receptor coupling to G proteins (biochemical uncoupling) and appears to occur rapidly (seconds to minutes) (Lefkowitz *et al.*, 1990). Agonist-induced sequestration of β -ARs occurs over a similar time-frame to the biochemical uncoupling, but β -AR phosphorylation does not apparently act as a trigger for sequestration (Hausdorff *et al.*, 1989; Lohse *et al.*, 1990). In contrast to uncoupling and sequestration, receptor down-regulation seems to require exposure to agonist for at least one hour (Lefkowitz *et al.*, 1990).

The role of sequestration in desensitization and down-regulation has yet to be determined. Receptor sequestration can be completely prevented using concanavalin A or phenylarsine oxide without affecting the rapid desensitization process (Toews *et al.*, 1984; Hertel *et al.*, 1985), which would appear to indicate that sequestration does not precede desensitization. However, if receptor phosphorylation is prevented by mutagenesis of the receptor (Hausdorff *et al.*,

1989) or by specific kinase inhibitors (Lohse *et al.*, 1990), a small amount of rapid desensitization still occurs, which can be blocked by concanavalin A. This suggests that under certain experimental conditions at least, β -AR sequestration may provide an additional mechanism for desensitization. In the case of receptor down-regulation, β -AR internalization may be a necessary step, but is not in itself sufficient to account for agonist-induced down-regulation. This was demonstrated by Mahan *et al.* (1985) who investigated down-regulation in human S49 lymphoma cells. 15 min coincubation of S49 cells with $1\mu\text{M}$ isoprenaline caused a 60% loss in surface β -ARs (internalization) without any loss in total receptor number (down-regulation).

The use of S49 clonal variants has helped to elucidate the part that the components of the β -AR-G-protein-adenylyl cyclase complex play in the processes of receptor internalization, down-regulation and desensitization. UNC cells have a defective interaction between Gs and the β -AR; *cyc*⁻ cells lack the α -subunit of Gs; H21a cells have a defective interaction between Gs and adenylyl cyclase; and *kin*⁻ cells have no cAMP-dependent protein kinase activity. Receptor sequestration in all the above S49 variants was equivalent to that in the wild-type cells, indicating that the α -subunit of Gs, activation of adenylyl cyclase, cAMP itself, and cAMP-dependent protein phosphorylation are not required for β -AR internalization in S49 cells (Mahan *et al.*, 1985). Receptor down-regulation and desensitization in response to 6 hour coincubation with isoprenaline in *kin*⁻ cells were similar to that in wild-type cells (Shear *et al.*, 1976), however there was attenuation of down-regulation in *cyc*⁻ cells in response to 2.5 hour isoprenaline coincubation (Shear *et al.* 1976). Similar results were obtained in the study of Su *et al.* (1980) who observed that whilst there was no β -AR down-regulation in response to isoprenaline in *cyc*⁻ cells, in UNC cells down-regulation did occur though it was significantly less than in the wild-type cells and only occurred after 24 hour isoprenaline coincubation. Mahan *et al.* (1985) demonstrated that receptor down-regulation in response to isoprenaline coincubation of up to 12 hours was altered in all the S49 variants except for *kin*⁻ cells, with *cyc*⁻ and to a lesser extent UNC variants having a blunted isoprenaline-mediated loss in β -ARs, and H21a cells showing a greater rate of down-regulation than wild-type cells. These results indicate that cAMP-dependent protein phosphorylation is not

required for either receptor down-regulation or desensitization of the adenylyl cyclase response, whereas an intact Gs is required for both processes. Agonist-occupancy alone is not sufficient to cause receptor down-regulation in these cells.

Further evidence of the separation of the rapid processes of agonist-promoted phosphorylation, sequestration and desensitization from uncoupling of Gs and the more slowly evolving process of down-regulation, has been provided by Hausdorff *et al.* (1991). β -AR mutants were constructed in which a cluster of four serine and threonine residues located in the proximal end of the carboxyl tail was replaced by alanine and glycine residues. These residues are potential sites of phosphorylation by β ARK. These mutant receptors were transfected into Chinese hamster fibroblasts and ovary cells. All rapid regulatory processes of the receptor were abolished, whilst functional coupling to Gs and down-regulation were unaffected. Complementary to these results are the findings of the same group (Campbell *et al.*, 1991) that mutant β -ARs with impairments in coupling to Gs showed blunted agonist-induced receptor down-regulation but normal sequestration. These results and the variable expression in the development of down-regulation in S49 variants imply that receptor-Gs interaction regulates the fate of receptors linked to the stimulation of adenylyl cyclase.

As receptor phosphorylation can either be mediated by PKA (cAMP-dependent) or by β ARK (cAMP-independent), studies have been carried out to determine whether the two mechanisms occur simultaneously or depend upon the conditions of agonist exposure (Hausdorff *et al.*, 1989; Lohse *et al.*, 1989 & 1990). β -AR mutants were constructed that lacked the potential sites of PKA phosphorylation (mutant A), the proposed region of phosphorylation by β ARK (mutant B) or all these phosphorylation sites (mutant AB) (Hausdorff *et al.*, 1989). These mutant receptors were transfected into Chinese hamster fibroblasts and were shown both to bind ligands normally and to activate adenylyl cyclase in a manner identical to wild-type receptors. Desensitization was induced by a 5 min incubation with either a high (2 μ M) or low (10nM) concentration of isoprenaline. At the high concentration mutants A and B each showed approximately 50% reduction in agonist-induced phosphorylation and a marked reduction in desensitization compared to wild-type receptors. Mutant AB showed

an almost 90% reduction of phosphorylation and a more dramatic attenuation of desensitization. At the lower isoprenaline concentration phosphorylation and desensitization were only diminished in mutants A and AB, but not in mutant B (Hausdorff *et al.*, 1989). These findings suggest that PKA is the major mediator of desensitization and β -AR phosphorylation at low agonist concentrations whereas both PKA and β ARK play a role at higher agonist concentrations.

The above results were supported by experiments investigating the effects of specific PKA inhibitors and relatively selective β ARK inhibitors on desensitization in response to high and low concentrations of isoprenaline (Lohse *et al.*, 1989; 1990). A431 epidermoid carcinoma cells were used for these studies, the cells being first permeabilized with digitonin in order to introduce the inhibitors into the cells. Permeabilization itself did not alter either the adenylyl cyclase response to agonist or the desensitization process. At high isoprenaline concentrations inhibitors of either kinase attenuated isoprenaline-induced desensitization, but at low concentrations only the inhibitor of PKA was effective.

Lefkowitz *et al.* (1990) have speculated that PKA is active and can phosphorylate and desensitize β -ARs at relatively low levels of fractional receptor occupancy by agonists. In contrast, β ARK may act primarily under circumstances where much higher levels of fractional occupancy are achieved. This would suggest that PKA might mediate desensitization *in vivo* at nanomolar agonist concentrations, as is the case for circulating catecholamine concentrations or during drug administration, whereas β ARK might be expected to be involved in desensitization at noradrenergic synapses where nerve stimulation results in the rapid increase of catecholamine levels to 10^{-5} M or higher. PKA would also be active at these high concentrations. Confirmation of this hypothesis awaits information on the relative kinetics of the two kinases and their distribution, however, northern blot analysis of mRNA from tissues probed with β ARK cDNA showed the highest levels of expression in brain, spleen and heart, with very little, if any expression in liver or muscle (Benovic *et al.*, 1989). Those tissues with the greatest levels of noradrenergic innervation had the highest expression of β ARK mRNA. This finding is compatible with the hypothesis that β ARK is selectively expressed at synapses.

1.2 Bipolar Depression

1.2.1 Description

The affective disorders are those in which there is a disturbance of mood, accompanied by a full or partial manic or depressive syndrome, that is not due to any other physical or mental disorder (American Psychiatric Association, 1980). In this context mood refers to a prolonged emotion that colours the whole psychic life.

The term manic-depressive psychosis was coined by Kraepelin and included all periodic and cyclical psychoses, all manias and most depressions, together with affective personality disorders and some cases of delirious states (Kraepelin, 1922). He based this synthesis on similarities in symptoms, on the good prognosis for the individual episode of illness, and on family history. Later, manic-depressive psychoses were divided into bipolar and unipolar disorders. This was based on Leonhard's classification of endogenous psychoses (1959) and the terms "monopolar" and "bipolar" were first suggested by Leonhard in 1962. This concept was also developed independently by Angst (1966) in Switzerland and by Perris (1966) in Sweden. Perris introduced the term "unipolar" in the interests of etymological purity. In this context "bipolar" referred to patients who had attacks of both mania and depressive psychosis, whilst "unipolar" referred to those patients who had recurrent attacks of mania alone or depressive psychosis alone. However, following family studies in America (Winokur *et al.*, 1969), the term "bipolar" came to apply to patients who have had an attack of mania, whether or not they have ever been depressed, and the term "unipolar" was restricted to patients with episodes of primary depressive illness alone. In this case "bipolar" is synonymous with Winokur's term "manic-depressive disease", and "unipolar" with "depressive disease".

Most individuals who have a disorder characterized by one or more manic episodes will eventually have a major depressive episode. In the general population unipolar disorder (UP) is more common with a morbid risk of around 3% compared with 0.2-1.0 % for bipolar disorder (BP) (Reich *et al.*, 1982).

Mania is characterized by elevation of mood, pressure of talk, over-activity, and expansiveness or grandiosity. Insomnia is a cardinal symptom of mania, with decreased duration of sleep often being the first symptom noted by the patient of an impending manic episode. Most patients are also irritable, with frustration leading to anger, hostility and even aggression (Gibbons, 1982). Patients have delusions of special abilities, grandiose identity, wealth, or of being on a special mission (Leff *et al.*, 1976). Delusions of persecution also occur. Some patients also report having auditory or visual hallucinations. A combination of pressure of activity, expansiveness, grandiosity and disinhibition often leads to extravagant behaviour, of which overspending is typical. During a manic episode all the savings of patient and family may be spent and large debts accumulated in a matter of weeks (Gibbons, 1982). In the community, the manic patients extravagance can lead to ruin. Manic episodes typically begin suddenly, with a rapid escalation of symptoms over a few days.

Depression is among the most ubiquitous of psychiatric symptoms. It has been estimated, on the basis of a study in an inner-London suburb, that seven out of ten women and more than four out of ten men will have had at least one clinically significant depressive episode by the age of 65 (Bebbington *et al.*, 1989). According to the Diagnostic and Statistical Manual of Mental Disorders, Third Edition (DSM-III) (American Psychiatric Association, 1980), the essential feature is either a dysphoric mood, usually depression, or loss of interest or pleasure in all, or almost all, usual activities and pastimes. Other symptoms of the depressive syndrome include appetite disturbance, change in weight, sleep disturbance, psychomotor agitation or retardation, decreased energy, feelings of worthlessness and guilt, difficulty concentrating or thinking, and thoughts of death or suicide or suicide attempts. The onset of a major depressive episode is variable, with the symptoms usually developing over a period of days to weeks, though it may be sudden and precipitated by severe stress.

In BP disorder the initial episode is often manic. The first manic episode of BP disorder typically occurs before the age of 30. Major depression may begin at any age, with the age of onset being fairly evenly distributed throughout adult life. Both the manic and depressive episodes are more frequent and shorter than

the depressive episodes in UP disorder. Frequently a manic or depressed episode is immediately followed by a short episode of the other kind. Some individuals have episodes separated by many years of normal functioning, whilst others have clusters of episodes. Frequency of episodes may increase with age.

1.2.2 Genetics

BP disorder can clearly be seen to aggregate in families. Kraepelin (1922) noted that hereditary factors were evident in 80% of his patients, and most subsequent authors have also acknowledged the importance of genetic factors. It has been estimated that genes account for more than 80% of the variance in liability to BP disorder (McGuffin & Katz, 1986).

Problems are posed for genetic analysis of affective disorders by the all-or-none quality of psychiatric diagnosis, the low frequencies of disorder in relatives of ill probands, and the multiple forms of affective disorders found in families (Gershon *et al.*, 1976). In order to deal with these problems, several strategies have been used. Appropriate genetic models have been formulated and are applied to the family study data on the affective disorder. The majority of these models are "liability-threshold" models in which it is assumed that a variable termed "liability to develop the disorder" is continuously distributed within the population but that only those individuals whose liabilities exceed a certain threshold will manifest the disorder (McGuffin & Katz, 1986). The relatives of affected individuals will then on average have a higher liability than the general population mean and so more will lie beyond the threshold for exhibiting the disorder.

Multiple threshold-liability models are also possible in which a number of conditions are said to occupy the same continuum of liability with quantitative rather than qualitative differences such that those individuals whose liability exceeds a certain threshold, manifest the common, "broad" form of the illness, while those whose liability exceeds a second more extreme threshold exhibit a less

common, more severe, "narrow" form of the disorder. Further, more extreme thresholds can be added.

The two main models concerning the genetic component of liability are single major locus (SML) and polygenic-multifactorial (MF) models. Under the SML model it is postulated that the liability is due to a single gene locus at which there are two alleles (or alternative genes). Under the MF model it is assumed that the liability is contributed by the predominantly additive effects of genes at multiple loci which together with environmental effects will result in a normally distributed liability within the population. A third "mixed" model is also possible in which liability is contributed by a major locus against a background of multifactorial/polygenic influences.

Another strategy is to define a sub-group from the affective disorders that is homogenous and which could then result in pedigrees or segregation ratios that fit classical Mendelian models. The subgroup may be defined in terms of clinical or biological characteristics, or on the basis of a distinctive mode of transmission, such as linkage to a known chromosomal marker.

The division of the affective disorders into UP and BP has been supported by studies into the family histories of these disorders. The first significant investigation along these lines was undertaken by Leonhard *et al.* (1962) who studied 104 female patients with affective psychoses and their families. They found that the parents, siblings (sibs) and sibs of parents of BP patients all showed higher incidences of affective psychoses than the corresponding relatives of UP patients. The incidence of affective disorders in the relatives of both BP and UP patients was greater than that of the general population. Angst (1966) reported on a family investigation into 331 psychotic-depressives observed from 1959 to 1963 and found essentially similar results. Perris (1966) conducted a family investigation into 294 patients with affective disorders. He also subclassified the relatives of the probands into UP and BP. He found that UP and BP were genetically distinct and tended to run true in the families in that there were very few UP relatives of BP probands (0.5% risk) and very few BP relatives of UP probands (0.3% risk). Subsequent studies have consistently found that BP

patients have more BP relatives than do UP patients (Perris, 1968; Reich *et al.*, 1969; Winokur *et al.*, 1969; Trzebiatowska-Trzeciak, 1977), although there was also considerable prevalence of UP illness in families of BP patients.

Gershon *et al.* (1976) reviewed and analysed six papers which presented the polarity of the probands and the secondary cases. These data satisfied the preconditions of transmissibility for a two-threshold model of liability for major affective disorder in that the relatives of BP probands had more BP illness than the relatives of UP probands, and that the risk of major affective disorder was greater among the relatives of BP probands than UP probands. Although this would suggest that a common genetic diathesis may account for both BP and UP illness, the opposite hypothesis of separate inheritance of the two disorders is also possible, particularly in the light of Perris' results. Some association between the two disorders may be the consequence of assortative mating. The morbid risk for affective disorders in the spouses of affectively ill patients has been shown to be much greater than the population prevalence suggesting that a great deal of assortative mating occurs (Reich *et al.*, 1982). It is not known, however, whether such assortative mating, even for many generations, is sufficient to explain the association of UP and BP disorders.

A preponderance females over males appears to be present in both UP and BP disorders (Gershon *et al.*, 1976). However, in BP disorder this sex distribution does not extend to the relatives of affected probands. Angst's study (1966) showed an equal sex distribution for relatives of BP probands, but a much higher incidence (16.4%) of affective psychoses in female relatives of UP patients.

Twin research has been very useful in evaluating the genetic contribution to affective disorders and any genetic distinction between UP and BP. Studies of monozygotic (MZ) and dizygotic (DZ) twins have provided evidence that strongly suggests that genetic factors have a significant influence in the aetiology of affective disorders. Price (1968) surveyed case reports in the literature of 12 MZ twin pairs who were reared apart and found a concordance rate of 67% for affective illness. Gershon *et al.* (1976) analysed six studies of MZ-DZ concordance rates for severely ill patients with affective disorders (1930-1974).

The overall concordance rate for MZ twin pairs was 69% and for DZ pairs it was 13%. Assuming that intrapair differences in environment are the same for MZ and DZ twins, then the considerably higher concordance rate for affective illness in MZ twin pairs than in DZ twins indicates a heritable disorder. The concordance rate for MZ twin pairs is comparable to that found by Price for twins reared apart. Gershon suggested that the presence of discordant MZ twin pairs (30.8%) indicated that non-genetic factors may influence the vulnerability to affective disorders.

Bertelson *et al.* (1977) conducted a twin study using the Danish twin register, which included all the same-sex twins born in Denmark between 1870 and 1920. By comparing the twin and psychiatric registers, it was possible to ascertain at least 110 pairs of twins where at least one member of the pair had an affective disorder. The concordance rate for affective disorders in MZ twins was 67% and in DZ twins it was 20%. When these data were categorized as to the polarity of the affective disorder, concordance for BP disorder in MZ twins was 79% and in DZ twins was 19%. For UP disorder the concordance rates were 54% and 24% respectively. These results are in keeping with the previous findings, and also suggest that genetic factors may play a greater role in BP than in UP disorders. Two further studies are consistent in showing significantly higher concordance rates in MZ twin pairs than DZ twin pairs (Torgersen *et al.*, 1986; McGuffin & Katz, 1989).

The concordance of polarity (UP or BP) in MZ twins has also been investigated. Zerbin-Rüdin (1969) examined the clinical information published both in systematic twin series and in single case reports given in other systematic investigations, from 1941 to 1966. In 83 MZ pairs reviewed, 21 were concordant for BP illness, 22 for UP illness, and 7 pairs had one member UP and one BP. Similarly, in Bertelson's study (1977), out of 46 concordant MZ pairs, 21 were concordant for BP illness, 15 for UP illness and 10 pairs had one member UP and one member BP. Since the genotypes of MZ twins are identical, these data indicate that both genetic and non-genetic factors play a role in determining polarity, and are ambiguous as regards determining whether or not there is a genetical difference between UP and BP syndromes.

Adoption studies have also provided useful data as regards the genetic contribution to affective disorders. Mendlewicz & Rainer (1977) studied the families of adult BP probands who had been adopted early in life. The incidence of BP and UP affective disorders in the biological parents was much higher than in the adoptive parents. This study lends support to the view that BP and UP disorders can be transmitted by a common genetic mechanism. Cadoret (1978) conducted a similar study whose results also supported the hypothesis of a genetic factor, primarily in UP disorder.

Gershon *et al.* (1976) concluded from their critical analysis of the literature that autosomal SML inheritance could account for both the UP and BP forms of affective disorder in some but not all family studies, using the multiple threshold model of liability. However, MF inheritance of a shared diathesis could also account for the data. Reich *et al.* (1982) concluded from their review of family, twin and adoption studies, that the data supported the view that UP and BP affective disorders are at least two independently transmissible genetic illnesses, but that the psychopathological mechanism for producing a BP illness may sometimes produce a UP one.

As the data on polarity and inheritance satisfy the rules for multiple threshold models of inheritance, results have been examined for evidence of sex-related liability thresholds and for evidence of sex-linked dominant transmission. The hypothesis of sex-related liability thresholds is that an autosomally transmitted genetic predisposition is associated with a lower threshold for the disorder in the sex with higher prevalence (i.e. female). To manifest the disorder a male would have to have passed a higher threshold of liability and would therefore have a higher liability than an ill female. This would predict that the total number of secondary cases for the sex with higher threshold probands (i.e. males) would be significantly higher than that for the opposite sex. In other words, female secondary cases of male probands should outnumber female secondary cases of female probands, and male secondary cases of male probands should outnumber the male secondary cases of female probands. Gershon *et al.* (1976) analysed eight studies that gave details of the sex and polarity of both the probands and the secondary cases and found that the above conditions were not satisfied by either

the UP or BP family data, or by both groups taken together. Therefore, the hypothesis of sex-related liability thresholds was not supported by the literature.

The hypothesis of X-linked dominant transmission implies that a male proband will not in general have an ill father or son but may have an ill mother or daughter. From Gershon's study of the literature it was clear that in BP disorder there was an overall excess of ill mothers and daughters of male probands, which is compatible with X-linkage. However, as there was still an appreciable number of male probands with ill fathers and sons, the observed excess may have been due to the aforementioned preponderance of female affected patients over male. Goetzl *et al.* (1974) similarly found four occurrences of father to son transmission out of 39 probands. Gershon's group therefore compared the number of ill fathers and sons to the number of ill brothers of male BP probands, since the hypothesis of X-linkage implies that more brothers will be ill than fathers or sons and this comparison avoids any effects of the sex ratio. They found that there was no significant difference between the two groups so the X-linked dominant transmission of BP disorder was not supported by this study.

A central goal of genetic studies of affective disorders is to identify distinct entities that are genetically transmitted. Biological and pharmacological characteristics of these disorders are a means of identifying such entities. Studies have been carried^{out} to discover genetic markers that could be applied to each individual in a pedigree to predict who is at risk and who is not. An ideal situation would be the identification of a biological variant, such as an altered protein, that could be assigned a specific locus on a particular chromosome. According to Nurnberger & Gershon (1982) a characteristic can be investigated as a possible genetic marker of vulnerability to an illness if it meets a number of qualifications: the characteristic must be associated with an increased likelihood of the illness; it must be heritable; it must not be a secondary effect of the illness; and it must be observable or evocable in the well state, so that it is possible to determine its presence independently of the illness and to evaluate well relatives. To determine that a biological characteristic is a necessary or contributing genetic factor in an illness it must be demonstrated that the transmission of the illness and the transmission of the characteristic must be related within pedigrees, that is,

there must be non-independent assortment with the illness within pedigrees. However, since twin and family data strongly suggest that some persons with the genetic tendency to mood disorder will be phenotypically well, and since there may be biological heterogeneity in the BP disorder, then this non-dependent assortment may not necessarily be absolute.

The most straightforward form of genetic marker study is that of association, where the frequency of a marker in a particular patient group is compared to that of healthy controls. In 1979, Comings reported the identification of a mutant protein, Pc 1 Duarte, with an increased frequency in brain specimens taken at autopsy from individuals with affective disorder and/or alcoholism. This protein was most common in the caudate, putamen, thalamus, pons and brainstem. Controls showed a frequency of 32% carrying the abnormal protein, while depressed and alcoholic individuals showed a frequency of 62%. There were 11 individuals with BP disorder, in whom the frequency of the Duarte protein was 73%. No increased frequency of the Duarte protein was found in individuals with schizophrenia. However, there was an association with multiple sclerosis (53% frequency). Whilst euphoria and depression are not uncommon symptoms in multiple sclerosis and psychiatric disorders of clinical significance are more common in multiple sclerosis than in the general population, there is no evidence that patients with affective disorders are more likely to have multiple sclerosis (Nurnberger & Gershon, 1982). It may be that other vulnerability factors in addition to the Duarte protein are required for expression of these disorders.

Human leukocyte antigen (HLA) associations with BP disorder have been reported (Bennahaum *et al.*, 1975; Shapiro *et al.*, 1976 & 1977a; Stember & Fieve, 1977) but without statistical significance. Other reports have not supported the presence of any association (Beckman *et al.*, 1978; Smeraldi *et al.*, 1978; Targum *et al.*, 1979; Temple *et al.*, 1979). The possibility of genetic heterogeneity accounting for these differences was ruled out by Targum *et al.* (1979) who carried out a pedigree study of linkage of HLA antigens to BP disorder.

Associations with the ABO blood group system have also been investigated. There has been some agreement that blood group O is increased in BP patients

compared with controls (Masters, 1967; Mendlewicz *et al.*, 1974) and in BP patients compared with UP patients (Shapiro *et al.*, 1977b). However, in one study, group O was found to be associated with involuntional melancholia, a diagnostic subtype which seemed to contain only UP patients (Irvine & Miyashita, 1965), while in another study group A was increased in both BP patients and the total sample of patients with affective disorders (Flemenbaum & Larson, 1976) whereas in Shapiro's study (1977b) group A was decreased in BP patients compared to UP patients and to controls. These discrepancies suggest that no important association is present.

Linkage studies aim to determine whether a particular genetic marker assort with the illness within families. Linkage occurs when two genetic loci on the same chromosome are sufficiently close to one another for alleles at those loci not to be inherited independently. Recombination is the exchange of genetic material between pairs of homologous chromosomes during meiosis by rearrangement of alleles due to cross-overs. The distance between loci can be inferred from the number of recombinations as the closer the loci, the less likely is recombination to occur. Thus, if the recombination fraction, which is a measure of the amount of recombination, is close to zero, linkage is likely. If the two loci are far apart or on different chromosomes, the recombination fraction is 0.5. The criteria for linkage are that there be greater than random association of the two traits within pedigrees and that the odds favouring this maximum be statistically significant. Statistical significance is measured using the odds ratio which is the ratio between the probability of there being linkage at a given recombination fraction from 0 to 0.5 and that of there being no measurable linkage. This ratio is expressed as a logarithm and is known as the lod score. For example, a lod score of 3 represents odds of 1000:1 that the observations are the result of linkage at the specified recombination frequency rather than independent segregation.

Certain traits, such as protan colour blindness, deutan colour blindness, glucose-6-phosphate dehydrogenase (G6PD) deficiency and Xg blood group, are known X chromosome markers. Linkage of these markers to BP disorder has been reported by Angst (1966), Reich *et al.* (1969), Winokur & Tanna (1969), Mendlewicz *et al.* (1972, 1979 & 1980), Fieve *et al.* (1973), Mendlewicz & Fleiss

(1974), Baron (1977), Del Zompo *et al.* (1984) and Baron *et al.* (1987). However, as it is now known that the Xg and colour blindness loci are far apart on the X chromosome, it is unlikely that these markers can both be linked to BP disorder (McGuffin & Katz, 1986) and in fact, close linkage to the Xg locus was ruled out in two studies (Leckman *et al.*, 1979; Risch & Baron, 1982). When the data from those of the above studies written before 1974 were combined, linkage criteria were not satisfied (Gershon *et al.*, 1976), although in some of the reported families linkage did seem to be present. Gershon's group concluded that femaleness was a random rather than a familial component of liability to affective illness, although X-linkage may be present in an indeterminate proportion of cases in some populations studied.

Baron *et al.* (1987) investigated linkage of BP disorder to the markers for colour blindness and G6PD deficiency, excluding families with male-to-male transmission. They found good evidence for linkage to colour blindness in four pedigrees (lod score of 4.75 at a recombination fraction of 0.04) and for linkage to G6PD deficiency in one pedigree (lod score of 2.94 at a recombination fraction of 0.00). They concluded that the most probable location of the disease locus is close to and possibly between the two marker loci and they estimated that in general about one third of the BP population could carry this X-linked gene. The study of Mendlewicz *et al.* (1987) provided further support for the hypothesis of an X-linked gene. This group demonstrated linkage to coagulation factor IX situated at q27 on the X-chromosome in ten pedigrees out of twenty-four investigated (lod score 3.10; recombination fraction 0.11). There was no male-to-male transmission of BP disorder in these ten pedigrees. However, the findings of three other studies were not consistent with X-linkage (Gershon *et al.*, 1979; Kidd *et al.*, 1984; Berrettini *et al.*, 1990) even though the studies of both Gershon & Berrettini investigated pedigrees in which there was no male-to-male transmission. It would seem, therefore, that although BP disorder is linked to X-chromosome loci in some pedigrees, only a small proportion of the BP population carry this X-linked gene.

An investigation (Egeland *et al.*, 1987) into the genetically isolated Old Order Amish population identified a multigenerational pedigree of 81 members,

19 of which had major affective disorders. Of these 19 subjects, 14 had some form of BP affective illness. Linkage analysis provided evidence of linkage to two markers, the cellular oncogene *Ha-ras-1* (HRAS1) and insulin (INS) both localized close to the distal end of the short arm of chromosome 11. These results were potentially very interesting as the structural gene encoding tyrosine hydroxylase, one of the principal enzymes in the synthesis of catecholamines, has been mapped to the same segment of chromosome 11 as HRAS1 and INS (Craig *et al.*, 1985). However, two studies published in the same issue of *Nature*, found no evidence of linkage to these markers in either three Icelandic pedigrees or three North American pedigrees (Hodgkinson *et al.*, 1987; Detera-Wadleigh *et al.*, 1987). Furthermore, when Egeland's original study was extended and reanalysed to include 39 additional members of the pedigree and changes in diagnoses for two previously unaffected individuals, there was no longer evidence of linkage to either HRAS1 or INS (Kelsoe *et al.*, 1989).

In summary, the evidence for a genetic contribution to BP disorder is consistent and compelling, although no candidate gene responsible for or associated with BP disorder has yet been identified. It provides one of the firmest foundations for the conviction that there is a biological basis for depression.

1.3 Biochemical Investigations of Affective Disorders

It has long been accepted that biochemical changes are important in the aetiology of affective disorders (Coppin, 1967). A biochemical aetiology implies that there are certain biochemical changes in the brain that need to be restored to normal before the patient's clinical condition will improve. These biochemical differences could either be inherent or precipitated and maintained by psychological and environmental events. As described above, for BP disorder at least, it would seem that inherent or genetic factors have a major contribution, accounting for more than 80 % of the variance in liability to BP disorder (McGuffin & Katz, 1986).

The most cogent argument in favour of a biochemical basis to affective disorders is the success of physical methods of treatment of these illnesses, such as antidepressants and electroconvulsive therapy (ECT). Two major approaches have been used to investigate the biochemical changes that might occur in depression and mania. The first involves the examination of tissues obtained from patients with UP and BP disorder. Those normally taken are blood (plasma, platelets and lymphocytes), cerebrospinal fluid (CSF), and urine. These tissues can be examined either when patients are euthymic or when they are suffering an episode of mania or depression, and the biochemical effects of treatment can be assessed. *Post-mortem* brain tissue, usually from suicide victims, has also been used in some studies. The second approach is to study the ways that antidepressants, lithium and ECT alter the biochemical functioning of the brain in experimental animals.

1.3.1 Biochemical differences in patients with affective disorders

The first indication that monoamines may be involved in depression came in the 1950's when it was noticed that reserpine caused depression in a small proportion of patients prescribed the drug as an antihypertensive (Achor *et al.*, 1955; Muller *et al.*, 1955; Harris *et al.*, 1957). Furthermore, it was observed that the effects of reserpine when administered to rats (sedation, reduced locomotion,

ptosis and hypothermia) could be prevented by prior treatment with iproniazid, imipramine and desipramine which were then novel antidepressants (Chessin *et al.*, 1957; Sulser *et al.*, 1964). Reserpine prevents the intraneuronal storage of monoamines (NA, 5-hydroxytryptamine (5-HT) and dopamine (DA)) allowing them to be metabolized by monoamine oxidase (MAO). This results in a gradual depletion of monoamines from the brain. Iproniazid, an MAO inhibitor, causes the leakage of monoamines from neurones resulting in excitation. Imipramine, a tricyclic antidepressant, inhibits the neuronal uptake of NA and 5-HT, thereby potentiating the effects of any NA or 5-HT released (Hertting *et al.*, 1961; Axelrod & Inscoc, 1963). These observations led to the formulation of the monoamine theories of affective disorder which state that depression in man is associated with a decrease in noradrenergic and/or serotonergic function and that, conversely, mania may be associated with an excess of these amines (Schildkraut, 1965; Coppen, 1967; Lapin & Oxenkrug, 1969).

As a consequence of the proposal of the monoamine hypothesis, researchers have attempted to identify deficits in the activity of monoamine transmitters in depressed patients by the measurement of the concentration of these transmitters and their metabolites in plasma, CSF or urine, and in the brain.

NA and DA are synthesised from tyrosine with DA being a precursor of NA. NA is metabolized by MAO and/or catechol-O-methyltransferase (COMT) to 3-methoxy,4-hydroxy-phenyl-ethylglycol (MHPG), 3,4-dihydroxyphenylethylglycol (DHPG), normetanephrine, and vanillylmandelic acid (VMA). MHPG is thought to be the major product from within the CNS, though recent animal studies have suggested that perhaps DHPG or normetanephrine may be more reliable indicators of central NA activity. VMA is thought to be major product in the periphery. DA is metabolized by MAO to 3,4-dihydroxyphenyl-acetic acid (DOPAC) and by MAO and COMT to homovanillic acid (HVA).

Measurement of CSF catecholamines and metabolites have produced conflicting results, with some groups reporting decreases in MHPG (Gordon & Oliver, 1971; Post *et al.*, 1973; Jimerson *et al.*, 1975), VMA (Jimerson *et al.*, 1975) and HVA (Bowers *et al.*, 1969; Papeschi & McClure, 1971; van Praag & Korf,

1971; Mendels *et al.*, 1972; Wilk *et al.*, 1972; Ashcroft *et al.*, 1973). The decrease failed to reach significance in some of the HVA studies. Other groups found no changes in MHPG in depressed patients (Wilk *et al.*, 1972; Shaw *et al.*, 1973; Shopsin *et al.*, 1975) although Wilk's study did show significantly higher levels of MHPG in patients with mania which were reduced after lithium therapy. Mendels *et al.* (1972) reported that HVA increased on recovery in three out of four patients, whereas Ashcroft *et al.* (1973) found no increase in HVA on mood improvement. The decrease in CSF HVA observed by Ashcroft's group was only seen in UP patients, with BP patients, both manic and depressed, having HVA values similar to controls.

There is considerable debate as to the origin of NA metabolites within lumbar CSF as there are active NA neurones within the spinal cord. The concentration of NA metabolites in lumbar CSF is probably a more accurate reflection of noradrenergic activity in the spinal cord than in the brain. This means that decreases in NA metabolites are likely to be a result of the reduced motor activity often associated with depression. With regard to HVA, there seems to be fair agreement that the concentration of this metabolite in lumbar CSF reflects brain DA metabolism as there are few DA neurons in the spinal cord (Green & Costain, 1979). Brain *post-mortem* levels of both NA and DA are generally agreed to be unchanged in depressed patients (Beskow *et al.*, 1977; Elliott & Stephenson, 1989).

Although it is difficult to assess what proportion of urinary MHPG arises from central NA metabolism (Maas *et al.*, 1972a), measurement of urinary MHPG has produced some reports of decreased levels in depressed patients and in patients with a brief episode of depression due to amphetamine-withdrawal (Maas *et al.*, 1968; Schildkraut *et al.*, 1971a & 1972; Watson *et al.*, 1972). In other studies either not all the patients excreted less MHPG (Maas *et al.*, 1972b) or no decrease in urinary MHPG was observed (Bunney *et al.*, 1972; Shopsin *et al.*, 1973). In Shopsin's study, four out of eleven patients with mania had elevated MHPG levels which fell within the control range when the patients recovered or were depressed. When patients were subdivided into UP and BP type (Schildkraut, 1978), it became apparent that low levels of MHPG were observed

in those patients with BP disorder during episodes of depression and were increased during episodes of mania. These changes in NA activity could be related to the level of activity associated with a particular mood.

Another approach in investigating noradrenergic deficits is to test peripheral noradrenergic function in patients with depressive disorders. The rationale behind this type of study is that since similarities exist between central and peripheral neural noradrenergic mechanisms, the more readily accessible peripheral nervous system may provide insights into the CNS (Esler *et al.*, 1982). Also some patients with depressive illness have clinical symptoms suggesting autonomic nervous system dysfunction (Matussek *et al.*, 1981). Results from such studies do not support the hypothesis of catecholamine deficits in depression, and in fact several have provided evidence of increased noradrenergic functioning. Plasma NA levels have been shown to be elevated in some depressed patients (Wyatt *et al.*, 1971; Esler *et al.*, 1982; Lake *et al.*, 1982; Roy *et al.*, 1985). In Lake's study, high plasma NA levels were seen in both UP and BP patients and were associated with tachycardia, but not hypertension, in both groups. Roy *et al.* (1985) found elevated plasma NA levels only in UP patients who had either a current diagnosis or a past history of melancholia. BP patients had tendency towards lower plasma NA levels than controls. There was evidence of peripheral noradrenergic dysregulation or instability in BP patients as there was a greater percentage change in plasma NA levels from lying down to standing up in these patients. Esler's group (1982) studied plasma NA kinetics, measuring the rates of NA release to and removal from the plasma. The NA spillover rate, that is the rate at which endogenous NA, released from sympathetic neurons, spills over into plasma, was found to be elevated in five out of eleven patients with depressive illness compared to controls. The half-time of the rapid component of NA disappearance from plasma was shortened in depressed patients. This half-time is determined mainly by neuronal uptake of NA. The half-time of the slow component of removal, which may represent extraneuronal uptake and metabolism, was lengthened. The overall steady state clearance of NA from plasma was normal in depressed patients. These results suggest that uptake of NA by sympathetic neurons is increased in depressed patients.

Although there is therefore some evidence for a decrease in noradrenergic transmission in depression from studies of NA metabolites in CSF and urine, this evidence is far from consistent or conclusive. In addition, there is little indication that noradrenergic transmission is increased in mania, and in fact some studies suggest that any change in noradrenergic function is in the same direction in both mania and depression. Furthermore, the results of several studies of peripheral noradrenergic function suggest that this is increased in depression.

5-HT is metabolized by MAO to 5-hydroxyindole acetic acid (5-HIAA). Measures of plasma and urine 5-HT and 5-HIAA predominantly reflect peripheral metabolism and are therefore inappropriate as indicators of central 5-HT. Although some studies of CSF samples suggested that the concentrations of 5-HT and 5-HIAA were lower in depressed patients than in euthymic or control subjects (Ashcroft *et al.*, 1966; Dencker *et al.*, 1966; Coppen *et al.*, 1972) other investigations have been unable to confirm this as a consistent finding (Bowers *et al.*, 1969; van Praag *et al.*, 1970; Wilk *et al.*, 1972). Ashcroft *et al.* (1973) examined 5-HIAA concentrations in the CSF of UP and BP patients, both depressed and manic. They found that only the UP patients had lower 5-HIAA concentrations than controls. In contrast, Coppen *et al.* (1972) found diminished 5-HIAA levels in both depressed and manic patients.

One interpretation of these inconsistencies was suggested by Asberg *et al.* (1976) who observed that CSF 5-HIAA concentrations in depressed patients followed a bimodal distribution, one group having normal values, the other low concentrations and this latter group had a significant association with suicidal tendencies, particularly with suicide by violent means. This finding has since been replicated (Asberg *et al.*, 1986; Roy *et al.*, 1989) although the effect has also been associated with more generalized impulsive aggressive behaviour (Brown *et al.*, 1979). However, as in the measurement of NA metabolites in CSF, measurement of 5-HT and its metabolites in CSF does not necessarily measure central 5-HT activity as there are 5-HT neurons in the spinal cord. There is also evidence of wide diurnal variation in the content of 5-HIAA in lumbar CSF. Again, reduced concentrations of indoleamine in lumbar CSF from depressed patients may simply reflect reduced motor activity.

Post-mortem brain studies have also produced conflicting results. Shaw *et al.* (1967) found significantly lower 5-HT concentrations in the hind brain tissue of depressed suicides than in controls and Pare *et al.* (1969) found lowered 5-HT but normal 5-HIAA, although the significance of Pare's findings were offset by a positive correlation between 5-HT concentration and age. Lloyd *et al.* (1974) also noted a significant decrease in the lower brainstem 5-HT content in suicides, particularly in the raphe nucleus dorsalis where the serotonergic cell bodies are located. Brainstem 5-HIAA concentrations were normal in this study. In contrast, Bourne *et al.* (1968) found normal 5-HT but low 5-HIAA concentrations in depressive suicides. A subsequent study did not find any deficits in either 5-HT or 5-HIAA (Beskow *et al.*, 1976).

Post-mortem studies can be difficult to interpret (Cooper *et al.*, 1985) as often little is known of the psychiatric history or previous drug treatment of the patient before death, particularly in suicide victims. Indeed, suicide victims may not necessarily have been depressed. A study of psychiatric diagnoses of suicides documented a variety of diagnoses, such as schizophrenia, drug abuse and alcoholism (Rich *et al.*, 1986). Careful matching of the test group to the control group is important, particularly in terms of the agonal state at the time of death. Minimal *post-mortem* delay is also essential to ensure that changes in neurotransmitter levels do not occur.

It would seem therefore that as in the case of noradrenergic functioning, the evidence for decreased 5-HT functioning in depression is unconvincing as much of the data are contradictory, particularly from the *post-mortem* studies.

In addition to measurement of monoamines and their metabolites, studies of the metabolic enzymes themselves have also been carried out. The approach in these studies was to investigate peripheral enzyme activity as routine assay of brain enzymes was not possible, and the assumption was that peripheral enzyme activity reflects the activity of the centrally located enzyme. No significant differences between depressed patients and controls have been observed in the activity of COMT located in erythrocytes or CSF, or plasma dopamine beta-hydroxylase. Some studies suggested that platelet MAO may have increased

activity in depressed patients (Sandler *et al.*, 1981), but further study refuted this finding and many studies have found that platelet MAO activity is decreased in BP patients compared to UP patients or controls (Sandler *et al.*, 1981; Fowler *et al.*, 1982; Wirz-Justice, 1988). It has also been suggested that platelet MAO activity is a predictor of response to lithium, with BP patients with normal MAO activity being responsive to lithium and those with low MAO activity being lithium-refractory (Sullivan *et al.*, 1977). MAO activity was found to have a close familial association which was independent of any affective disorder (Wirz-Justice, 1988). Differences in platelet MAO activity may also be related to particular syndromes such as suicidal behaviour (low activity) and anxiety (high activity) and to variables such as age, sex and stage of menstrual cycle (Wirz-Justice, 1988).

To summarize the above studies, clinical findings so far lend little support to the hypothesis of deficits in neuronal monoamines as the primary biochemical basis of depression. There are other contradictions of this hypothesis, such as the fact that the antidepressants iprindole and mianserin do not inhibit monoamine uptake into neurones, whilst cocaine and amphetamine which are monoamine uptake inhibitors have no antidepressant activity. Furthermore, tricyclic antidepressants cause blockade of monoamine uptake within hours of a single dose, whereas the onset of the therapeutic response can take two to three weeks (Oswald *et al.*, 1972). With this latter point in mind, recent research has been concerned with the long-term changes in monoamine systems due to daily administration of antidepressants or ECT, with the hope that the elucidation of the mechanism(s) of action of antidepressant treatment will lead to the discovery of the biochemical basis (bases) of depression.

1.3.2 Biochemical effects of chronic administration of antidepressants to animals

Chronic administration of tricyclic antidepressants to rats results in a decrease in the level of NA in the brain (Schildkraut *et al.*, 1971b; Roffler-Tarlov *et al.*, 1973) which does not occur after a single dose (Sulser *et al.*, 1964). Segal *et al.* (1974) reported adaptive changes in the activity of tyrosine hydroxylase that were inversely correlated with changes in the availability of catecholamines. Thus,

administration of the tricyclic antidepressant desipramine (DMI) for eight days produced a significant decrease in the activity of the enzyme in the locus coeruleus and hippocampus-cortex area, while reserpine caused a marked increase in enzyme activity. This is a possible explanation of the decrease in the level of NA seen by Schildkraut and Roffler-Tarlov's groups, although a decrease in brain DA levels would also have been expected and this did not occur.

Prolonged administration of MAO inhibitors to rats resulted in a 35-55% reduction in NA-stimulated cAMP accumulation in the limbic forebrain (Vetulani *et al.*, 1975). Vetulani and Sulser (1975) went on to investigate the effect of 4-6 week administration of DMI, iprindole or 8 days of ECT. They found that NA-stimulated cAMP accumulation in the rat limbic forebrain was reduced by 70% with DMI and iprindole and by 30-50% by ECT. These studies suggested that antidepressant treatments cause a decrease in noradrenergic receptor function which is central to their mechanism of antidepressant action and that this is a common mode of action at postsynaptic noradrenergic receptor sites regardless of their action at presynaptic sites (Sulser *et al.*, 1978). Furthermore, this suggests that supersensitive β -ARs may be an aetiological factor in some forms of depressive illness (Sulser *et al.*, 1978). A decrease in NA-stimulated cAMP accumulation of about 40% was observed in the rat cerebral cortex by Schultz (1976) after six days administration of imipramine and by Frazer *et al.* (1978) after seven or ten days administration of DMI lending further support for the above hypothesis. In both studies, no reduction was seen after one or three days administration of the antidepressant.

Banerjee and Kung (1977) demonstrated that six week administration of DMI, iprindole or doxepin to rats resulted in a 66%, 42% and 31% decrease respectively in β -AR binding in a microsomal brain suspension. They suggested that the decrease in cAMP accumulation found by Vetulani's group was due to a decrease in the number of β -ARs. β -AR down-regulation after chronic DMI possibly resulted from desensitization following an increase in the level of catecholamines at postsynaptic sites as a result of uptake inhibition. β -AR occupancy by agonists results in desensitization due to phosphorylation of the receptor by PKA and/or β ARK and its uncoupling from Gs. If receptor

occupancy is prolonged, phosphorylation and uncoupling will lead to β -AR down-regulation (see pages 30-34). However, this does not explain the β -AR loss observed with iprindole, which has a minimal effect at presynaptic sites. β -AR down-regulation was also observed by Sarai (1978) in homogenates of rat cerebral cortex after repeated injections of DMI; by Dibner and Molinoff (1979) in rat cortical tissue slices after 10 days DMI administration; and by Pandey *et al.* (1979) in rat cerebral cortical homogenates after twelve days ECT. Wolfe *et al.* (1978) conducted a simultaneous investigation of the effect of five to twenty-one days DMI treatment on both β -AR-coupled cAMP accumulation and β -AR density in rat cerebral cortex. A 35-45 % decrease in cAMP accumulation was observed, which was accompanied by a 35-40 % decrease in β -AR number. Decreases in isoprenaline-stimulated cAMP accumulation and β -AR density were temporally related, with maximal increases being observed by five to seven days of treatment. They found similar results with pargyline, an MAO inhibitor, and iprindole was shown to decrease β -AR binding after eleven days administration.

The weight of evidence of β -AR down-regulation and desensitization in response to chronic antidepressant treatment in the rat supports Sulser's hypothesis that this is the mechanism of antidepressant action and that β -ARs are supersensitive in depression, but does not prove it, partly because these studies were all carried out in "normal" rats. This hypothesis would seem to be in contradiction of the catecholamine hypothesis that noradrenergic functioning is decreased in depression.

Studies been carried out to elucidate the mechanism of antidepressant-induced β -AR down-regulation and desensitization. Frazer *et al.* (1978) noted that there was no reduction of the response to catecholamines after chronic DMI administration in the rat diaphragm which is devoid of adrenergic innervation. Wolfe *et al.* (1978) and Schweitzer *et al.* (1979) investigated the role of adrenergic innervation in the development of β -AR subsensitivity in response to antidepressants using 6-hydroxydopamine (6-OHDA) administered to newborn rats to destroy adrenergic terminals. Chronic treatment with DMI had no effect on isoprenaline-stimulated cAMP accumulation and β -AR density in these rats lending support to the concept that blockade of the neuronal uptake system with

a concomitant increase in the effective amount of NA in the synapse causes the decrease in β -AR subsensitivity. However, *in vitro* coincubation with DMI of rat glioma C6 cells, a system lacking presynaptic neurons, has been shown to result in β -AR down-regulation, which suggests that DMI has a direct action on the β -AR-adenylyl cyclase complex (Fishman & Finberg, 1987; Manji *et al.*, 1991). Furthermore, in the study of Wolfe *et al.*, 6-OHDA treatment also abolished the down-regulation previously observed with iprindole, which is neither an uptake blocker nor an MAO inhibitor. These results of Wolfe *et al.* are open to criticism as 6-OHDA treatment alone caused marked β -AR sensitization.

Maggi *et al.* (1980) extended the above findings to include examination of the effect of chronic treatment with various antidepressants on 5-HT₁, cholinergic muscarinic and α -adrenergic receptor binding in the rat brain. Chronic administration of imipramine led to a decrease in β -AR binding in the hippocampus and frontal cortex and a decrease in [³H]-5-HT binding in the corpus striatum, hippocampus and frontal cortex. DMI treatment caused significant β -AR loss in the frontal cortex and cerebellum and also significant 5-HT₁ receptor loss in the frontal cortex. Nisoxetine and fluoxetine, which are more specific inhibitors of NA and 5-HT uptake respectively than the tricyclic antidepressants, had no effect on β -AR binding in any of the brain areas studied, either alone or in combination. Nisoxetine had been previously shown not to cause β -AR down-regulation in rat cortex, even when higher multiple daily doses, that induced subsensitivity of the NA-stimulated adenylyl cyclase, were used (Mishra *et al.*, 1979). Nisoxetine and fluoxetine also had no effect on [³H]-5-HT binding in any brain area when administered separately. However, when administered in combination, these drugs caused a significant reduction in [³H]-5-HT binding sites in the frontal cortex, but not in the hippocampus, suggesting that 5-HT₁ receptor losses may be related to a combined inhibition of both 5-HT and NA uptake. Cholinergic muscarinic receptor binding was not significantly altered by any of the treatments.

The potencies of a wide range of antidepressants on α -adrenergic binding was also assessed in this study. Blockade of presynaptic α_2 -autoreceptors would be expected to increase the release of NA into the synapse by inhibition of the

negative feedback effects of NA, which would presumably lead to β -AR down-regulation in a manner comparable to the blockade of NA uptake. All the compounds studied displayed some activity in inhibiting [^{3} H]-clonidine binding to both high and low affinity α_2 -ARs. However, with the exception of mianserin, all of the antidepressants examined had weak affinity for both α_2 -AR subtypes, with K_i values in the 1-10 μ M range, comparable to that of prazosin, which is pharmacologically inactive at the α_2 -AR. It therefore would seem unlikely that antidepressants in general cause down-regulation via selective blockade of α_2 -ARs. The classical antidepressants, such as imipramine, DMI and amitriptyline, were all shown to have high affinity for the α_1 -AR and to be 20-100 times more potent at the α_1 -AR than the α_2 -AR (U'Prichard *et al.*, 1978; Maggi *et al.*, 1980). Blockade of α_1 -ARs has been suggested as the mechanism of the sedative effects associated with these drugs, which are particularly effective in patients with psychomotor agitation (U'Prichard *et al.*, 1978). Nisoxetine, fluoxetine and iprindole were at least 10-fold weaker inhibitors of α_1 -AR binding than all the other antidepressants examined in the aforementioned study. Mianserin, a tetracyclic antidepressant which does not cause β -AR down-regulation (Mishra *et al.*, 1980; Sellinger-Barnette, 1980), was shown to have high affinity for both the α_1 - and α_2 -ARs.

Estimations of the effects of chronic antidepressant administration on α_2 -AR populations have produced conflicting results, probably due in the main to methodological differences, particularly in terms of whether agonist or antagonist radioligand binding was measured (Elliott & Stephenson, 1989). Sugrue (1983) found that pargyline reduced [^{3} H]-clonidine binding in the rat, whereas several other antidepressants, including DMI, imipramine, amitriptyline, mianserin and ECT, were without effect. In contrast, decreased [^{3} H]-clonidine binding was reported in the rat cortex after imipramine (Campbell & McKernan, 1982), in the rat limbic system after amitriptyline (Smith *et al.*, 1981) and in several brain regions following ECT (Stanford & Nutt, 1982).

A variety of antidepressant drugs, including MAO inhibitors and iprindole, have been shown to reduce the density of 5-HT₂ receptors with prolonged administration (Peroutka & Snyder, 1980; Kellar *et al.*, 1981). This effect seems

to be specific to antidepressants as it is not seen with structurally related compounds that are devoid of antidepressant activity (Peroutka & Snyder, 1980). However, amphetamine also has this effect (Nielsen *et al.*, 1980) and 10 days ECT was shown to produce an increase in 5-HT₂ receptor binding (Kellar *et al.*, 1981), so the relevance of 5-HT₂ receptor density decrements to antidepressant activity is questionable. It has also been questioned whether [³H]-spiroperidol, the ligand used in all the above studies, binds to a receptor specific to 5-HT (Middlemiss *et al.*, 1980).

Iprindole, which does not seem to increase the availability of NA, nevertheless reduces both the sensitivity to NA and the density of β -ARs (Vetulani and Sulser, 1975; Banerjee and Kung, 1977; Wolfe *et al.*, 1978). Furthermore, zimeldine, which is a selective 5-HT uptake inhibitor, produces a diminished cAMP response to NA in brain slices (Mishra *et al.*, 1980). Conversely, cocaine, an uptake blocker, increases the amount of NA in the synapse, but does not change the density of β -ARs (Sethy & Harris, 1981). In order to try to account for these paradoxical findings, and since the terminal fields of 5-HT projections in the cortex overlap those of NA projections, the role of 5-HT neurons in the regulation of β -ARs and the β -AR-coupled adenylyl cyclase system has been investigated.

Janowsky *et al.* (1982) examined the consequences of selective lesioning of serotonergic neurones on the effects of DMI in the rat cortex. They found that chronic DMI administration failed to decrease the density of β -ARs in the absence of serotonergic input, while still reducing the sensitivity of the cAMP-generating system to NA and isoprenaline, leading to the suggestion that 5-HT is required for β -AR down-regulation in response to antidepressants. However, such lesions of 5-HT axons ^{have} themselves been shown to cause an increase the density of β -ARs in the cerebral cortex and hippocampus (Janowsky *et al.*, 1982; Stockmeier *et al.*, 1985), with or without a concomitant increase in isoprenaline-stimulated cAMP production (Stockmeier *et al.*, 1985; Gillespie *et al.*, 1988).

5-HT *in vitro* reversed the increased density of β -ARs induced by serotonergic lesions (Gillespie *et al.*, 1988). Gillespie's study also showed that it

was the density of β -ARs in the low affinity conformation that was increased by 5-HT lesions and subsequently returned to control values by *in vitro* 5-HT. This would explain the lack of change in β -AR-coupled cAMP production seen by this group, inasmuch as the ability of an agonist to activate adenylyl cyclase correlates closely with the amount of the high affinity state of the receptor formed in the presence of the agonist (Kent *et al.*, 1980). As chronic DMI or ECT have been shown to decrease the number of [^{3} H]-DHA binding sites that have high affinity for isoprenaline, and this decrease is unaffected by serotonergic lesions (Manier *et al.*, 1987; Stockmeier & Kellar, 1988), then it is likely that the serotonin lesion-induced increase in [^{3} H]-DHA binding sites with low affinity for isoprenaline offset the DMI- and ECT-induced decrease in the high affinity sites. This would suggest that 5-HT axons do not play a critical role in the down-regulation of β -ARs by antidepressant treatments. However, elevated 5-HT levels have been shown to accelerate the rate of β -AR down-regulation in rat cerebral cortex in response to DMI (Baron *et al.*, 1988). Synaptic 5-HT levels were increased by administration of fluoxetine, which in itself did not alter β -AR number. When fluoxetine was administered in combination with DMI, a significant decrease in β -AR density was seen after only four days, at which time DMI treatment alone had no effect, and after fourteen days the extent of down-regulation was greater than was seen with DMI alone. This synergistic effect of fluoxetine and DMI was dependent on serotonergic transmission as it was abolished by 5-HT depletion with p-chlorophenylalanine. So it would seem that whilst 5-HT has a limited role in the mechanism^{or} antidepressant-induced β -AR down-regulation, it can act synergistically with DMI to accelerate the rate and increase the extent of down-regulation.

1.3.3 Biochemical effects of administration of antidepressants to depressed patients

All the above studies on the effects of antidepressant treatments on central adrenergic and serotonergic receptors have been carried out in the rat. The relevance of such studies to the effects of antidepressants in the human brain, particularly as regards patients with affective disorders, must always be questioned.

Certainly, the extrapolation of such results to hypotheses of the aetiology of affective disorders involves many assumptions, not least of which are that antidepressant treatments have the same biochemical effects in rats as they do in humans and that these effects are related to their antidepressant action. Species differences are to be expected, and an example of such a difference is that chronic administration of DMI, phenelzine and ECT did not cause β -AR down-regulation in the cerebral cortex of guinea pigs (Hu *et al.*, 1980). Confirmation of the proposed hypersensitivity of β -ARs in depressed patients is limited by lack of endocrine or behavioural models of central β -AR function.

There are several ways to investigate central receptor density and/or function and the effects of antidepressants in patients with affective disorders. One method, which will be discussed in the next section, is to use peripheral models of CNS receptor systems, such as the platelet α -AR and the lymphocyte β -AR. Other ways include examining receptors in *post-mortem* brains of suicides, and measuring the secretion of melatonin as a marker of β -AR function.

Post-mortem receptor-binding measures in brain tissue are less susceptible than monoamine levels to the effects of both *post-mortem* delay and acute fluctuations in neuronal activity and provide a direct approach to the study of CNS receptor differences in depression. Mann *et al.* (1986) investigated 5-HT₁ and 5-HT₂ receptor binding in the frontal cortex of suicide victims dying mainly of violent, non-pharmacological means compared to control subjects matched for age, gender, *post-mortem* delay and the degree of violence associated with death. They found a significantly higher density of 5-HT₂ receptors (28%) in brains of suicide victims but no difference in 5-HT₁ receptor binding. These results, however, are concerned with suicide by violent means, which as previously mentioned is specifically associated with low 5-HIAA concentrations in the CSF. No assessment was made as to whether the suicide victims were depressed and whether either suicides or controls had been drug-treated, which is particularly relevant to the suicides who might have been taking antidepressants. 5-HT₂ and 5-HT₁ binding sites were examined in the brains of suicides for whom a firm diagnosis of depression in the absence of symptoms of other psychiatric and neurological disorders could be made (Cheetham *et al.*, 1988; 1989). Both drug-

free and antidepressant-treated subjects were studied. No significant differences in the number or affinity of 5-HT binding sites were found in the frontal or temporal cortex between drug-free or antidepressant-treated suicides and controls. However, there was a significantly lower number of 5-HT₂ (Cheetham *et al.*, 1988) and 5-HT₁ (Cheetham *et al.*, 1989) binding sites in the hippocampus, but not the amygdala, of drug-free suicides. No differences in 5-HT₁ sites were found when antidepressant-treated suicides were compared to matched controls, but when compared to drug-free suicides the number of 5-HT₁ binding sites was significantly higher (40%) and the binding affinity was lower (31%). The change in binding affinity was probably due to residual drug effects. These results suggest that 5-HT₁ and 5-HT₂ receptors in the hippocampus are lower in subjects with depression than in controls, but are increased to control values by antidepressant treatment.

The above group have examined α_2 - and β -AR binding sites in drug-free and antidepressant-treated depressed suicides (De Paermentier *et al.*, 1990; 1991; Horton, 1991). The number of α_2 -ARs was higher in drug-free suicides than controls, and significantly so in the temporal cortex (De Paermentier, 1991) and the frontal cortex (Horton, 1991) of suicides who died by violent means. These results confirmed those of Meana & Garcia-Sevilla (1987), who found that frontal cortical α_2 -AR density was significantly higher in five depressed, violent suicides than in controls. The lowest α_2 -AR densities among the suicides were found in the two amitriptyline-treated suicides. Conversely, there was a small, but significant, decrease in α_2 -ARs in the hippocampus of non-violent drug-free suicides (Horton, 1991).

The number of β -ARs in cortical areas were generally lower for depressed drug-free suicides than controls (De Paermentier, 1990). The differences were significant in the temporal cortex and the occipital cortex (Horton, 1991). There was no difference in β -AR binding in the hippocampus, amygdala and thalamus. These results are similar to those of Stockmeier & Meltzer (1991) which did not show any significant difference in β -AR binding in the frontal cortex in suicides compared to matched controls. However, they are in contrast to two previous studies that showed that β -ARs were markedly higher in the frontal cortex of

suicides than matched controls (Mann *et al.*, 1986; Biegon and Israeli, 1988). As none of these three studies presented any psychiatric assessment, it is impossible to know for sure whether or not their suicides were depressed. Subjects receiving antidepressant drugs were excluded in Biegon & Israeli's study, but not from that of Mann's or Stockmeier and Meltzer's groups. Both Mann *et al.* and Biegon and Israeli examined suicides dying mainly from violent means. In De Paermentier's study, depressed suicides who died by violent means had significantly lower numbers of β -ARs in the frontal cortex than controls, which is the opposite of the results of Mann *et al.* and Biegon & Israeli, whereas Stockmeier and Meltzer found no difference in frontal cortex β -AR binding between violent suicides and controls. Non-violent suicides had significantly lower β -ARs in the occipital cortex than controls (De Paermentier *et al.*, 1990). Antidepressant-treated suicides had decreased β -AR binding in the temporal cortex and the thalamus compared to controls, though overall there was no clear indication that antidepressants had caused β -AR down-regulation in the brain of depressed suicides. These studies suggest that β -AR density in some brain areas is reduced in depression, which supports the catecholamine hypothesis of decreased noradrenergic function in depression, and contradicts Sulser's hypothesis of β -AR supersensitivity.

The secretion of melatonin is controlled by noradrenergic innervation which terminates on post-synaptic β_1 -ARs in the pineal: the blockade of these with propranolol or atenolol inhibits the nocturnal secretion of melatonin in man (Hanssen *et al.*, 1977; Cowen *et al.*, 1983). If β -AR supersensitivity is present in depression and normalized by antidepressants, then it would be expected that melatonin secretion would be increased in depressed patients and decreased by antidepressant treatments. In fact, several groups have reported that nocturnal melatonin secretion is reduced in depression (Wetterberg *et al.*, 1979; Branchey *et al.*, 1982), although these studies did not control the effects of body weight, season of year and psychotropic drugs on melatonin secretion. A carefully controlled study compared 24 hour melatonin secretion in UP patients to that in matched control subjects and in UP or BP patients when depressed to that when euthymic (Thompson *et al.*, 1988). In contrast to the previous studies, no significant difference was found between depressed patients and controls, and there was in fact a tendency for the nocturnal melatonin values of the depressed

patients to be higher than controls. When the depressed condition was compared against euthymia, there was shown to be a significantly greater secretion of melatonin during depression. Paradoxically, DMI treatment has been reported by the same group to increase the secretion of melatonin in depressed patients but not in controls (Thompson *et al.*, 1983 & 1985). These results perhaps suggest that melatonin secretion is not a reliable measure of β -adrenergic function. No evidence of functionally significant pineal β -AR down-regulation by antidepressants has yet been found in humans.

1.4 Peripheral Models in Bipolar Depression

The difficulty of *post mortem* or cerebrospinal fluid studies compared with the ease of blood sampling and its possibility of sequential observations, has led to the use of different blood cells as possible peripheral representations of central processes. The aim of such studies in affective disorders is twofold: to provide a convenient and easily manipulated model of a particular central process implicated in the disorder with which to investigate that process in patients and controls, with and without drug treatment; and to thus find a peripheral biochemical marker of the affective disorder.

A biochemical marker is any altered biochemical system or function in a patient which indicates either a diagnostic entity or a specific brain process or the function of a neuroreceptor (Checkley, 1985). A peripheral marker of affective disorder is an alteration in a peripheral system in the disorder that may reflect similar changes in that system, or a related system, located centrally. For example, platelet MAO activity as a model of MAO activity in the brain has been investigated as a putative peripheral marker for UP and BP disorder (see page 52) and receptors located on blood cells have been used as peripheral models of neuroreceptors (see below).

Peripheral markers may be state or trait dependent. State dependent markers are present only in the active state of the illness whereas trait dependent markers are also present in euthymia and so indicate the trait of being

predisposed towards the development of the illness. State dependent markers are useful in the monitoring of response to treatment whilst trait dependent markers are of value in genetic studies. A peripheral marker may not necessarily reflect similar changes in the brain but rather be a result of other changes and so may be a secondary biochemical marker.

As alterations in the adrenergic system have been implicated in affective disorders, two peripheral models of this system have been investigated, the platelet α_2 -AR and the lymphocyte β_2 -AR.

1.4.1 The platelet α_2 -adrenoceptor

Chronic administration of some, but not all, antidepressants to the rat reduces α_2 -AR number in the brain with associated changes in behaviour (see page 56). In depressed patients neuroendocrine and cardiovascular responses to the α_2 -AR agonist clonidine are muted and may be modified following chronic antidepressant treatment (Katona *et al.*, 1987).

The human platelet expresses α_2 -ARs which are qualitatively similar in biochemical terms to those found in human brain tissue (Summers *et al.*, 1983). The platelet α_2 -AR binding characteristics in depressed patients have been examined and compared to those in healthy controls in the hope that differences in the density and affinity of this receptor binding site in the platelet reflects similar differences in the α_2 -AR in the brain.

Using the selective α_2 -AR antagonists, [^{3} H]-yohimbine or [^{3} H]-rauwolscine, most studies have shown that receptor number is not significantly different to controls in either UP or BP patients (Stahl *et al.*, 1983; Katona *et al.*, 1989; Elliott, 1991). This is in contrast to the above results from *post-mortem* brain, where [^{3} H]-rauwolscine binding sites tend to be increased in depressed suicides. However, using the partial agonist [^{3} H]-clonidine, several groups have reported increased binding capacity in the platelets of depressed patients than in those of controls, which was in the main associated with the high affinity site (Garcia-

Sevilla *et al.*, 1981 & 1986; Doyle *et al.*, 1985; Takeda *et al.*, 1989). In two of these studies (Garcia-Sevilla *et al.*, 1981; 1986) increased binding was not associated with the severity of the illness, but Takeda *et al.* found a positive correlation between the binding capacity and depression rating score.

The effects of antidepressant treatment on platelet α_2 -AR binding has also been studied. Using [^{3} H]-yohimbine or [^{3} H]-rauwolscine most studies found no significant changes following 1-6 weeks treatment with antidepressants (Stahl *et al.*, 1983; Pimoule *et al.*, 1983; Wolfe *et al.*, 1989; Katona *et al.*, 1989) or ECT (Cooper *et al.*, 1985) even though clinical improvement was seen in the majority of patients. However, using [^{3} H]-dihydroergocryptine ([^{3} H]-DHE), Healy *et al.* (1983) reported a significant reduction in binding only in those patients who showed clinical improvement. In contrast, when Wood and Coppen (1983) compared [^{3} H]-DHE binding in untreated depressed patients, euthymic BP patients receiving lithium and controls, they found that there was no significant difference in binding between the two patient groups, both of which had significantly lower binding than controls. In the aforementioned studies of Garcia-Sevilla, patients with initially high [^{3} H]-clonidine binding were shown to have decreased binding after antidepressant treatment, although the extent of receptor decrease did not correlate with the degree of clinical improvement. This would suggest that the change in binding was a direct biochemical response to the presence of the drug rather than a state-dependent change. Overall, these results do not correspond well with the α_2 -AR down-regulation seen in rats treated with antidepressants.

It is clear that there is little consistency in the platelet α_2 -AR studies in affective disorders. Experiments that employed the radioligands [^{3} H]-yohimbine and [^{3} H]-rauwolscine, technically the most suitable ligands, indicated no difference in binding between depressed and normal patients. These results would suggest that the platelet α_2 -AR is of limited use as a marker in depression and that reports of its use must be carefully assessed in terms of the radioligand employed.

1.4.2 The lymphocyte and lymphoblast β -adrenoceptor

The human lymphocyte is a small spherical cell (7-12 μm diameter) with scanty cytoplasm and a round, slightly indented and eccentrically located nucleus, containing coarse masses of chromatin (Klein, 1988). The main and unique function of lymphocytes is to initiate an immune response by reshuffling their genes in such a way that they can then produce receptors for antigens. These receptors provide the basis for distinguishing two types of lymphocyte: T lymphocytes differentiating in the thymus; and B lymphocytes differentiating in the bone marrow. The receptor of the T lymphocyte is able to recognize both an antigen (non-self) and a molecule encoded in the genes of the major histocompatibility complex (self) at the same time. In contrast, the receptor of the B lymphocyte, the immunoglobulin molecule, recognizes only the foreign antigen (non-self). The main physiological function of human lymphocytes is to distinguish self from non-self and mount a specific attack on non-self via the development of B cells into effector cells which secrete antibodies.

Another characteristic of lymphocytes is their ability to pause in the middle of their differentiation and begin to cycle. Each cycle involves the transformation of the small, resting lymphocyte into a large, active blast cell, followed by several rounds of cell division and then a gradual return to the resting phase. This burst of activity can be repeated a number of times.

As described earlier (page 26), the human lymphocyte expresses β_2 -ARs that can be easily characterized by radioligand binding techniques and which are positively coupled to adenylyl cyclase. β -Adrenergic agents can alter various elements of the immune response. Catecholamines can inhibit or augment antibody production and can inhibit mitogen-induced lymphocyte proliferation, T-cell mediated cytotoxicity, release of lysosomal hydrolases from neutrophils, and the release of mediators from mast cells (Khan & Melmon, 1988). The majority of these actions are mediated by activation of adenylyl cyclase and the subsequent accumulation of cAMP. It has been hypothesized that cAMP has a general inhibitory action on the immunological and inflammatory functions of lymphocytes so regulating the character and intensity of immune and inflammatory responses.

This suggests that cAMP serves to protect the host from the dangerous consequences of an unregulated immune response (Bourne *et al.*, 1974). cAMP also plays a role in the human B cell cycle, with elevated cytoplasmic cAMP levels inhibiting the late phase of the cycle (Muraguchi *et al.*, 1984). The presence of β -ARs on lymphocytes, neutrophils and mast cells may indicate that the sympathetic nervous system and the adrenal medulla exert some control over immunological responses. Researchers in the field of neuroimmunology have even suggested that certain cells of the immune system may serve as "free-floating nerve cells", with these cells collectively representing a "mobile brain" (Blalock & Smith, 1985).

The lymphocyte β -AR model has been used extensively to monitor β -AR properties in less accessible tissues, such as the heart and lung, and thus to investigate (patho)physiological and drug-induced alterations in the β -adrenergic receptor system, particularly in heart disease and asthma (Motulsky & Insel, 1982).

As alterations in lymphocyte β -ARs are thought to reflect changes in central noradrenergic function this model has been used to study the β -AR in a number of diseases involving the brain (Werstiuk *et al.*, 1990). Several studies have used the lymphocyte β -AR to investigate β -AR binding and function in UP and BP patients, both drug-free and antidepressant-treated.

Ten studies have compared lymphocyte β -AR density in depressed patients to that in controls, with conflicting results. Two studies observed elevated binding capacity in depressed patients (Healy *et al.*, 1983; 1985); three groups reported no difference in binding between patients and controls (Zohar *et al.*, 1983; Mann *et al.*, 1985; Jeanningros *et al.*, 1991); and five studies demonstrated reduced β -AR density in depressed patients (Extein *et al.*, 1979; Wood *et al.*, 1986; Carstens *et al.*, 1987; Pandey *et al.*, 1987; Magliozzi *et al.*, 1989). There was no correlation between the reduction in binding capacity and severity of depression as indicated by the Hamilton depression rating scale (Mann *et al.*, 1985; Pandey *et al.*, 1987). Of these ten studies, only that of Magliozzi *et al.* reported any change in binding

affinity. They observed a trend, that was not statistically significant, for lower binding affinity to [^{125} I]-CYP in UP patients compared to controls.

These differing results cannot be accounted for by the radioligands used, as of the five studies that found lower binding in depressed patients, two used [^{3} H]-DHA and three used [^{125} I]-CYP. Broken cell preparations were used in all five of these studies, whereas Healy *et al.* (1983 & 1985), Mann *et al.* (1985) and Jeanningros *et al.* (1991) used intact cells. Binding studies on intact cells can yield misleading results as during the course of a binding experiment receptors may be lost from the surface due to recycling and newly synthesized receptors may appear on the cell membrane. Kinetic experiments on intact lymphocytes have suggested that even small changes in the specific conditions of the binding assay (radioligand concentration range, temperature, assay incubation time) may lead to considerable variation in the binding parameters (Graafsma *et al.*, 1988).

Seven studies have investigated lymphocyte adenylyl cyclase activity in depression, five of which found a reduced response to isoprenaline (Extein *et al.*, 1979; Pandey *et al.*, 1979; Mann *et al.*, 1985; Ebstein *et al.*, 1988; Halper *et al.*, 1988). Extein *et al.* (1979) and Mann *et al.* (1985) simultaneously assayed β -AR binding and function. Extein *et al.* found that both isoprenaline-stimulated cAMP accumulation and β -AR number were decreased in depression, whereas Mann's group found that the cAMP response was significantly reduced in depressed patients without a significant decrease in receptor number. Mann's study also found a significant negative correlation with psychomotor agitation score and the isoprenaline-induced cAMP increase, but this was not replicated in a later study (Halper *et al.*, 1988). Kanof *et al.* (1989) found no difference in cAMP response between depressed patients and controls. The only difference between this study and those finding a reduction in cAMP response is the fact that polymorphonuclear, as opposed to mononuclear, leukocytes were used in Kanof's study. Klysner *et al.* (1987) also found no difference in isoprenaline-stimulated cAMP accumulation in depressed patients, but they found an increased response to a combination of NA and phentolamine. A possible problem with this study is that subjects were drug-free for a minimum of only one week whereas most of the other studies had a two week minimum washout period. A minimum of two

weeks drug washout has been recommended to remove possible effects of previous treatments and to obtain consistent results (Werstiuk *et al.*, 1990). As in the binding studies, there was no correlation between the degree of the reduced cAMP response and the severity of illness as assessed by the Hamilton scale (Mann *et al.*, 1985; Ebstein *et al.*, 1988; Halper *et al.*, 1988).

All of the above studies compared patients undergoing an episode of depression to normal controls, though two groups also investigated manic patients (Extein *et al.*, 1979; Pandey *et al.*, 1979). In studies where the polarity was described, the patient groups were either all or mainly UP (Mann *et al.*, 1985; Carstens *et al.*, 1987; Halper *et al.*, 1988; Magliozzi *et al.*, 1989) or a combination of UP and BP (Extein *et al.*, 1979; Pandey *et al.*, 1979; Wood *et al.*, 1986; Klysner *et al.*, 1987; Ebstein *et al.*, 1988; Jeanningros *et al.*, 1991). In some of these studies the UP and BP subgroups were compared individually to controls and/or to each other (Pandey *et al.*, 1979; Extein *et al.*, 1979; Ebstein *et al.*, 1988; Jeanningros *et al.*, 1991). Comparisons have also been made between depressed and euthymic patients (Extein *et al.*, 1979). Pandey's group (1979) found that both UP depressed patients and BP manic patients had lower isoprenaline-stimulated adenylyl cyclase activity than controls, and that there was no significant difference between the two patient groups. Extein's study found that β -AR density and isoprenaline-stimulated cAMP accumulation were reduced in both depressed and manic patients compared to both controls and euthymic patients, although the reduction in cAMP response was only significant when the data from depressed and manic patients were pooled. Ebstein *et al.* (1988) similarly found no difference in cAMP response between UP and BP patients. In contrast, Jeanningros *et al.* (1991) found significantly lower β -AR binding in UP patients than in BP patients. Apart from this most recent study, these results suggest that decreased β -AR number and function is not dependent on whether the patient is undergoing a depressive or manic episode, or on the polarity of the affective disorder.

Buckholtz *et al.* (1988) provided further evidence of this latter point when they compared β -AR binding in UP and BP patients and found no difference in binding capacity or affinity. This study also measured plasma NA concentrations

and found them higher in the BP than the UP depressed group. Higher plasma NA but equal β -AR number suggests perhaps that there was a deficit in agonist-induced down-regulation in the BP group. Analysis of the binding curves and of isoprenaline competition studies done in the absence and presence of GppNHp revealed a significantly greater K_L/K_H ratio in the BP group, whereas one would have expected high plasma NA to be associated with a lower K_L/K_H ratio as this ratio is a measure of the agonist affinity state of the β -ARs, with a higher ratio indicating higher β -AR sensitivity to NA. In other words, there is a dissociation of plasma NA concentration from the K_L/K_H ratio measure of sensitivity in lymphocytes from BP patients.

The aforementioned results also suggest that decreased β -AR number and function is state-dependent, as depressed and manic patients had lower β -AR number and function than euthymic patients. However, as state comparisons were not made from the same patients when depressed or manic and then when recovered, these results are perhaps questionable. A reduction in β -AR density was apparent in euthymic BP patients receiving lithium (Wood *et al.*, 1986) and β -AR number in depressed patients was not altered by ECT even though there was considerable clinical improvement (Cooper *et al.*, 1985). DMI and lithium treatment were not associated with any significant change in β -AR number or function in UP and BP patients tested before and after drug treatment (Extein *et al.*, 1979). These results indicate that β -AR number is not affected by antidepressant treatment, so presumably decreased β -AR number in affective disorders is not state-dependent. However, in the two studies which reported increased β -AR binding in depressed patients, antidepressant treatment did result in a decrease in receptor binding (Healy *et al.*, 1983; 1985). Antidepressant therapy was not associated with any change in β -AR number in Buckholtz's study (1988). Interestingly, Buckholtz *et al.* found that antidepressant therapy increased the K_L/K_H ratio, suggesting an increase in receptor sensitivity to NA.

The lymphocyte β -AR model has an important disadvantage in that this receptor is sensitive to modulation by catecholamines, drugs and hormones in the plasma (Fraser *et al.*, 1981; Feldman *et al.*, 1983), which will be discussed further in Chapter Five. Lymphocytes can be transformed to immortal lymphoblast cell

lines using the Epstein-Barr virus (Nietzel, 1986). Lymphoblasts are genetically stable (Povey, 1973; 1980) and though their β -AR density is lower than that seen in lymphocytes, their β -AR affinity for radioligands is similar (Ebstein *et al.*, 1985; Elliott, 1987). The use of cultured cells avoids the problems of receptor modulation by plasma catecholamines and hormones and any effects of the phase of illness and drug treatment. The kinetic parameters of the lymphoblast β -AR have been shown to be a stable characteristic of a cell line (Berrettini *et al.*, 1987). Any persistent defect can therefore be presumed to be genetically coded (Wright *et al.*, 1984) and changes in intrinsic β -AR density should be revealed, that will be trait-dependent.

According to the two threshold-liability model described previously, BP and UP can be seen as "narrow" and "broad" versions of the same condition occupying the same continuum of liability. Therefore it would seem probable that the genetic or biological contribution to BP will be greater than for UP. As lymphoblast studies investigate the β -AR as a possible genetic susceptibility marker, BP patients have been used because the genetic loading in this disorder is greater than that in UP disorder.

β -AR density has been studied in lymphoblast cell lines derived from BP patients and normal controls by two groups (Wright *et al.*, 1984; Berrettini *et al.*, 1987). Wright *et al.* used BP probands with severe illness of early onset and a family history of affective disorder in two generations. Samples from unaffected members of the proband's family were used as controls in addition to non-psychiatric controls. Cell lines from three out of five BP families (four out of six patients) had significantly lower β -AR number in affected members than in unaffected members or non-psychiatric controls. Only one out of nine familial controls had this receptor defect. In contrast to these results, Berrettini *et al.* (1987) found no difference in binding capacity or affinity between 17 BP subjects and 14 unrelated controls. There was no suggestion of a patient subgroup with lower β -AR binding. β -AR binding capacity in controls agreed well with that of Wright *et al.*. The main difference between the two studies was the radioligand used as Wright's group used [^{125} I]-HYP whereas Berrettini's group used [^{125} I]-CYP. The binding characteristics of these two radioligands have been

compared in a lymphoblast cell line, with differences in binding characteristics of these ligands being proposed as the reason for the conflicting results (Friend *et al.*, 1988).

Overall, studies of the lymphocyte β -AR would seem to suggest that β -adrenergic activity is decreased in depressed patients compared to normal controls. This β -AR defect was similar both in patients with depression and in those with mania, was not related to the severity of depression and did not alter after treatment, which suggests that it is not dependent on the phase of the illness. As binding affinity is unaltered in affective disorders, it would seem that reduced β -AR density is due to a defect in β -AR regulation as opposed to a defect in the receptor itself. This could either be a decreased synthesis or an increase in uncoupling, desensitization or down-regulation. However, the findings of Buckholtz *et al.* (1988) indicate that sensitivity to NA is reduced in BP patients, which would suggest that a decrease in down-regulation and desensitization would be expected in these patients.

Nurnberger and Gershon (1986), have stated that for genetic progress to be made in affective disorders, our interest must move from changes in the acute manic or depressive episode to the chronically present constitutional vulnerability. They suggest that the most valuable findings will be those abnormalities that do not change from the ill to the well state, and those that can be unmasked in the well state, possibly by pharmacological challenge methods. Abnormalities of the lymphoblast β -AR in patients with affective disorders would be independent of affective state. Furthermore, cultured cells allow controlled manipulation of the conditions that may reveal abnormalities in receptor regulation and function.

1.4.3 Validity of the lymphocyte and lymphoblast β -adrenoceptor models

Since peripheral blood cells are easily accessible, human lymphocytes are frequently used as a model to study physiological and pharmacological alterations in the β -AR system. Fraser *et al.* (1981) found that there was a good correlation between human lymphocyte β -AR density and cardiac sensitivity to isoprenaline

which they felt validated the use of studying the lymphocyte β -AR as a model of adrenergic function. Maisel *et al.* (1988) compared lymphocyte and cardiac β -AR sensitivity to isoprenaline in the guinea pig. They also found that lymphocyte β -ARs respond to circulating isoprenaline in a manner generally similar to that of β -ARs on the myocardium. However, their data indicated that the lymphocyte β -AR does not reflect changes promoted by β_1 -AR selective agonists and/or changes that occur at synapses where the catecholamine concentration greatly exceeds that to which the lymphocytes are exposed in the plasma. This would suggest that the lymphocyte model is useful in investigating correlations between plasma concentration of catecholamines and hormones and β -AR number and function, whereas the lymphoblast, which is grown in culture under controlled conditions, is the preferred model to examine intrinsic differences in the β -AR and its regulation.

The extrapolation from changes in lymphocyte β_2 -ARs to changes in occurring in other tissues was discussed by Brodde *et al.* (1987). They concluded that changes in lymphocyte β_2 -ARs can only be taken as representative of changes in other human tissues either when these changes are caused by non-selective β -adrenergic agents such as isoprenaline, adrenaline or propranolol, or when the other tissue also has a homogenous population of β_2 -ARs. As to the extrapolation of the results obtained with the lymphocyte or lymphoblast β -AR model to neuronal β -ARs, it is interesting to note that comparisons between the immune system, viewed as a functional network open to stimulation from the outside, and the nervous system have been made (Jerne, 1974). These systems are both able to respond to an enormous variety of signals and to receive as well as transmit signals, either excitatory or inhibitory. As in the nervous system, modulation of the immune network by foreign signals represents its adaptation to the outside world. Both systems learn from experience and build up a memory that is sustained by reinforcement and that is deposited in persistent network modifications. It was hypothesized by Jerne that these phenotypic analogies between the immune system and the nervous system may result from similarities in the sets of genes that govern their expression and regulation.

The aim of this study was to investigate the binding characteristics and adenylyl cyclase activity of lymphoblast β -ARs derived from patients with BP affective disorder and from age and sex matched control subjects. From the studies of the lymphocyte β -AR, it was expected that lymphoblast β -AR density and adenylyl cyclase activity would be lower in BP cell lines than in controls, which would confirm and extend Wright's findings as opposed to those of Berrettini.

Most clinical investigators have looked for alterations in receptor number in disease and due to drug administration. However, it is not clear how much impact the relatively small changes observed have on physiological function. In studies on normal persons, the variations in the numbers and affinities of lymphocyte β -ARs are often very large (Motulsky & Insel, 1982). It has been suggested that future clinical studies should search for more than just changes in receptor number, but should also examine the effects of modulators and the potential for receptors to be dynamically regulated (Motulsky & Insel, 1982). This is particularly relevant in the case of the β -AR in depression as many antidepressants cause β -AR down-regulation and/or desensitization and this has been hypothesized as their mode of action (Sulser *et al.*, 1978).

With this in mind, β -AR down-regulation and desensitization in response to coincubation of the lymphoblasts with isoprenaline was examined, first in controls in order to establish the effect of a range of isoprenaline concentrations and coincubation times on β -AR binding and function, and then in BP and matched control cell lines at an appropriate isoprenaline concentration and coincubation time.

The effects of coincubation with lithium, the major drug therapy in use in BP affective disorder (see Chapter Six), and with DMI, were studied. This was to investigate whether any previously observed differences in β -AR binding, function and agonist-induced down-regulation between BP cell lines and control cell lines were still apparent after lithium or DMI coincubation. Normalization of these parameters would suggest that such differences are of significance in the aetiology of BP affective disorder and/or in the mechanism of action of these drugs.

CHAPTER TWO

MATERIALS AND METHODS

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2.1 Materials

2.1.1 Cell culture equipment and media

All cell culture work was carried out in a M.A.T. Class II Microbiological safety cabinet with negative pressure and vertical laminar downflow of sterile air in the working area to ensure that air could not leak out of the cabinet and also to provide protection from external contamination. The cells were incubated in a LEEC Automatic CO₂ GA2 incubator at 37°C in a 95% air/5% CO₂ atmosphere and 99% humidity in an open culture system allowing gas exchange with the environment. A Mistral 3000 centrifuge (MSE Scientific Instruments, Sussex, UK) was used to harvest the cells. All cell culture flasks and centrifuge tubes were supplied by Sterilin (Cel-Cult) Ltd., Hounslow, UK and were tissue culture treated and sterilised by Gamma irradiation. 30ml Sterile Universal tubes were also supplied by Sterilin. Cell density was counted using a Coulter Counter, model ZF (Coulter Electronics Ltd., Luton, UK).

The cell culture medium routinely used was RPMI 1640 Medium buffered with 2 g/l sodium bicarbonate. When indicated the medium was buffered with 20mM HEPES (RPMI 1640 (HEPES)) instead of sodium bicarbonate. Buffering was to enable the medium to equilibrate to pH 7.4 in 5% CO₂. The culture medium was supplied by Gibco Life Technologies Ltd., Paisley, Scotland, as was phosphate buffered saline (PBS). Lymphocyte Separation medium was supplied by Flow Laboratories Ltd., Rickmansworth, Hertfordshire, UK. All media used was supplemented with a cocktail of antibiotics - 20 µg/ml gentamicin, (Cidomycin injectable preparation, 80mg/2ml, Roussel Laboratories Ltd., Wembley, UK), 100 µg/ml flucloxacillin sodium, (Ladopren for injection, Berk Pharmaceuticals Ltd., UK) and 200 µg/ml piperacillin, (Pipril for injection, Lederle Laboratories Ltd., Gossport, UK) - to inhibit bacterial contamination. The medium was supplemented with foetal calf serum (FCS) (Flow Laboratories) which contains all the necessary growth factors for the cells. Heparin sodium B.P. (preservative free, 5000 U/ml) was supplied by Paines and Byrne Ltd., Greenford, UK. Cyclosporin A was kindly donated by the Department of Biochemistry, St Mary's Hospital Medical School. Epstein-Barr virus was generously supplied by Jackie

Pontin of the Department of Pharmacology. Dimethylsulphoxide (DMSO) was supplied by BDH Chemicals Ltd., Poole, UK.

2.1.2 Other equipment

Other equipment used in this study were as follows:- Shimadzu multipurpose recording spectrophotometer, MPS-2000, connected to a Shimadzu graphic printer, PR-3 (Shimadzu Corp., Kyoto, Japan); Sorvall RC-5B Refrigerated Superspeed Centrifuge with an SA600 rotor, from Sorvall Instruments, DuPont Co., Biomedical Products, Wilmington, USA; Braun Potter S homogenizer (B. Braun, Biotechnics Division, Melsungen, Germany); MSE ultrasonic disintegrator (soniprobe); Skatron cell harvester (Skatron Instruments Ltd., Newmarket, UK.); Packard Cobra *gamma* counter and Minaxi *beta* counter (Packard Instrument Co., Meriden, Connecticut, USA); Techne SB-16 shaking water bath (Techne Ltd., Cambridge, UK); Econo Mini columns for column chromatography (Bio-Rad Laboratories, California, USA); Biofuge B microcentrifuge (Heraeus Sepatech, Germany); Sorvall centrifuge tubes; 1.5ml microtest tubes (Eppendorf, Hamburg, Germany); Macrowell tube strips (1ml), 12 tubes/strip, from Skatron Instruments Ltd.; 3.5, 5 and 13ml plastic test tubes, from Starstedt, Numbrecht, Germany; Whatman GF/C glass microfibre filter paper (Whatman Int. Ltd., Maidstone, UK); Packard 20ml scintillation vials (Packard Instruments, Groningen, Holland).

2.1.3 Chemicals and radiochemicals

Suppliers of chemicals and radiochemicals used in this study were as follows:-

BDH Chemicals Ltd., Poole, UK:- sodium chloride (NaCl); Folin-Ciocalteu reagent;

Bio-Rad Laboratories:- analytical grade cation exchange resin, 200-400 mesh, hydrogen form (Dowex 50);

Boehringer Ingelheim Ltd., Bracknell, Berkshire, UK: fenoterol;

CIBA-Geigy, Horsham, UK:- phentolamine; CGP 12177; CGP 20712; oxprenolol;
 Coulter Electronics Ltd:-Isoton II balanced electrolyte solution;
 Duphar Laboratories Ltd., West End, Southampton, UK: isoxsuprine;
 Fisons Scientific Apparatus, Loughborough, UK:- d-glucose (anhydrous); calcium chloride (CaCl_2);
 FSA Laboratory Supplies, Loughborough, UK:- magnesium chloride (MgCl_2);
 diaminoethanetetraacetic acid disodium salt (EDTA); sodium hydroxide pellets (NaOH); sodium fluoride (NaF); orthophosphoric acid; potassium chloride (KCL);
 ICI Pharmaceuticals, Alderley Edge, Cheshire, UK:- ICI 118551; l-propranolol; d-propranolol; practolol; atenolol;
 Janssen Pharmaceutical Ltd., Oxfordshire, UK: ketanserine; haloperidol;
 National Diagnostics, New Jersey, USA:- Ecoscint biodegradable scintillation solution;
 Pierce and Warriner (UK) Ltd., Chester, UK:- sodium dodecyl sulphate (lauryl) (SDS);
 Sigma Chemical Co. Ltd., Poole, UK:- adenosine 3':5'-cyclic monophosphate (cAMP); adenosine 5'-triphosphate (ATP); alumina; l-ascorbic acid; atropine; bovine serum albumin (BSA), RIA grade; copper sulphate, pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$); creatine phosphokinase; deoxycholic acid sodium salt (NaDOC); digitonin (50%); dopamine; (-)-epinephrine (+) bitartrate (adrenaline); ethyleneglycol-bis-(β -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA); forskolin; guanosine-5'-triphosphate, sodium salt from equine muscle (GTP); 5'-guanylylimidodiphosphate, sodium salt (GppNHp); imidazole, crystalline grade 1; 5-hydroxytryptamine (5-HT); 3-isobutyl-1-methylxanthine (IBMX); (-)-isoprenaline, (+)-bitartrate salt; (+)isoprenaline; (-)-norepinephrine hydrochloride (noradrenaline); phosphocreatine; prostaglandin E_1 (11,15-dihydroxy-9-oxoprost-13-en-1-oic acid) (PGE_1); sodium carbonate; sodium tartrate; trichloroacetic acid; Trizma hydrochloride (Tris[hydroxymethyl]-aminomethane hydrochloride) (Tris); Amersham International, Green End, Buckinghamshire, UK:- (-)(3)[^{125}I]-iodocyanopindolol in methanol solution ([^{125}I]-CYP), specific activity approximately 2000 Ci/mmol, 100 mCi (3.7 kBq) in 0.1ml, used within 6 weeks of activity date; cyclic AMP assay kit containing Tris/EDTA buffer; binding protein purified from bovine muscle; [8- ^3H]-adenosine 3'5'-cyclic phosphate ([^3H]-cAMP),

180pmol containing approximately 5 μ Ci, 185kBq; adenosine 3'5'-cyclic phosphate (cAMP) standard, 1600pmol; and charcoal adsorbent; NEN-Dupont (UK) Ltd, Hertfordshire, UK:- [α -³²P]ATP specific activity approximately 600Ci/mmol.

2.2 Cell Culture

2.2.1 Establishment of lymphoblast cell lines

Lymphoblast cell lines were established by separating lymphocytes from fresh blood by the use of a Ficoll-Hypaque gradient (Boyum, 1968; Williams *et al.*, 1976) and then transforming them using the Epstein-Barr virus (EBV) according to the method of Neitzel (1986).

20ml blood samples were taken from subjects and placed in sterile Universal tubes containing 0.2ml 1000 U/ml heparin sodium to prevent coagulation. All the following procedures were carried out in the safety cabinet. Each blood sample was diluted with 10ml RPMI 1640 (HEPES), to control the degree of aggregation of red blood cells so that they would sediment easily, with a minimum of lymphocyte entrapment. The diluted blood was then overlaid onto Lymphocyte Separation Medium in Universal tubes in a ratio of 15ml of blood to 10ml of the separation medium. The tubes were then centrifuged at 400g and 22°C for 30 min with a minimal brake applied during deceleration. Following centrifugation the blood separated into layers above and below the separation medium, the bottom layer being red blood cells and the top layer plasma, with a thin layer of lymphocytes at the interface of the plasma and the separation medium. Using a bright light for illumination, the lymphocyte layer was carefully aspirated avoiding the separation medium as that would have caused contamination with granulocytes. The lymphocytes were transferred to clean Universal tubes and washed by making up to 25ml with RPMI 1640 (HEPES), supplemented with 5% FCS, and centrifuged at 400 g, 22°C, for 15 min. The resulting pellet of mononuclear cells typically comprised 85% small lymphocytes, 13% monocytes and less than 2% polymorphonuclear leukocytes (Williams *et al.*, 1976). This pellet was resuspended in 3ml of RPMI 1640 (Bicarb.) supplemented

with 15% FCS and pipetted into a 25cm³ culture flask. 0.5ml EBV isolated from the B95-8 monkey kidney cell line was added. Cyclosporin A to a final concentration of either 0.1 or 0.5 µg/ml was also added to stimulate the growth of B-type lymphocytes and to inhibit T-cells which would otherwise suppress the transformation of the B cells (Bird, 1981).

The flasks were incubated for 5-7 days at 37°C/98% humidity without disturbance. Expired medium was replaced with fresh RPMI 1640 (Bicarb.)/15% FCS as required. After about four weeks aggregates of lymphoblastoid cells could be seen and the volume of medium was increased gradually to 30ml, moving the cell suspensions to 75cm³ culture flasks when necessary. Once cell lines were established the concentration of FCS was reduced to 10%.

The cell lines were subcultured every 3-5 days, depending on the growth rate of the cells, by removing 20ml of medium and cells and replacing with 20ml fresh RPMI 1640/10% FCS. Lymphoblast cell lines were not used for experiments until at least 6-8 weeks after transformation and were continuously grown in culture for periods extending to 3 years. Lymphoblast cell lines of "normal" as opposed to "malignant" origin grown over such time periods have been shown to be genetically stable (Povey *et al*, 1973; 1980).

When necessary cell counts were carried out in duplicate on 100µl samples diluted in 10ml isoton using a Coulter Counter with an aperture of 100 microns, a sensitivity setting of 16 and an attenuation setting of 0.707. Cells were harvested for assay by centrifugation at 400g for 10 minutes.

2.2.2 Storage of lymphoblast cell lines in liquid nitrogen

As soon as cell lines were established a sample of lymphoblasts was frozen down and stored in liquid nitrogen. 40ml of confluent cell suspension were poured into a 50ml centrifuge tube and spun at 400g for 10 minutes. The resulting cell pellet was resuspended on ice with 4ml of ice cold RPMI 1640 medium with 10% FCS and 10% DMSO and the suspension pipetted into 4 x 2ml

cryogenic vials. These were then placed on the cooling tray within a liquid nitrogen dewar to slowly cool down to approximately -184°C. After 4 hours the tubes were put into the liquid nitrogen where they were stored at -196°C.

When it was necessary to re-establish a cell line, the frozen lymphoblast suspension was rapidly thawed after removal from the liquid nitrogen and poured into a Universal tube. 20ml of RPMI 1640 medium/20% FCS was added. The tube was then centrifuged at 400g for 10 min and the pellet resuspended in 5ml RPMI 1640/20% FCS. As the cell numbers increased the volume was increased to a volume of 30ml as before. Cell lines varied in their rate of growth, but were generally used for experiments 1-2 weeks after thawing.

2.2.3 Culture of A431 human epidermoid carcinoma cell line

The human epidermoid carcinoma cell line, A431, was also grown in culture. This cell line has a high number of β -ARs which are functionally coupled to adenylyl cyclase (Delavie-Klutchko *et al.*, 1984; Levitzki & Kashles, 1987). During this investigation, the A431 cell line was used mainly as a standard with which to compare the lymphoblast cell lines when setting up the adenylyl cyclase assay. A431 cells grow as a monolayer on the surface of the cell culture flask. When about 80% of the surface of a culture flask was covered with A431 cells, the cells were harvested or subcultured. The medium was poured off and the cells washed with PBS. The cells were then briefly exposed to a dilute trypsin solution (0.1%) and the excess poured off. The flask was then put into the incubator for a few minutes to allow the trypsin to hydrolyse the proteins responsible for adhesion of the cells to the culture flask. The flask was then vigorously agitated to promote the detachment of the cells from the surface of the flask. 20ml RPMI 1640 containing 10% FCS was then added to the flask to inactivate the trypsin and to wash off the cells and the cells and medium pipetted into a Universal tube. After centrifuging at 400g for 10 min the pellet was either used for biochemical assay or subcultured by resuspension in 1ml RPMI 1640/10% FCS using a syringe after which 0.2ml of the cell suspension was added to 10ml of RPMI 1640/10% FCS and the cells maintained in 95% air/5% CO₂.

2.3 Radioligand Binding

The basis of a radioligand binding assay for the study of receptors is as follows:- the radioligand is incubated with the tissue, the unbound radioactivity is removed by filtration or centrifugation, and the radioactivity bound to the tissue is measured. Binding to non-specific sites is determined by incubating the radioligand and tissue in the presence of a non-radioactive drug that will bind to ~~virtually~~ all the receptors being studied. With all the receptors occupied by this unlabelled compound, the radioligand should bind only to non-specific sites. Specific binding is calculated by subtracting the radioactivity observed in the presence of the unlabelled compound (non-specific binding) from that observed in its absence (total binding).

2.3.1 Preparation of lymphoblast membranes

The lymphoblast β -AR was characterized by radioligand binding using [^{125} I]-iodocyanopindolol ([^{125} I]-CYP). The protocol was adapted from the method of Brodde *et al.*, (1981) developed for lymphocyte membranes. Lymphoblast cells were harvested by centrifugation and the pellet ~~either~~ resuspended in 25ml of ice cold hypotonic medium (Tris 5mM, MgCl₂ 1mM, titrated to pH 7.4 with NaOH solution). Hypotonic medium was used to promote cell lysis. The cell suspension was homogenized on ice using a Braun Potter S homogenizer and then sonicated for 30 sec with an ultrasonic disintegrator at 15 microns. Nuclei and unbroken cells were sedimented by slow centrifugation (700g, 5 min, 4°C) and then discarded. The supernatant was spun at 30 000g for 15 min at 4°C. The resulting pellet was washed in 25ml of the same medium and centrifuged as before. Generally assays were carried out on fresh tissue, but for those experiments when it was necessary to freeze the tissue either the cell pellet or the membrane pellet were stored at -20°C until required. The membrane pellet was finally resuspended in assay medium (Tris 50mM, MgCl₂ 10mM, phentolamine 1 μ M, pH 7.4) at a final density 0.1-0.4mg protein/ml. The membrane suspension was kept on ice until use which was usually within 5 mins. Samples of the membrane suspension were stored at -20°C for determination of the protein concentration.

2.3.2 Radioligand binding protocol

200 μ l aliquots of tissue were incubated in triplicate with 50 μ l [^{125} I]-CYP in 1ml Skatron Macrowell tubes. Radioligand binding assays were carried out at 6 concentrations of [^{125} I]-CYP ranging from 5-100 pmol/l for 90 min at 37°C. Specific binding was defined by 1 μ M CGP 12177, a potent hydrophilic non-selective β -AR antagonist. Displacement studies used 25 μ l of a fixed concentration of [^{125} I]-CYP of about 40pM plus 25 μ l of either incubation buffer (total binding), CGP 12177 to give a final concentration of 1 μ M (non-specific binding) or one of a range of ten concentrations of inhibitor. The assay was terminated by filtration through Whatman GF-C filters with a Skatron cell harvester using a cycle of 10 secs wash with ice-cold buffer (Tris 50mM; MgCl₂ 10mM; EGTA 1mM; pH 7.4) and 10 secs air to dry the filter paper and membranes. The filter paper was punched through into 3ml Starstedt tubes for counting. Total radioactivity added to each tube was determined using 50 μ l aliquots in triplicate for each of the six concentrations of [^{125} I]-CYP. Bound and total radioactivity for each incubate were estimated by a Packard Cobra gamma-counter with an 81% efficiency using a 5 min count time.

2.3.3 Data analysis

Specific binding for each incubate was determined by subtracting the non-specific binding in the presence of CGP 12177 from the total binding in the absence of CGP 12177. The results were analysed by Scatchard analysis, which plots the ratio of bound over free radioactivity against the bound radioactivity, and computerized non-linear regression of the amount of bound radioactivity against the total concentration of [^{125} I]-CYP for each incubate (Duggleby, 1981). The initial values for the concentration of receptor, B_{max}, and its affinity, K_d, for the ligand, [^{125} I]-CYP, are derived from Scatchard analysis and are then used for the construction of a binding curve based on the law of mass action. The actual values for the B_{max} and the K_d are derived by successively iterating fits to the data. The goodness of fit is determined by finding the smallest residual variance

(ratio of the sum of squares of residuals divided by the degrees of freedom) achievable in successive iterations.

Displacement of specific [^{125}I]-CYP binding was analysed using the EBDA program which is a commercial computer program (McPherson, 1985).

2.4 Adenylyl Cyclase Assay

Adenylyl cyclase catalyses the conversion of ATP to 3'5'-cyclic AMP and pyrophosphate in the presence of GTP and magnesium ions. In the lymphocyte and lymphoblast this reaction is stimulated by β -AR agonists, prostaglandins, forskolin and sodium fluoride. Two different methods of measuring adenylyl cyclase activity were investigated. That of Salomon (1973; 1979) quantifies the conversion of [^{32}P]-ATP to [^{32}P]-cAMP and is therefore a direct measure of enzyme activity. In contrast the Amersham cAMP radioimmunoassay kit which is based on Brown's method (1971) measures the amount of [^3H]-cAMP in a sample. The assays were run initially on both a control lymphoblast cell line and the A431 cell line.

2.4.1 Salomon method of adenylyl cyclase assay

Salomon's method measures the formation of [^{32}P] cyclic AMP from [^{32}P]-ATP. Separation of the reaction product was achieved using sequential chromatography on Dowex 50 cation exchanger and on neutral alumina which permits almost complete separation of [^{32}P]-cAMP formed from the substrate and other [^{32}P]-containing compounds. The Dowex column was prepared by washing with 2 x 3ml water and the alumina column by washing with 2 x 3ml imidazole (0.1M).

Cells were harvested as previously described and homogenized in 25ml lysate buffer (Tris 5mM, EDTA 5mM, pH 7.5). After a 20 min centrifugation at 15 000g and 4°C, the pellet was resuspended in the lysate buffer and the

centrifugation repeated. The pellet was resuspended in approximately 1.5ml TME buffer (Tris 50mM, MgCl_2 5mM, EDTA 1mM, pH 7.5). An assay cocktail was prepared in advance and stored in 1ml aliquots at -20°C until required. This cocktail comprised 53 μM GTP, 0.1mM cAMP, 0.12mM ATP, 40 $\mu\text{g}/\text{ml}$ creatine phosphokinase, 8mM phosphocreatine, 67mM Tris and 2mM MgCl_2 . [α - ^{32}P]ATP was added to the assay cocktail to give $1\text{--}2 \times 10^6$ cpm/tube. Eppendorf tubes were set up with 40 μl of the ^{32}P cocktail and 10 μl agonist to stimulate adenylyl cyclase. The ~~agonists~~^{stimulants} used were 1mM sodium fluoride, 0.01 and 10 μM forskolin and 0.01 and 10 μM isoprenaline. 10 μl TME was used in place of agonist to give the basal level of cAMP formation. All tubes were set up in triplicate.

The assay was started by the addition of 50 μl tissue to the tubes which were incubated at 37°C for 20 min. The assay was stopped by the addition of 1ml cold stopping solution which was made up of 200mg/l ATP, 100mg/l cAMP and 25 $\mu\text{l}/\text{ml}$ ^3H -cAMP solution (0.03 $\mu\text{Ci}/\text{ml}$). The tubes were centrifuged in a Biofuge centrifuge for 2 min at 11 000g.

1ml of supernatant from each incubate was added onto the Dowex columns and allowed to drain. The columns were then washed with 2 x 1.5ml water allowing each addition to drain through. The Dowex columns were then placed above the alumina columns and eluted with 2 x 2.5ml water. The Dowex columns were removed and the alumina columns were washed with 0.5ml 0.1M imidazole. After placing the alumina columns over scintillation vials, a further 3ml of imidazole was added to elute the cAMP into the vials.

Scintillation vials containing 3ml imidazole as a blank, 1ml ^3H -cAMP + 2ml imidazole to give a ^3H -cAMP standard, and 40 μl ^{32}P cocktail + 3ml imidazole to give a ^{32}P -ATP standard were also set up in triplicate. 16ml of Ecoscint scintillation cocktail were added to the scintillation vials and the vials were counted in scintillation counter on a dual $^3\text{H}/^{32}\text{P}$ channel for 5 min. The columns were regenerated after use by the addition of 2 x 3ml 1M HCL followed by 2 x 3ml water to the Dowex columns and 2 x 3ml 0.1M imidazole to the alumina columns.

2.4.2 Radioimmunoassay of cAMP content

The second method of measuring adenylyl cyclase activity is based on the competition between unlabelled cAMP and a fixed quantity of [^3H]-labelled cAMP for binding to a protein with high specificity and affinity for cAMP. The amount of labelled protein-cAMP complex formed is inversely proportional to the amount of unlabelled cAMP in the assay sample. The method for preparation and stimulation of the cells was adapted from those of Ebstein *et al.* (1985 & 1988) and Mann *et al.* (1985).

Cells were harvested and resuspended in balanced salt solution (BSS) (NaCl 0.13M, Glucose 0.01%, BSA 1%, CaCl_2 0.005mM, MgCl_2 0.098mM, KCl 0.54mM, Tris 0.015M, pH 7.6) to a concentration of about 6×10^6 cells/ml. This cell suspension was then preincubated at 37°C for 30 min. Eppendorf tubes were prepared with 50 μl IBMX 5mM (final concentration 0.25mM) and 50 μl BSS (basal) or agonist (stimulated). IBMX is a potent phosphodiesterase inhibitor. The agonists used were isoprenaline, forskolin, prostaglandin E_1 (PGE_1) and sodium fluoride (NaF) in varying concentrations. 900 μl aliquots of the cell suspensions were added to the tubes, which were then incubated at 37°C for 10 min. The reaction was stopped by centrifugation at 11 000g for 2 min. The supernatant was rapidly discarded and the tubes placed on ice. 200 μl of ice cold Tris-EDTA buffer (Tris 50mM, EDTA 4mM, pH 7.5) was added and the cells were disrupted by sonication. The protein was precipitated by boiling for 5 min and spinning at 11 000g for 2 min. The resulting supernatant was then assayed for cAMP content.

Standard cAMP solutions were prepared by serial dilutions of the stock cAMP solution (320 pmol/ml) with Tris-EDTA buffer to give a range of standards from 0.25-16 pmol/tube. Eppendorf tubes were prepared in a rack at 0°C in an ice/water bath. In duplicate, 50 μl aliquots of the standard cAMP solutions or the unknown samples were pipetted into the eppendorfs. 50 μl of [^3H]-cAMP was added to each assay tube, followed by 100 μl of binding protein (purified from bovine muscle). Eight more eppendorfs were also included to give estimates of the instrument blank, the assay blank, binding in the absence of unlabelled cAMP

and the total radioactivity added to each tube. A summary of the protocol is shown in Table 2.1. All tubes were vortexed for 5 sec and the ice bath and tubes were placed in a cold room at 4°C for a minimum of 2 hours. 100µl charcoal suspension was then added to all the tubes which were vortexed briefly and then centrifuged at 11 000g for 2 min to sediment the charcoal. Without disturbing the sediment, 200µl aliquots were removed from each tube and placed in scintillation vials. 2.5ml Ecoscint was added to each vial and the vials were then counted in a Packard liquid scintillation counter for 4 min.

Table 2.1 Cyclic AMP assay kit protocol

Tris/EDTA buffer µl	cAMP vol µl	sample conc pmol/ml	[³ H]cAMP µl	binding protein µl	
250	-	-	-	-	total
200	-	-	-	-	activity
150	-	-	50	-	instrument
50	-	-	50	100	blank
-	50	0.25	50	100	assay
-	50	0.5	50	100	blank
-	50	1.0	50	100	zero dose
-	50	2.0	50	100	standard
-	50	4.0	50	100	"
-	50	8.0	50	100	"
-	50	16.0	50	100	"
-	50	?	50	100	unknown

2.4.3 Data analysis

The counts per minute (cpm) for each pair of duplicates were averaged and the cpm for the assay blank subtracted to give the cpm bound in the presence of standard or unknown unlabelled cAMP (C_x). A standard curve was plotted of % radioactivity bound to the binding protein against the concentration of the standard using a SPLINE curve fit program. The % bound was calculated as follows:

$$\% \text{ bound} = \frac{C_x}{C_T} \times 100$$

where C_T is the total activity.

The amount of unlabelled cAMP in the unknown samples was found by first calculating the % bound for the samples and then using the standard curve to estimate the amount of cAMP. The results were then normalised in terms of cell density or protein concentration to give either pmol cAMP/ 10^6 cells or pmol cAMP/mg protein.

2.5 Protein Determination

Two methods of determining the protein concentration of the membrane homogenates were used. Initially the method of Bradford (1976) was used as this was the method currently in routine use by the laboratory.

2.5.1 Protein assay by Bradford's method

Bradford's reagent was prepared at least 24 hours before running the assay. To make up 250ml of the reagent, 25mg of Coomassie Blue dye was weighed out and 12.5ml of absolute ethanol added. After mixing to dissolve as much of the dye as possible, 25ml of concentrated (85%) orthophosphoric acid and 212.5ml of distilled water were added. This mixture was stirred for a minimum of 4-5 hours. It was then filtered under gravity through Whatman No.1 filter paper and stored at 4°C.

Protein standards (0, 25, 50, 75 and 100 $\mu\text{g/ml}$) were prepared using Bovine Serum Albumin (BSA) dissolved in incubation medium. The tissue samples were thawed and then sonicated to resuspend the precipitated protein. Dilutions of the tissue, usually 1:3, were made with incubation medium to bring the protein concentrations into the range of the standards. 100 μl aliquots of the standard

BSA solutions and of the tissue samples were pipetted into 5ml tubes in triplicate and 1ml of the Bradford's reagent added to each.

The absorption of monochromatic light at 595nm was quantified using a spectrophotometer, at a slit width of 1nm. The standards were read first and a standard curve was calculated, which was then used to determine the protein concentration of the samples.

2.5.2 Protein assay by Peterson's method

The second method, that of Peterson (1977) was an adaptation of the method of Lowry *et al.* (1951) in that the protein is first precipitated from the tissue suspension. This precipitation step prevents any interference from the incubation buffer and allows samples from different sources, that is made up in various incubation buffers, to be assayed together using a single protein standard curve. Standards and tissue samples were prepared by sonication and dilution as for the Bradford assay but distilled water was used in the place of incubation medium. 0.1ml 0.15% NaDOC was added to 1ml of either standard or sample, vortexed and incubated at room temperature for 10 min. 0.1ml 72% trichloroacetic acid was then added and the tubes were spun at 3000g for 30 min at 4°C. The supernatant was poured off and the tubes inverted and spun at 300g for 7 min to completely remove any remaining supernatant. Meanwhile the reagents for the assay were prepared. Reagent A was made up of equal volumes of copper-tartrate-carbonate solution (CTC), 10% SDS, 0.8M NaOH and distilled water. CTC solution was 0.1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.2% sodium tartrate and 10% sodium carbonate. Reagent B was 1 volume Folin-Ciocalteu phenol to 5 volumes distilled water. 1ml of distilled water was then added to the protein pellets, followed by 1ml of Reagent A. The tubes were vortexed and after 10 min at room temperature 0.5ml of Reagent B was added and the tubes vortexed. After 30 min the absorption at 750nm was quantified using the spectrophotometer and the protein concentrations determined as before.

2.5.3 Comparison of protein assays

As previously mentioned, the Bradford assay was initially used as it had been in regular use in the laboratory. However, when the Peterson method was tried, it was found to give more accurate and reproducible results. Consequently, the Peterson method supplanted the Bradford method. The Peterson method was used for all experiments on cell lines from patients with bipolar depression and from their sex and age matched controls. All comparisons of data were made using results from one method alone.

2.6 Statistical Tests

Differences between groups were analysed using either the Student's t-test or one-way or two-way analysis of variance, with a probability (p) level of less than 0.05 as the limit of significance. The t-test was used to compare two groups when the effect of only one parameter was being investigated. When the effect of, for example, two concentrations of a single drug on a particular response, was being examined, one-way analysis of variance was used. If the effects of two parameters were being tested, such as the concentration of a drug and the subject status, then two-way analysis of variance was used. Paired and unpaired statistical tests were used depending on whether matched data or unmatched data were being examined. Data were matched, necessitating the use of a paired test if comparisons were being made within the same group of cells, that is either control or bipolar cell lines. When comparing the results between different cell types, the data were unmatched, therefore an unpaired test was appropriate.

CHAPTER THREE

CHARACTERISATION OF THE LYMPHOBLAST β -ADRENOCEPTOR

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3.1 Introduction

In order to establish whether the lymphoblast β -AR was an appropriate model of β -AR function and regulation, the receptor binding characteristics first had to be ascertained. β -AR identification depends upon the fulfilling of stringent criteria (Lefkowitz, 1974; Cuatrecasas *et al.*, 1974). The binding should be rapid and reversible; it should be saturable indicating a finite number of binding sites; β -AR agonists and antagonists should compete for the binding sites in a manner that closely parallels the known specificity of physiological β -adrenergic responses and (-)stereoisomers of adrenergic agonists and antagonists should be considerably more potent than the (+)stereoisomers, reflecting the stereospecificity observed in physiological β -adrenergic responses. It was also important to investigate whether the process of transformation of the lymphocytes to lymphoblasts had affected the binding characteristics. The cell cycle of the lymphoblast is made up of the preconfluent phase which is an exponential growth phase, when the cells are rapidly dividing, and the confluent phase when the maximal cell density of about $1-2 \times 10^6$ cells/ml has been reached. In order to ensure that repeated assays on an individual cell line would yield consistent results the binding characteristics were investigated at different stages of the cell cycle. The stability of the binding characteristics over long periods of culture and after freezing and storing in liquid nitrogen were also studied.

As the lymphocyte β -AR is coupled to the adenylyl cyclase system, any investigation into β -AR function in the lymphoblast must include assessment in terms of adenylyl cyclase stimulation. The basal and stimulated levels of cAMP were determined for lymphoblast cell lines for a range of agonists.

3.2 Methods

3.2.1 Binding characteristics

Association and dissociation time courses were constructed to establish the binding kinetics. Lymphoblast membrane homogenate was prepared as described in Chapter Two. For the association time course a single concentration of [^{125} I]-CYP of 60pM was used and the tissue and radioligand were incubated in triplicate at 37°C for 10-180 min. For the dissociation time course free concentration of [^{125} I]-CYP was 30pM and the tubes were incubated for 60 min and then dissociation was studied over the next 10-150 min. Specific binding was defined by 1 μ M CGP 12177.

3.2.2 Displacement studies

Displacement of specific binding of [^{125} I]-CYP by various compounds was investigated to identify the receptor type. The protocol for the displacement studies was as described in Chapter Two. The agonists and antagonists used as inhibitors and their respective concentrations were as listed in Table 3.1. (-)Isoprenaline was used both alone and in combination with either 10 μ M GTP or 10 μ M GppNHp. Analysis of the data was carried out using the EBDA program as described in the previous chapter.

3.2.3 Comparison of lymphocyte and lymphoblast binding characteristics

20 ml blood samples were taken from 7 subjects whose lymphocytes had been successfully transformed to lymphoblastoid cell lines. Lymphocytes were isolated from heparinised blood as previously described. The cell pellet obtained after washing the lymphocyte layer was resuspended in hypotonic medium and the cell density counted. The lymphocytes were then prepared exactly as for harvested lymphoblast cells and the radioligand binding assay carried out.

Table 3.1 Compounds used to displace specific binding of [¹²⁵I]-CYP to lymphoblast membrane preparations

Inhibitor	Concentration (nM)
CGP 12177	0.1 - 10000
l-propranolol	0.1 - 1000
d-propranolol	0.1 - 10000
ICI 118551	0.1 - 1000
Oxprenolol	1 - 10000
(-)Isoprenaline	10 - 100000
(-)Isoprenaline + GTP (10 μ M)	10 - 1000000
(-)Isoprenaline + GppNHp (10 μ M)	10 - 100000
(+)Isoprenaline	10 - 100000
CGP 20712	1 - 10000
* Phentolamine	1000
* Atropine	1000
* Ketanserin	1000
* Haloperidol	1000
Practolol	1 - 10000
Atenolol	1 - 10000
Adrenaline	10 - 100000
Fenoterol	10 - 100000
Isoxsuprine	10 - 100000
Noradrenaline	10 - 100000
* Dopamine	10000
* 5-HT	10000

* A single concentration only was used.

3.2.4 Reproducibility

To investigate whether the binding characteristics varied with the stage of the cell cycle, confluent cell cultures were subcultured down to a cell density of about 0.3×10^6 cells/ml and then assayed 1, 2, 3 and 7 days after subculture. Inter-assay and intra-assay variation of the binding characteristics were also studied. Repeated radioligand binding assays on individual cell lines from 7 subjects were carried out weekly for 4-12 weeks. In three subjects blood samples were taken and the lymphocytes transformed on three separate occasions and radioligand binding studies were carried out on these cell lines. This was to ensure that the β -AR characteristics of a cell line were consistent whenever the cell line was transformed and so were determined by genetic factors. Repeated

analyses were also carried out on 3 subjects on 6 occasions over a period of 9 months of continuous culture. Cell lines were also assayed before and after freezing and storage in liquid nitrogen. It was expected that in some circumstances it would be necessary to freeze cell homogenates before assaying, so 4 cell lines were assayed using fresh and frozen homogenates in parallel. Cells were harvested and homogenised, and centrifuged twice as usual, but the final pellet was frozen at -20°C before the final resuspension step. A week later cells from the same cell line were harvested and the membrane homogenate prepared as normal. The frozen membrane pellet was thawed and resuspended in incubation medium and the radioligand binding assay was carried out on both tissue preparations.

3.2.5 Adenylyl cyclase assay

Initially the formation of [^{32}P]-cAMP from [$\alpha^{32}\text{P}$]-ATP was measured in A431 cells using Salomon's method to ascertain if the separation methods were working. Two 75cm³ flasks of confluent A431 cells were harvested and the cell pellet was frozen and stored at -20°C until use. When required the cells were thawed and then assayed as described. The assay was then repeated on a lymphoblast cell line with a high β -AR capacity. The assay was first carried out on cells that had been frozen as the A431 cells had been, but the assay was then repeated with fresh cells. In both cases three flasks containing 30ml of confluent cells were harvested. A further assay was carried out with a different method of preparing the tissue. As opposed to homogenization, a permeabilized cell preparation was used in order to minimize perturbation of the system. This has been shown in lymphocytes to result in a significant increase in isoprenaline stimulated adenylyl cyclase activity (Feldman, 1985 & 1989). The pellet was resuspended in 30ml TME buffer which included 10 $\mu\text{g/ml}$ digitonin to permeabilize the cell membrane. This was incubated for 15 min at 4°C and then centrifuged at 400g for 10 min at 4°C. The pellet was then resuspended in 30ml TME buffer alone and centrifuged at 400g for 10 min at 4°C to wash off the digitonin. The cells were maintained at 4°C during the permeabilization to inhibit

ex vivo alterations in lymphoblast β -AR properties (De Blasi *et al.*, 1986). The pellet was resuspended in 1.5ml TME and assayed as for broken cell preparations.

The second method of assaying for adenylyl cyclase activity (using the Amersham cAMP assay kit) was then tried, initially running it with A431 and lymphoblast cells concurrently, but afterwards with lymphoblast cells only. Cells were harvested from one 30cm³ flask of confluent A431 cells and two flasks of 30ml confluent lymphoblast cells. As a trial run the adenylyl cyclase was stimulated by 10⁻⁵M isoprenaline, 6 x 10⁻⁵M forskolin, 10⁻⁶M prostaglandin E₁ (PGE₁) (concentrations shown by Ebstein *et al.* (1984, 1985) to be maximally stimulating in lymphocytes under similar assay conditions) and 10⁻³M NaF and the cell suspensions and agonists were incubated for 15 min at 37°C. Time and dose response curves were then carried out for all the agonists, with incubation times of 5, 10 and 15 min being used. The agonist concentrations used were as follows: isoprenaline 10⁻⁹-10⁻⁴ M; Forskolin 6 x 10⁻⁶-10⁻⁴ M; PGE₁ 10⁻⁹-10⁻⁶ M and NaF 10⁻⁴-10⁻² M.

3.3 Results

3.3.1 Binding characteristics

Incubation of lymphoblast membranes with 60pM [¹²⁵I]-CYP at 37°C for 10-180 min showed specific binding to be saturable with respect to time (Fig. 3.1), with an association half time, $t_{1/2}$, of 23 min. A routine incubation time of 90 min was therefore adopted, which was 4 x $t_{1/2}$, by which time approximately 95% of equilibrium binding would have been acquired. Dissociation was very slow, with only slight dissociation in the 150 min time course studied.

Specific binding of [¹²⁵I]-CYP to lymphoblast membranes was saturable in the range 5-100 pmol/l and Scatchard analysis of the data closely approximated a straight line, indicating the presence of a single binding site (Fig. 3.2).

Fig. 3.1

Association time curve:-

Specific binding of 60pM [^{125} I]-CYP to lymphoblast membranes against time. Each point represents the mean $\pm$ sem from triplicate estimates in a single cell line.

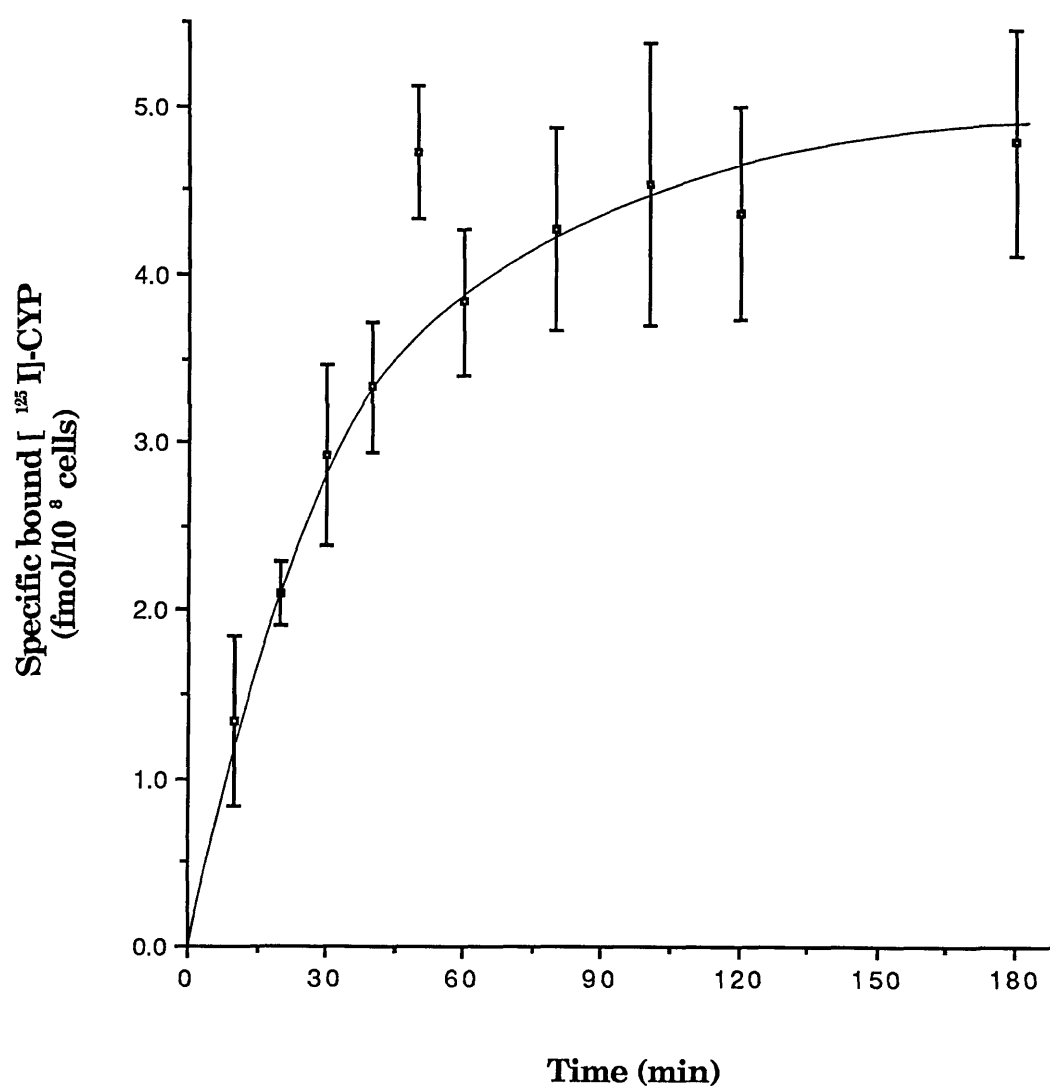
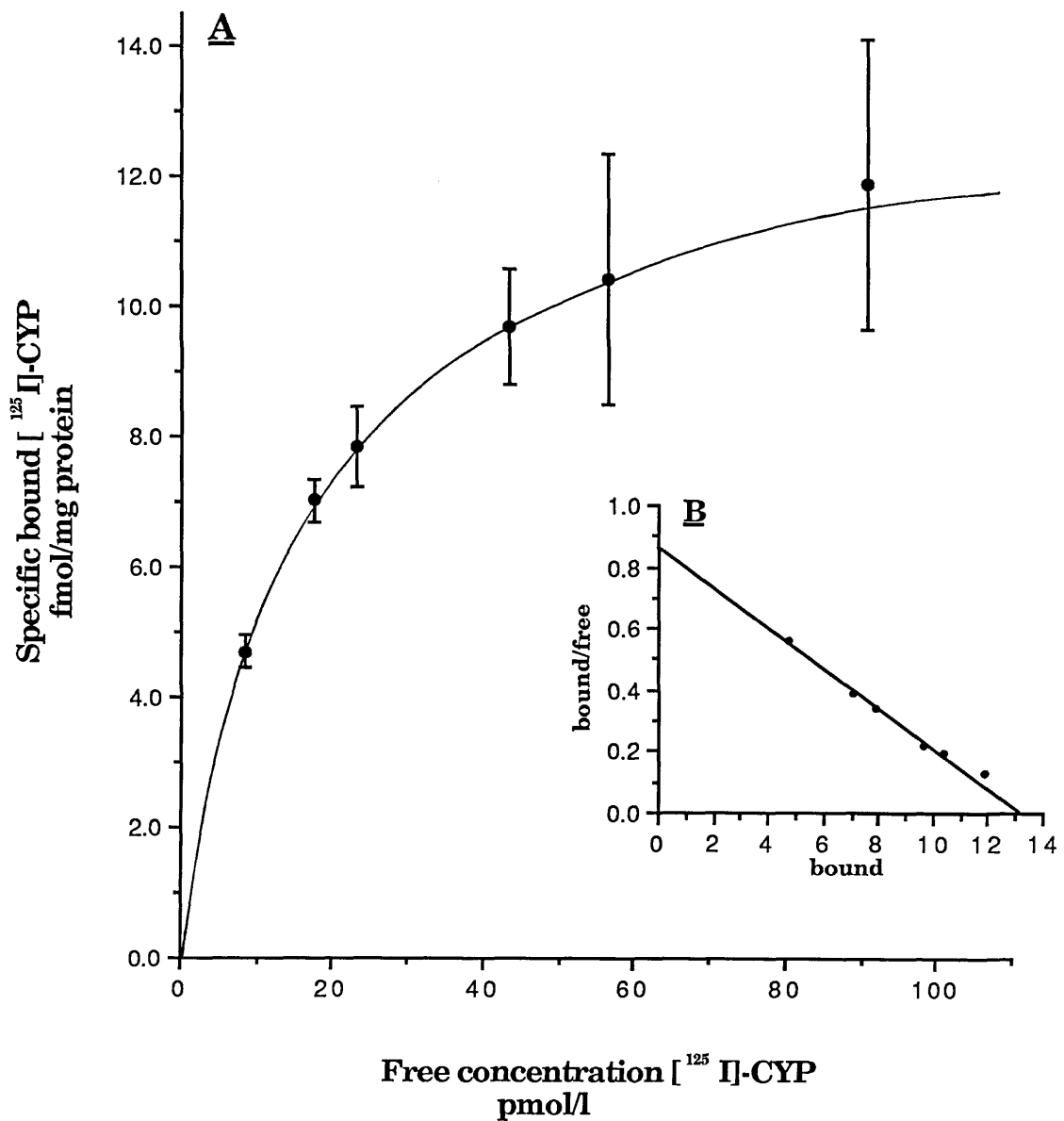


Fig. 3.2 **A** Specific binding of [^{125} I]-CYP to lymphoblast membranes. Each point represents the mean $\pm$ sem from triplicate estimates in a single cell line.

B Scatchard plot of the data.



Bmax = 13.15 fmol/mg protein
Kd = 15.26 pM
r = 0.972

3.3.2 Displacement studies

Displacement of specific binding of [^{125} I]-CYP by various compounds demonstrated an inhibition profile typical of the β -AR (Table 3.2 and Fig. 3.3). Substantial differences were observed in the potencies of the stereoisomers of propranolol and isoprenaline, the biologically active enantiomer being the more potent inhibitor of binding. Within the group of β -AR antagonists, compounds with selective affinity for the β_1 subtype (CGP 20712, atenolol and practolol) were less potent than non-selective compounds (propranolol and CGP 12177) or the β_2 selective compound, ICI 118551 (Fig. 3.3A). This indicates that the β -AR on the lymphoblast cell is of the β_2 type, similar to that reported on the parent lymphocyte cells (Brodde *et al.*, 1981).

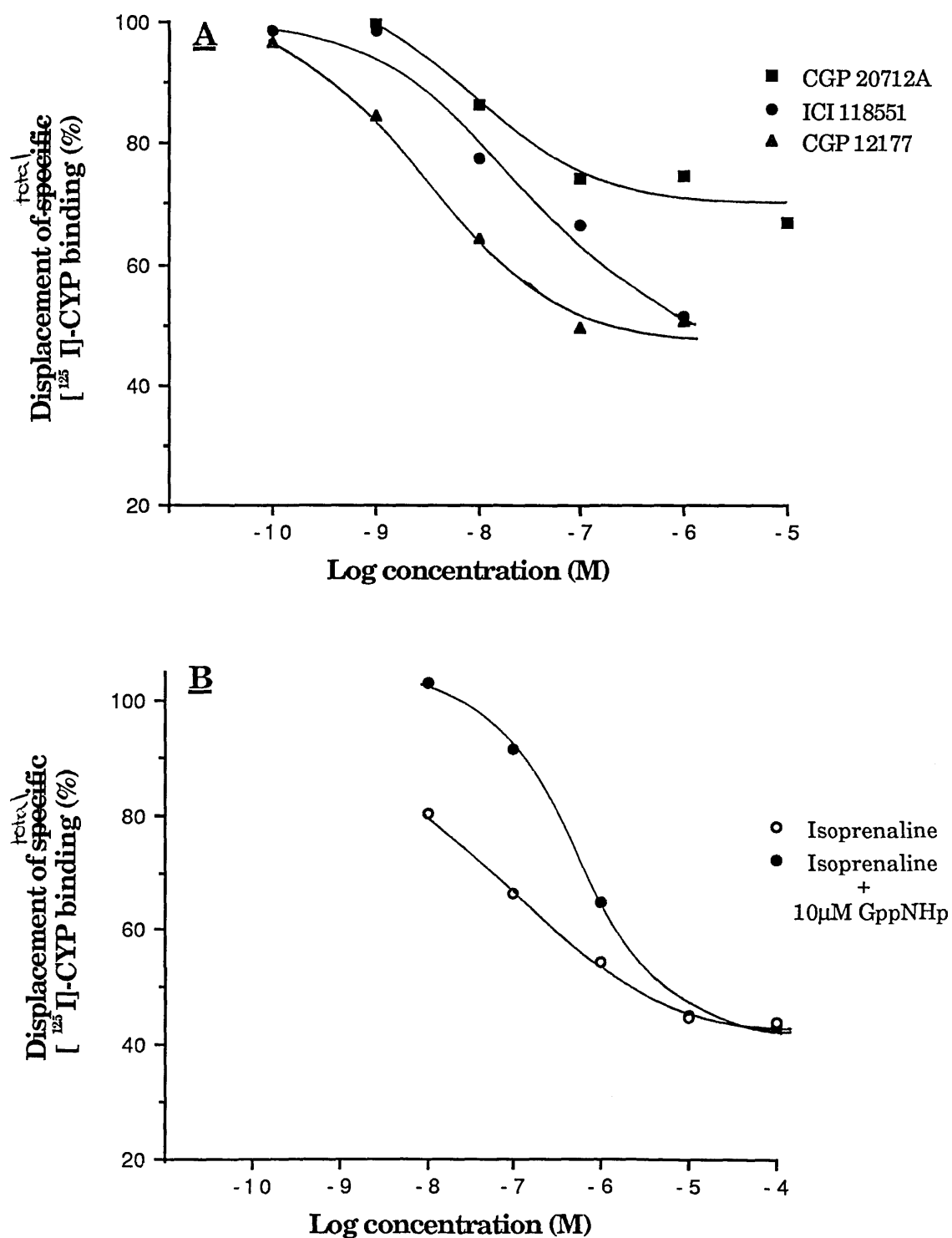
Displacement of the radiolabel by antagonists occurred with Hill coefficients close to unity, whereas displacement by agonists resulted in Hill coefficients significantly less than unity. In the case of isoprenaline, addition of GTP or GppNHp resulted in an increase in the IC_{50} value and in the Hill coefficient, suggesting coupling of the receptor to nucleotide binding proteins associated with adenylyl cyclase (Fig. 3.3B).

Table 3.2 Inhibition profile for displacement of specific binding of [^{125} I]-CYP to cultured lymphoblast cells. Results are expressed as IC₅₀ (nmol/l) and represent means of 2 - 7 individual estimations.

Inhibitor	IC ₅₀
CGP 12177	3
l-propranolol	2
d-propranolol	334
Oxprenolol	14
ICI 118551	36
(-)Isoprenaline	59
(-)Isoprenaline + GTP (10 μ M)	232
(-)Isoprenaline + GppNHp (10 μ M)	634
(+)Isoprenaline	33000
CGP 20712	>1000
* Phentolamine	>1000
* Atropine	>1000
* Ketanserin	>1000
* Haloperidol	>1000
Practolol	1418
Atenolol	1605
Adrenaline	3685
Fenoterol	3693
Isoxsuprine	6510
Noradrenaline	7500
* Dopamine	>10000
* 5-HT	>10000

* A single concentration only was used

Fig. 3.3 Displacement of specific [^{125} I]-CYP binding to lymphoblast membrane by the β -AR antagonists CGP 12177, ICI 118551 and CGP 20712A (A) and by isoprenaline, with and without 10 μ M GppNHp (B). Each point represents the mean $\pm$ sem from triplicate estimates in a single cell line.



3.3.3 Comparison of lymphocyte and lymphoblast binding characteristics

The results of the comparison of the binding characteristics of [^{125} I]-CYP on isolated lymphocyte preparations and on lymphoblast preparations from cell lines grown from the same individuals are shown in Table 3.3. Comparison of the meaned data showed no significant difference in the binding affinity (Kd) but significantly lower binding capacity (Bmax) in the lymphoblast cells whether expressed in terms of protein content or cell number. Correlation between the individual binding capacity in lymphocytes and in lymphoblasts was statistically significant when expressed in terms of cell number ($r = 0.91$, $p < 0.05$), but not in terms of protein content.

Table 3.3 Binding characteristics of [^{125} I]-CYP on membranes from cultured lymphoblasts and parent lymphocytes. Results are expressed as mean $\pm$ sem (n = 7).

Binding characteristics	LYMPHOCYTE	LYMPHOBLAST
Kd (pM)	13.3 $\pm$ 0.7	14.9 $\pm$ 0.6
Bmax (fmol/mg protein)	78.0 $\pm$ 12.1	5.7 $\pm$ 0.3
Bmax (sites/cell)	444 $\pm$ 55	39 $\pm$ 4

3.3.4 Reproducibility

Cell density increased with time over the 7 day period studied, with the growth curve showing a typical exponential form (Fig. 3.4). When expressed in terms of receptor sites/cell, the binding capacity increased during the preconfluent phase of rapid growth, and then returned to the initial levels by day 7. However, when measured in terms of receptors/mg protein, the binding capacity showed little change throughout the cell cycle. This suggested a gradual increase in cell size during growth, which was supported by calculation of the cell protein content which mirrored the binding capacity identified in terms of receptor sites/cell.

Fig. 3.4 Lymphoblast cell density in a single cell line measured for a seven day period after subculturing. Table 3.4 shows the results for one cell line. These results indicated that calculation of receptor density with respect to protein content was less prone to variation than that expressed in terms of sites/cell.

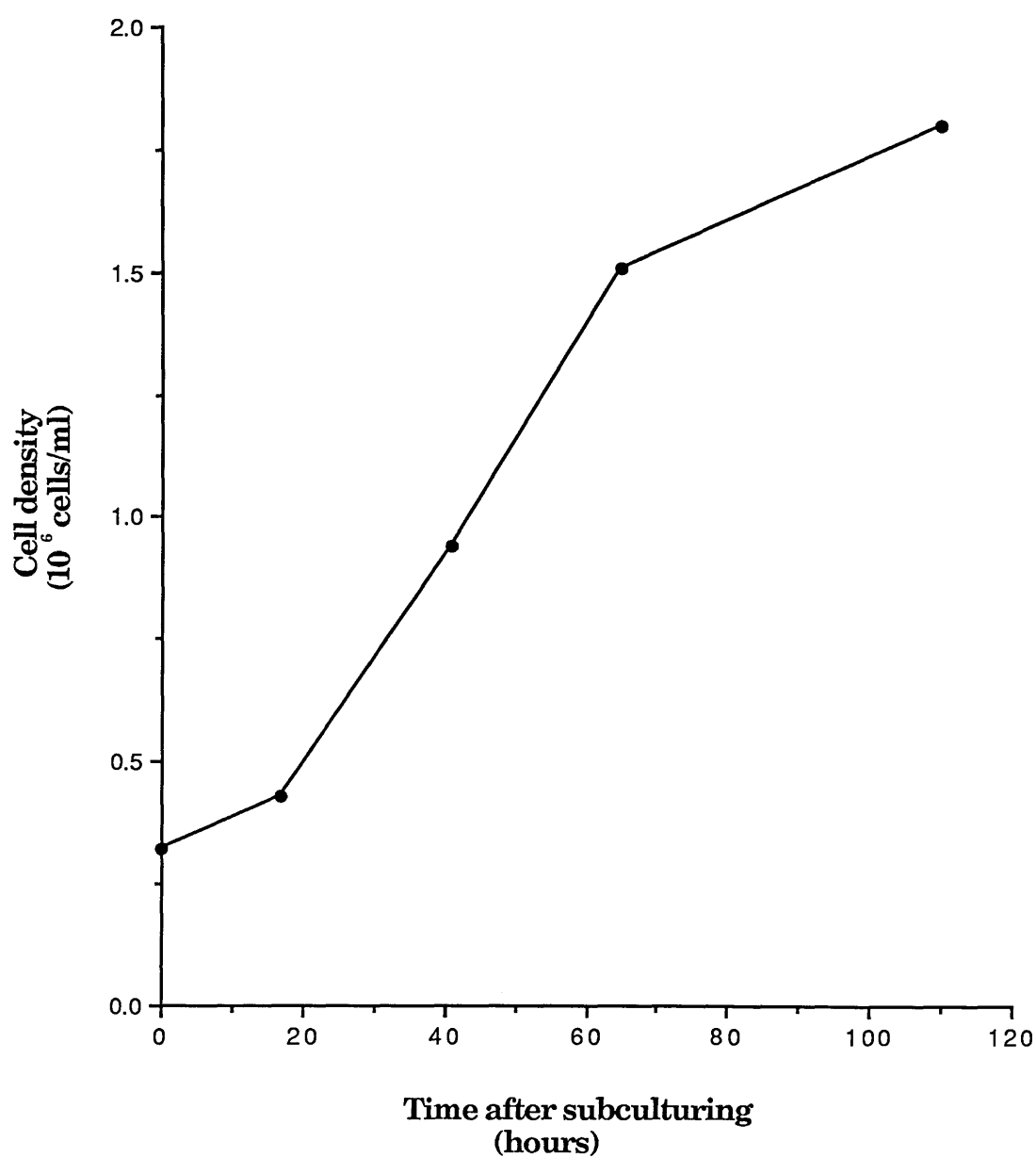


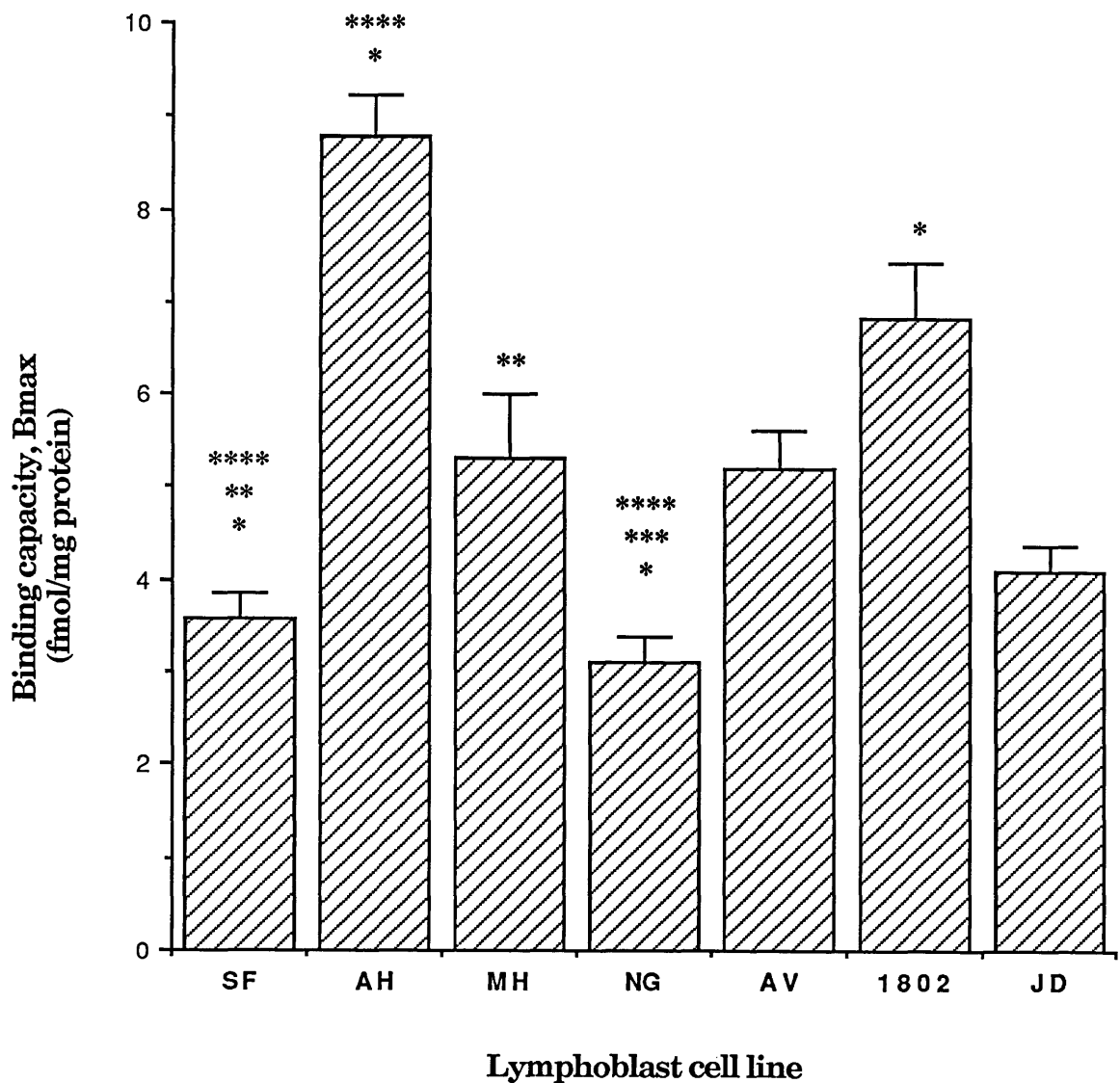
Table 3.4 Cell density and β -AR density on an individual lymphoblast cell line over a 7 day period.

	Days After Subculturing				
	0	1	2	3	7
Cell density (10^6 cells/ml)	0.3	0.4	0.9	1.5	2.1
Bmax - fmol/ 10^8 cells	-	19.1	7.3	50.1	17.4
fmol/mg protein	-	7.1	6.0	9.8	7.6
Cell Size (mg protein/ 10^8 cells)	-	2.7	4.6	5.1	2.3
Kd (pM)	-	21.7	22.0	19.2	21.4

Repeated binding studies on 7 cell lines over 4-12 weeks demonstrated individual coefficients of variation of 5-13 % (mean 8.2 ± 0.9) for Bmax values and 7-19 % for Kd values. Significant variation in Bmax values was observed between individuals, when tested by Student's unpaired t test, with values ranging from 3.1-8.8 fmol/mg protein (mean 5.3 ± 0.8 fmol/mg protein), whereas there were no significant differences in Kd values (mean Kd value, 15.7 ± 1.0 pmol/l). The differences in receptor density in different cell lines can be seen clearly in Fig. 3.5.

In the three cell lines that were established on three separate occasions the differences in binding characteristics between cells transformed on each occasion were no greater than the normal inter-assay variation, and the differences between different individuals were maintained. Repeated analyses of [^{125} I]-CYP binding in three subjects on six occasions over a nine month period of continuous culture revealed no significant changes in binding capacity or affinity with time (coefficients of variation for the three cell lines were 12%, 7% and 10% for the Bmax values and 12%, 12% and 6% for the Kd values; $r < 0.34$ for both Bmax and Kd values against time for all three cell lines).

Fig. 3.5 Binding capacity (Bmax) in 7 lymphoblast cell lines. Results are expressed as the mean $\pm$ sem of 4-16 experiments.



* p<0.05 SF v. AV; AH v. 1802; JD v. NG, 1802;
 ** p<0.01 MH v. SF, NG;
 *** p<0.005 NG v. AV;
 **** p<0.001 SF v. AH, 1802; AH v. MH, AV, JD;
 NGv1802.

Similarly, freezing and storage of the cells in liquid nitrogen for three months did not effect the binding characteristics when tested after recovery of the cells in culture. Freezing the membrane homogenate before carrying out the binding assay also had no significant effect on the Bmax, the Kd or the protein content of the tissue when tested by Students' unpaired or paired t test (Table 3.5).

Table 3.5 Comparison of Bmax and Kd values in fresh and frozen tissue. Results are expressed as the mean $\pm$ sem for 4 cell lines.

	FRESH TISSUE	FROZEN TISSUE
Tissue Content (mg protein/ml)	0.21 $\pm$ 0.05	0.21 $\pm$ 0.03
Bmax (fmol/mg protein)	5.97 $\pm$ 1.22	6.47 $\pm$ 1.36
Kd (pM)	19.04 $\pm$ 2.10	19.85 $\pm$ 1.03

3.3.5 Adenylyl cyclase assay

In the initial adenylyl cyclase assay on A431 cells using Salomon's method, the cell homogenate had a basal activity of 56.3pmol [^{32} P]-cAMP/mg protein/min. Stimulation of the A431 adenylyl cyclase was induced by forskolin, isoprenaline and NaF in that order of potency (Table 3.6). The enzyme activity induced by 10 μ M isoprenaline was 331.5pmol/mg protein/min which was a stimulation of 489%. As the separation techniques were working efficiently, the experiment was repeated using a lymphoblast cell line with a high binding capacity for [^{125} I-CYP] of 8.8 fmol/mg protein. Although there was a measurable basal enzyme activity of 5.23pmol/mg protein/min, neither isoprenaline nor NaF induced any stimulation of the enzyme and 10 μ M forskolin caused only slight stimulation (44%) (Table 3.6). The assay was then repeated twice using cells immediately after harvesting. In neither experiment was any stimulation induced by forskolin, isoprenaline or NaF. When digitonin was used to permeabilize the cell membranes instead of

homogenization, similar results were obtained, though there was limited stimulation with 10 μ M isoprenaline (Table 3.6).

As Salomon's method seemed to be unsuitable for use in lymphoblast cells, estimation of adenylyl cyclase activity was attempted using the Amersham radioimmunoassay kit. The first attempt was run concurrently with A431 cells and lymphoblasts to ensure that the experimental technique was working properly. Isoprenaline, forskolin and PGE₁ all stimulated the lymphoblast adenylyl cyclase to produce increased amounts of cAMP over the basal level. 15 min incubation with 10 μ M isoprenaline caused a two-fold increase, with PGE₁ an eleven-fold and with forskolin a three-fold increase in cAMP levels over basal (Table 3.6). The basal level of cAMP in A431 cells was about 10 times that of the lymphoblast cells. As the assay gave satisfactory results with the lymphoblasts it was not necessary to continue with the A431 cell line.

Table 3.6 Adenylyl cyclase stimulation as a % increase over basal level in A431 cells and lymphoblast cells assayed either by Salomon's method or the Amersham cAMP assay kit.

	Forskolin		Isoprenaline		NaF	PGE ₁
	10nM	10 μ M	10nM	10 μ M	1mM	1 μ M
<u>Salomon method</u>						
frozen A431 cells	12	867	8	489	18	—
frozen lymphoblasts	0	44	0	3	2	—
fresh lymphoblasts	4	0	0	0	0	—
*fresh lymphoblasts	24	0	10	45	45	—
<u>Amersham kit</u>						
fresh lymphoblasts	231**		—	118	0	1006

* permeabilized membranes

** 60 μ M forskolin

Adenylyl cyclase stimulation by all four agonists was concentration and time dependent (Figs. 3.6 & 3.7). The maximal response to isoprenaline, a six-fold increase in cAMP compared to basal, was achieved at a concentration of 10⁻⁵M and a 10 min incubation time. However, the amount of stimulation decreased

with 10^{-4} M isoprenaline, and with 15 min incubations the maximum adenylyl cyclase stimulation was reached at 10^{-6} M isoprenaline and was only two-fold. This suggested that desensitization of the β -AR coupled adenylyl cyclase response was occurring during the 15 min exposure to isoprenaline. The EC_{50} for a 10 min incubation with isoprenaline was 10^{-7} M.

Forskolin showed a similar pattern of stimulation, with adenylyl cyclase stimulation being greater after 10 min incubation with forskolin than after either 5 or 15 min incubation (Fig. 3.7A). 10^{-4} M forskolin was maximally stimulating and caused a five-fold increase in cAMP over basal.

Neither PGE_1 nor NaF caused desensitization of the adenylyl cyclase response at the concentrations and incubation times used. The maximum stimulations observed for 10 min incubations with PGE_1 and NaF were a 50-fold increase in cAMP levels over basal with 10^{-6} M PGE_1 and a 10-fold increase with 10^{-2} M NaF.

Fig. 3.6 Concentration dependent stimulation of cAMP accumulation in lymphoblasts by 10 min coincubation with isoprenaline, forskolin and NaF (A) and with PGE₁ (B).

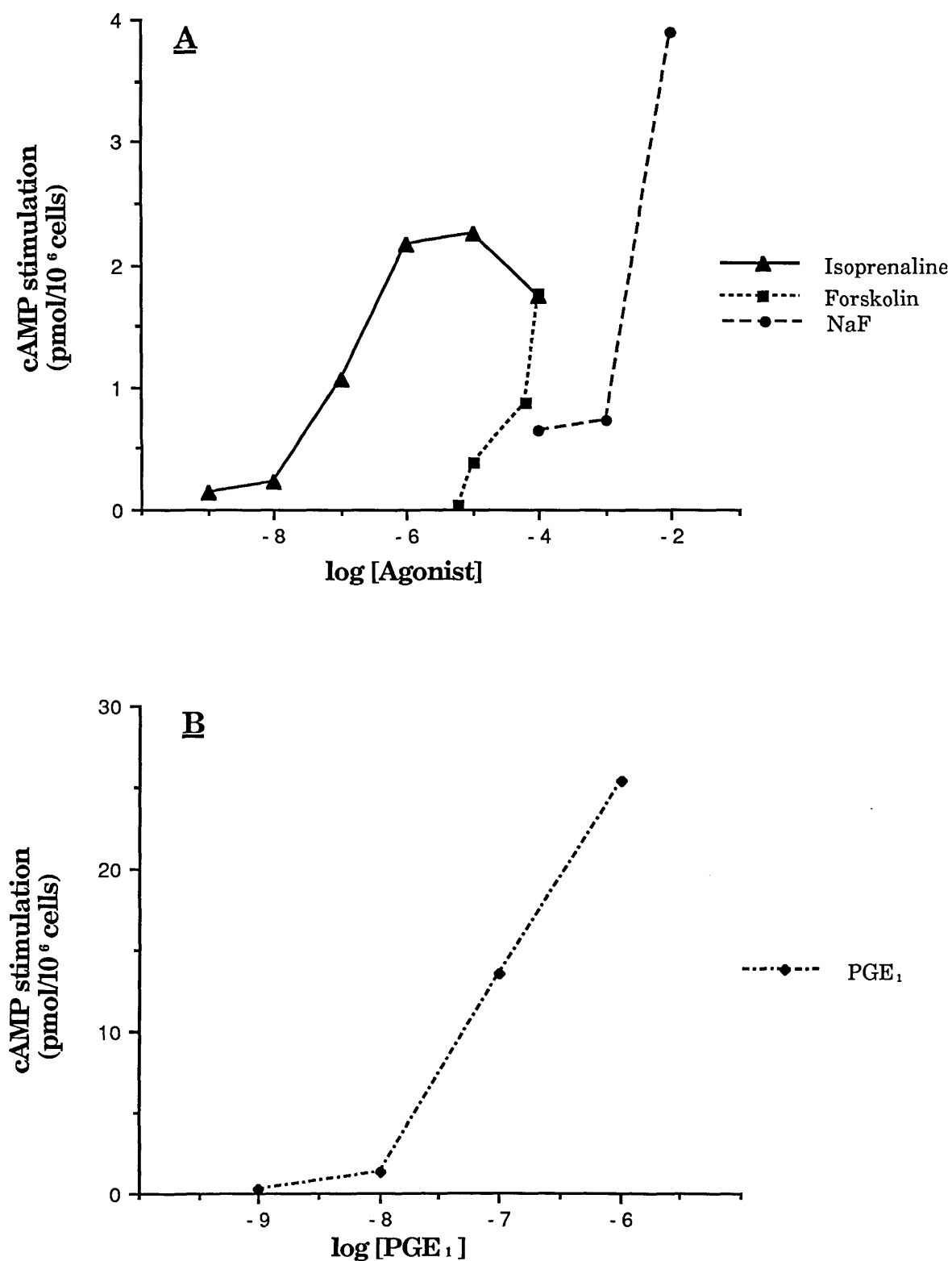
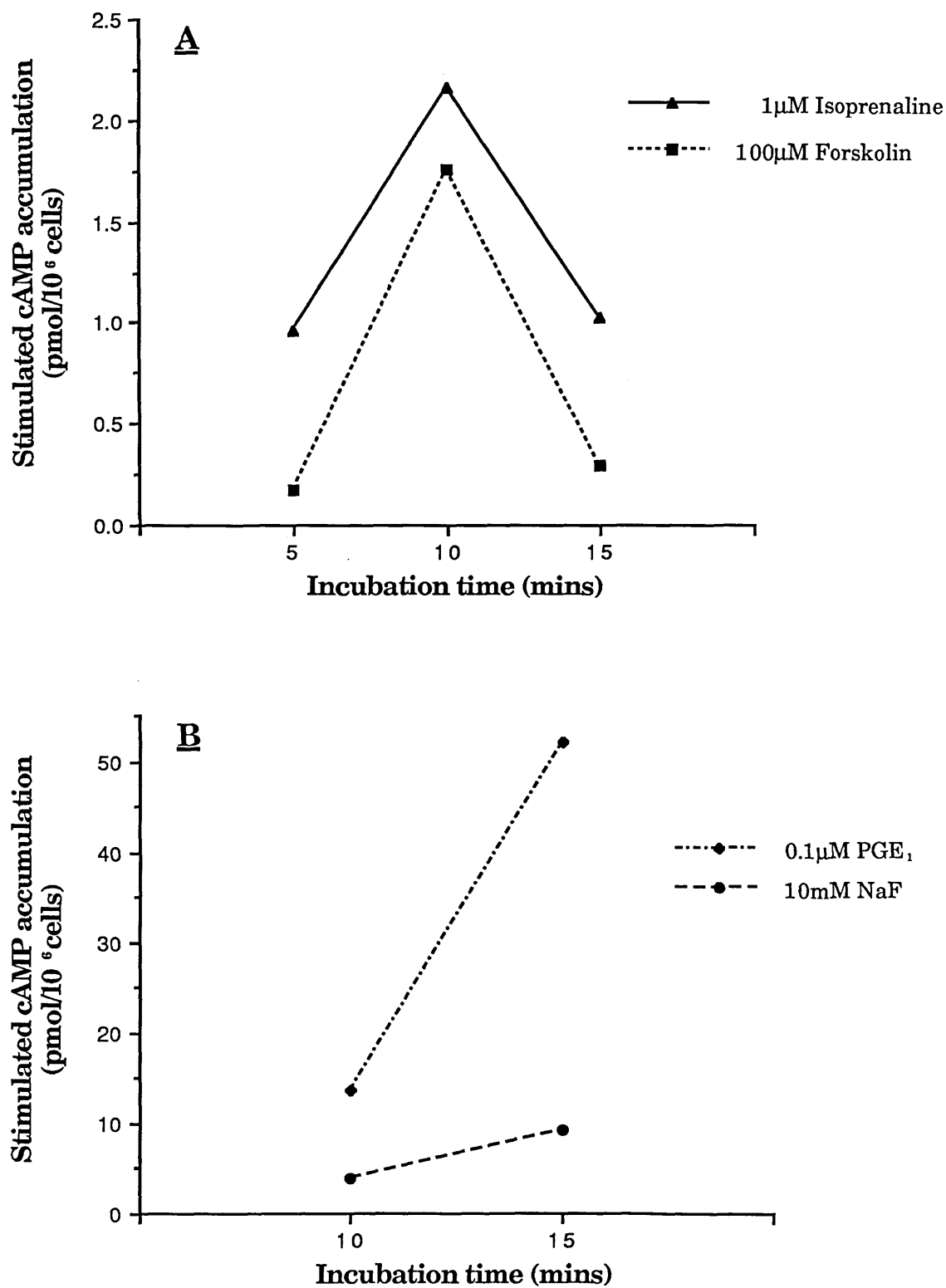


Fig. 3.7 Agonist-stimulated cAMP accumulation caused by 5, 10 and 15 min coincubation with isoprenaline and forskolin (A) and by 10 and 15 min coincubation with PGE₁ and NaF (B).



3.4 Discussion

Specific binding of [^{125} I]-CYP to lymphoblast membrane homogenates was rapid ($t_{1/2} = 23$ min) and saturable in the concentration range used (5-100 pM). Dissociation was slow, which attributed to the high affinity (K_d 20pM, approximately). Displacement of specific binding of [^{125} I]-CYP demonstrated an inhibition profile typical of the β -AR with an order of potency of: ICI 118551 > CGP 12177 = l-propranolol > atenolol > CGP 20712, which indicated the receptor was of the β_2 type. These results were in accordance with those for the lymphocyte β -AR using ($\pm$)[^{125} I]-CYP (Brodde *et al.*, 1981) or (-)[^{3} H]-Alprenolol (Williams *et al.*, 1976). There was a very good correlation between the IC_{50} values obtained in this study and both those seen by Brodde *et al.* and those seen by Williams *et al.* ($r = 0.998$ and 0.922 respectively).

The binding characteristics were qualitatively identical to the parent lymphocyte, with no change in binding affinity, but the receptor density was lower in the transformed lymphoblasts than in circulating cells as described by Ebstein *et al.* (1985) in a smaller study which also employed [^{125} I]-CYP as the radioligand. The data for binding capacity given by Ebstein *et al.* were not directly comparable to those of this study as the B_{max} values were expressed as fmol/ 10^6 cells. The binding capacity observed in this study of 5.3 ± 0.8 fmol/mg protein compared well to the published values of Wright *et al.* (1984) and Berrettini *et al.* (1987) of 6.8 ± 0.6 fmol/mg protein and 3.7 ± 1.4 fmol/mg protein, respectively. Ebstein's group also found that the binding affinity was reduced in lymphoblasts to 10.9pM compared to 17.6pM in lymphocytes, but these results were only from four cell lines and details of statistical significance were not given. The binding affinity seen in the present study, 15.7 ± 1.0 pM (7 cell lines), was closer to that observed by Ebstein for lymphocytes than for lymphoblasts, and was in accordance to that seen by Berrettini *et al.* (1987). Wright's group used a different radioligand, ([^{125} I]-iodohydroxybenzylpindolol. The similarity of both the inhibition profile and the binding affinity of lymphoblasts and of lymphocytes would seem to indicate that the β -AR itself is not altered by transformation, even though the receptor density is markedly reduced. Reduction in β -AR density leads to a reduction in isoprenaline-stimulated cAMP accumulation (Ebstein *et al.*, 1985 and the following

chapter). Cyclic nucleotides play an important role in many cell processes including cell division (Friedman *et al.*, 1976) and a number of agents that raise intracellular cAMP levels have been shown to inhibit immunological function and mitogenesis in lymphocytes (Muraguchu *et al.*, 1984). It may be that one stage in the transformation of lymphocytes to immortal cells is the reduced ability of the EBV-transformed cells to respond to hormones that stimulate cAMP synthesis.

Although lymphoblasts vary both in growth rate and the number of receptors/cell depending on the stage of the cell cycle, the binding capacity remained consistent when expressed in terms of receptors/mg protein. Davies and Lefkowitz (1980) also came to the conclusion that β -AR density in lymphocytes should be expressed in terms of protein content since this form of expression had a lower coefficient of variation than that for sites/cell. As the receptor density on lymphoblasts was relatively low, it was decided that cells should be harvested for biochemical assay when the cultures were confluent so that the high cell density would ensure that there would be sufficient cells for an accurate determination of Bmax values. Repeated studies of individual cell lines demonstrated consistent inter-assay binding characteristics but significant differences in the Bmax values between subjects. These differences were not gender-related as 6/7 of the cell lines were derived from male subjects. The reproducibility was maintained after freezing and storage of cell lines in liquid nitrogen, and after prolonged periods in continuous culture. The significant differences between subjects were also preserved in multiple cell lines derived from the same individual, indicating that the lymphoblast β -AR number in normal culture was dictated predominately by genetic factors.

Displacement of specific binding by agonists resulted in Hill coefficients significantly less than unity, indicating the presence of high and low affinity state binding sites. As the addition of either GTP or GppNHp resulted in an increase in the IC₅₀ and the Hill coefficient, it was evident that the lymphoblast β -AR was coupled to a guanine-nucleotide binding protein, which is an integral part of the β -AR/adenylyl cyclase complex.

Although many workers have investigated adenylyl cyclase stimulation in lymphocytes using the Salomon method, this method was found to be unsuccessful in lymphoblasts. This possibly was due in part to the low level of activity of adenylyl cyclase in lymphoblasts. However, as there was a measurable basal level of adenylyl cyclase activity but no induction of stimulation by agonists, it may also have been the result of a disruption of coupling between the GTP-binding proteins and the receptor and/or the adenylyl cyclase caused by the preparation of the membranes. The only other group to study the adenylyl cyclase complex in cultured lymphoblasts used the radioimmunoassay method (Ebstein *et al.*, 1985). Following an adaptation of Ebstein's method yielded satisfactory results. Isoprenaline caused time and concentration dependent stimulation of adenylyl cyclase confirming the presence of an adenylyl cyclase coupled β -AR. The increase in cAMP levels over basal caused by 10 μ M isoprenaline was similar to that seen by Ebstein. cAMP production was also increased in a time and concentration dependent manner by PGE₁, forskolin and NaF. Desensitization of the adenylyl cyclase response as detailed in the next chapter was seen with isoprenaline and forskolin at high concentrations and 15 min exposure time. An incubation time of 10 min was selected for further studies.

CHAPTER FOUR

DOWN-REGULATION AND DESENSITIZATION OF THE β -AR IN RESPONSE TO ISOPRENALINE

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4.1 Introduction

Down-regulation and desensitization of mammalian adrenergic receptors in response to agonists *in vitro* has been studied in several tissues, such as brain slices (Nukina & Labella, 1989), platelets (Cook *et al.*, 1987), reticulocytes (Yamashita *et al.*, 1987), astrocytoma cells (Toews *et al.*, 1987), fibroblasts (Berrettini *et al.*, 1987) and lymphocytes (Sandnes *et al.*, 1987). Most *in vitro* studies into the concentration and time dependency of the down-regulation and desensitization phenomena have been limited to acute studies by the survival time of the tissue.

Sandnes' group found that coincubation of intact human lymphocytes *in vitro* with isoprenaline caused β -AR down-regulation. The extent of the down-regulation depended on the concentration of isoprenaline and the duration of the coincubation. The coincubation time was only investigated for up to 4 hours, and the standard conditions adopted for the rest of the study of down-regulation and desensitization were coincubations with 1 μ M isoprenaline for 10 min. Acute studies such as this have a limited application to receptor down-regulation *in vivo*, which presumably involves very low concentrations of agonists and chronic exposure.

In order to investigate down-regulation in the lymphoblast in response to isoprenaline, coincubation times of 4 hours, 24 hours and 7 days were employed using isoprenaline concentrations ranging from 0.1nM to 10 μ M. The effects of changes in various parameters, such as the phase of the cell cycle and the length of time in continuous culture, were studied in order to optimise the protocol for isoprenaline coincubation. Inter-assay reproducibility within cell lines and individual sensitivity to isoprenaline down-regulation between different cell lines were investigated. The functional relevance of the β -AR down-regulation was investigated in terms of desensitization of adenylyl cyclase activity in response to isoprenaline coincubation for 4 hours, 24 hours and 7 days. Desensitization was determined by examining the β -AR coupled adenylyl cyclase response, the prostaglandin receptor coupled adenylyl cyclase, and direct stimulation of the

adenylyl cyclase by forskolin and NaF, so that it could be determined whether desensitization was homologous or heterologous.

4.2 Methods

4.2.1 Down-regulation of β -ARs on lymphoblast cells

Down-regulation of the β -AR in response to isoprenaline was investigated in 5 lymphoblast cell lines. RPMI 1640 medium containing isoprenaline (10^{-9} - 10^{-4} M) and ascorbic acid (10^{-3} or 10^{-2} M) was added to 3 flasks containing 30ml of confluent cells to bring the volume to 40ml and to give a final isoprenaline concentration of 10^{-10} - 10^{-5} M and a final ascorbic acid concentration of 10^{-4} or 10^{-3} M. As usual, all flasks were supplemented with 10% FCS. The ascorbic acid was included to inhibit oxidation of the isoprenaline. Cell lines were concurrently coincubated with 10^{-4} or 10^{-3} M ascorbic acid alone as controls and treated exactly as for the cells coincubated with isoprenaline. The flasks were then incubated at 37°C for 4 hours, 24 hours or 7 days and then the β -AR binding assay carried out. To ensure that ascorbic acid itself did not affect β -AR binding untreated cells were compared to those that had been coincubated with 10^{-3} M ascorbic acid for 24 hours.

The effect of the stage of the cell cycle on the extent of down-regulation was investigated in one cell line. Lymphoblast cells were subcultured on day 0 and the cell density measured. On day 1 or day 5, 10^{-7} or 10^{-6} M isoprenaline and/or 10^{-3} M ascorbic acid were added to the cells. The cells were coincubated with isoprenaline for 24 hours and then after measuring the cell density, the cells were harvested and assayed (day 2 or day 6).

For 7 day coincubations, the cells in initial experiments were subcultured by a ratio of 1:3 on either day 3 or day 4 (isoprenaline and/or ascorbic acid were added on day 0) and assayed on day 7. As a certain amount of oxidation of the isoprenaline may still have been occurring, resulting in an underestimation of the extent of down-regulation due to a particular isoprenaline concentration, the effect of replacing the medium daily during 7 day coincubations was investigated in all

5 cell lines. 10^{-9} M isoprenaline and 10^{-3} M ascorbic acid were added to the flasks as usual. Each day the flasks were stood upright to allow the cells to settle down to the bottom of the flask and then 30ml of the medium was aspirated off, taking care not to remove any cells, and replaced with 30ml fresh medium, FCS, isoprenaline and/or ascorbic acid. In subsequent 7 day coincubations the cells were subcultured 2 days before treatment by 1:3, subcultured 1:2 on day 2 and then 20ml of medium was replaced on day 5.

Another way of estimating the effect of any reduction in the concentration of isoprenaline due to oxidation was to assay the cells after a 24 hour coincubation with isoprenaline followed by incubation for 24 hours in isoprenaline-free medium. If the 24 hours in isoprenaline-free medium allowed recovery of some or all of the down-regulated receptors, then oxidation of isoprenaline during 7 days of coincubation would surely also result in a reduction in the extent of down-regulation. The cells from three cell lines were coincubated with 10^{-9} M isoprenaline and/or 10^{-3} M ascorbic acid for 24 hours and then all the medium was replaced with fresh medium without isoprenaline or ascorbic acid by centrifuging the cells and medium, discarding the supernatant and resuspending the cells. After a further 24 hours incubation the cells were harvested and assayed.

In order to determine whether the number of weeks in culture had any effect on the extent of down-regulation, and to see how reproducible the results were, 7 day coincubations with 10^{-9} M isoprenaline were carried out on five cell lines at 8, 10 and 12 weeks after lymphocyte transformation. After treatment the cells were harvested, homogenised, sonicated and spun twice. The pellet was then stored at -20°C until assayed. The β -AR binding assays for each cell line were then performed on consecutive days.

Initially 10^{-3} M ascorbic acid was used to inhibit isoprenaline oxidation but it was noted that in the 7 day coincubations the cell growth rate was reduced both in control and isoprenaline treated cells. This was particularly evident when the medium was changed daily. In order to ascertain what was the minimum concentration of ascorbic acid to prevent oxidation of isoprenaline whilst having no effect on cell growth, the growth of one cell line in the presence of 10^{-5} - 10^{-3} M

ascorbic acid was examined over 7 days and compared to that in the absence of ascorbic acid and the extent of down-regulation in response to 10^{-9} M isoprenaline without ascorbic acid compared to that with 10^{-3} and 10^{-4} M ascorbic acid.

Concentration and time response curves were constructed for 10^{-10} - 10^{-5} M isoprenaline for 4 hours, 24 hours and 7 day incubations using five cell lines. Inter-assay and intra-assay reproducibility were examined.

4.2.2 Down-regulation of β -ARs on A431 cells

As a comparison to lymphoblast cells, dose and time response curves were constructed for the effect of isoprenaline incubation on the β -ARs on A431 cells. Isoprenaline in concentrations of 10^{-10} - 10^{-6} M was incubated with A431 cells for 4 hours, 24 hours and 7 days.

4.2.3 Desensitization of the lymphoblast adenylyl cyclase response

The adenylyl cyclase response in the lymphoblast in response to various agonists was examined after incubation with isoprenaline in order to assess the functional relevance of β -AR down-regulation. Cell lines from 8 subjects were incubated with isoprenaline (10^{-9} - 10^{-7} M) and/or ascorbic acid (10^{-3} M) for 4 hours, 24 hours or 7 days. Incubations were stopped by centrifugation and the cells washed to ensure that all the isoprenaline was removed by resuspending in 30ml RPMI 1640 medium and centrifuging for a second time. The cAMP assay was then carried out on the cells using the method adapted from Ebstein *et al.* (1985) as described in Chapters Two and Three. Adenylyl cyclase was stimulated by isoprenaline (10^{-7} and 10^{-5} M), PGE₁ (10^{-7} and 10^{-6} M), forskolin (10^{-4} M) and NaF (10^{-2} M). These concentrations were those that in the dose response study of Chapter Three were either maximally stimulating (isoprenaline 10^{-5} M, PGE₁ 10^{-6} M, forskolin 10^{-4} M and NaF 10^{-2} M) or caused half the maximal response (isoprenaline 10^{-7} M and PGE₁ 10^{-7} M).

4.3 Results

4.3.1 Down-regulation of β -ARs on lymphoblast cells - preliminary studies

In the preliminary experiment to ascertain whether 10^{-3} M ascorbic acid had any effect on β -AR binding or affinity and whether any loss in β -AR binding could be seen when lymphoblasts were coincubated with isoprenaline, the lymphoblasts were divided into three treatment groups: untreated, 10^{-3} M ascorbic acid alone and 10^{-3} M ascorbic acid plus 10^{-6} M isoprenaline. After a 24 hour coincubation there was a 78% loss of β -ARs in the isoprenaline treatment group (Bmax values:- no treatment 4.4fmol/mg protein; 10^{-6} M isoprenaline 1.0fmol/mg protein) whereas the cells treated with ascorbic acid alone showed no loss of receptors (Bmax 4.3fmol/mg protein). Neither coincubation with ascorbic acid nor with ascorbic acid and isoprenaline had any effect on the binding affinity (Kd values:- 23.7, 23.7 and 25.9 pM for no treatment, ascorbic acid alone and ascorbic acid plus isoprenaline, respectively).

Lymphoblast cells incubated in the presence of 10^{-3} M ascorbic acid initially showed a slower growth rate than cells grown in the presence of 10^{-4} and 10^{-5} M ascorbic acid or cells grown in the absence of ascorbic acid. Three days after addition of 10^{-3} M ascorbic acid the cell density had increased by 29%, whereas with 10^{-4} M ascorbic acid it had increased by 45%, with 10^{-5} M ascorbic acid it had increased by 54% and in untreated cells the cell density had increased by 66%. After 7 days coincubation the cell densities in cells treated with 10^{-4} and 10^{-5} M ascorbic acid were the same as the cell density of the untreated lymphoblasts, however 10^{-3} M ascorbic acid had actually caused lymphoblast cell death, and the cell density was less than half of that before treatment.

7 day coincubation with 10^{-9} M isoprenaline in the absence of ascorbic acid did not result in any β -AR down-regulation, even though the medium was changed twice. Reduction of the amount of ascorbic acid routinely used to prevent isoprenaline oxidation to 10^{-4} M had no effect on the extent of down-regulation.

The results of the study of isoprenaline down-regulation in preconfluent and confluent cells are shown in Table 4.1. There was no difference in the extent

of down-regulation between cells coincubated with isoprenaline on day 1 and assayed on day 2 when the cells were preconfluent and those coincubated with isoprenaline on day 5 and assayed on day 6 when the cell culture was confluent, despite the fact that the cell density on day 6 was almost twice that on day 2.

Table 4.1 Down-regulation in response to 24 hour coincubation with isoprenaline in preconfluent and confluent cells. Cells of one cell line were subcultured on day 0 to a cell density of 0.5×10^6 cells/ml.

[Isoprenaline]M	DAY 2			DAY 6		
	0	10^{-7}	10^{-6}	0	10^{-7}	10^{-6}
Cell density (10^6 cells/ml)	0.6	0.7	0.6	1.1	1.1	1.2
Bmax (fmol/mg protein)	13.1	11.7	7.7	11.5	10.6	7.1
Kd (pM)	13.3	18.4	23.6	22.2	17.5	25.8
Down-regulation (% of control)	-	11.2	41.7	-	8.6	38.2

When five cell lines were coincubated with 10^{-9} M isoprenaline and/or 10^{-3} M ascorbic acid for 7 days with daily replacement of 30ml of medium, results were only obtained from two cell lines as three cell lines failed to grow and so there were not enough cells for an assay. Of the two cell lines that yielded results, one showed a down-regulation of 60% of its β -ARs which was identical to that seen when the medium was replaced only once during the 7 day coincubation. The extent of down-regulation in the other cell line was also approximately 60%, but this compared to a down-regulation of only 33% with a single medium change.

When three cell lines were coincubated with 10^{-9} M isoprenaline for 24 hours, followed by a further 24 hours incubation in RPMI 1640 alone before assaying, the degree of down-regulation was greatly reduced when compared to that in the same cell lines coincubated with 10^{-9} M isoprenaline for 24 hours as usual

(mean down-regulation 18% and 48% respectively) (Table 4.2). In other words, significant recovery of down-regulated β -ARs does occur, and within 24 hours, if the isoprenaline is removed.

Table 4.2 β -AR down-regulation after 24 hour coincubation with 10^{-9} M isoprenaline (iso) with or without a further 24 hour incubation in isoprenaline-free (iso-free) medium.

Treatment	24 hour iso	24 hour iso + 24 hour iso-free
Down-regulation (% of control)	48.0 $\pm$ 9.4	18.1 $\pm$ 10.6

Five cell lines assayed 8, 10 and 12 weeks after lymphocyte transformation and after 7 day coincubation with 10^{-9} M isoprenaline showed no significant effect of time in continuous culture on the extent of down-regulation. The β -AR down-regulation in response to isoprenaline 8 weeks after transformation was 61.2 ± 5.2 % (mean $\pm$ sem; n=5); after 10 weeks 61.6 ± 1.1 %; and after 12 weeks 61.5 ± 3.4 %. This also showed that the down-regulation caused by 7 day coincubation with 10^{-9} M isoprenaline was highly reproducible.

4.3.2 Down-regulation of β -ARs on lymphoblast cells - concentration and time dependence

β -AR down-regulation in response to isoprenaline was found to be concentration and time dependent (Table 4.3). When tested by two-way analysis of variance, both the interaction of receptor loss and isoprenaline exposure time (Fig. 4.1), and the interaction of receptor loss and isoprenaline concentration (Fig. 4.2), were highly significant ($p < 0.001$). Coincubations with low concentrations (10^{-9} M) of isoprenaline for 7 days caused a down-regulation of more than 50% of the β -ARs (Fig. 4.1).

Fig. 4.1 Lymphoblast β -AR down-regulation in response to coincubation with 1nM and 1 μ M isoprenaline for 4 hours, 24 hours and 7 days. Each point represents the mean $\pm$ sem of the results from 2-5 cell lines.

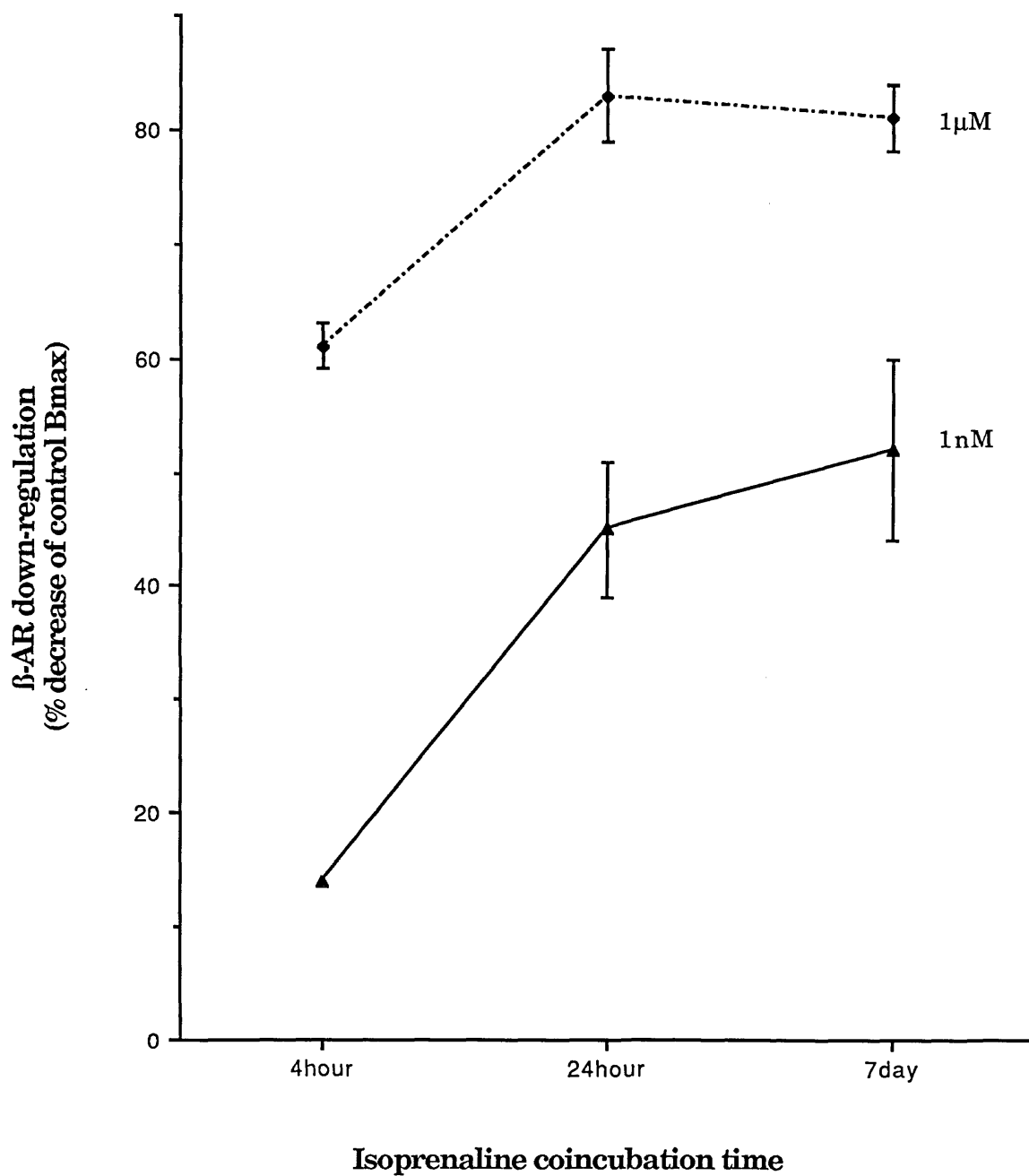
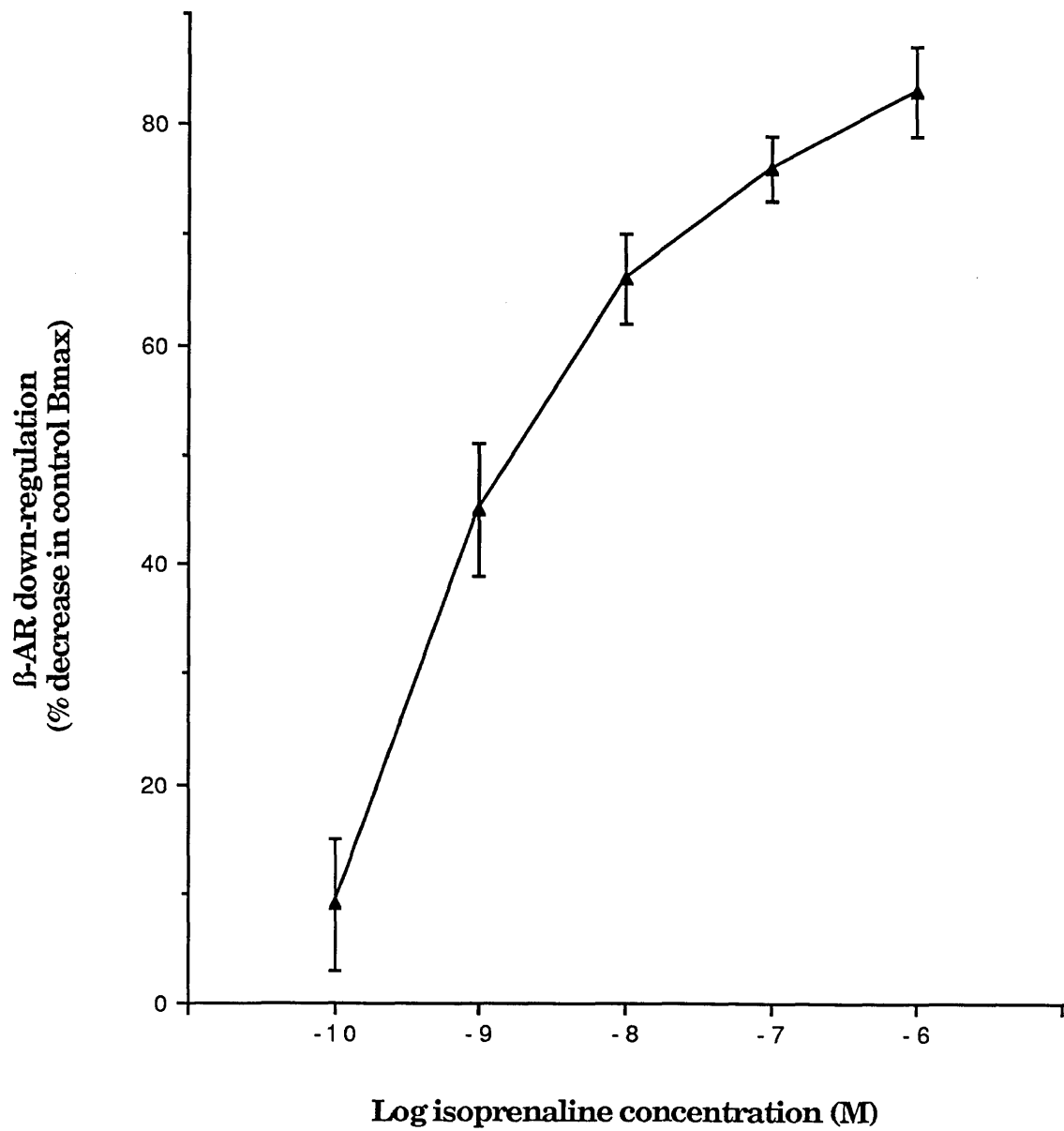


Fig. 4.2 Lymphoblast β -AR down-regulation in response to 24 hour coincubation with isoprenaline at concentrations of 10^{-10} - 10^{-6} M. Each point represents the mean $\pm$ sem of the results from 2-5 cell lines.



No difference in down-regulation was observed between 24 hour and 7 day coincubations at isoprenaline concentrations of 10^{-7}M and above, probably because the down-regulation had reached a maximum. Also, when the receptor loss exceeded 80%, accurate calculations of binding capacity were difficult and differences between experiments were not apparent. The maximum receptor loss after 24 hour and 7 day coincubation was approximately 80% whereas that after 4 hour coincubation was only 60%.

Table 4.3 Down-regulation of lymphoblast β -ARs by isoprenaline. Values are expressed as % decrease of control Bmax and are the mean $\pm$ sem of the results from 2-5 cell lines.

DURATION OF INCUBATION	ISOPRENALINE CONCENTRATION (M)					
	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}
4 hour	-	14	29	50 ± 5	61 ± 2	65 ± 2
24 hour	9 ± 6	45 ± 6	66 ± 4	76 ± 3	83 ± 4	-
7 day	20	52 ± 8	70 ± 5	81 ± 3	81 ± 3	-

The same pattern of down-regulation was seen with lymphoblasts as was previously reported by Sandnes *et al.* (1987) in isolated lymphocyte preparations with isoprenaline for up to 4 hours (Fig. 4.3). In both cases a maximum receptor loss of 60% was reached.

The coefficients of variation for repeated experiments within each cell line were low - less than 15 % - showing that there was good inter-assay reproducibility. Although the results with different cell lines were generally consistent, certain cell lines showed particularly high or low amounts of down-regulation. The results of two cell lines are compared in Table 4.4 and Fig. 4.4.

Fig. 4.3 Comparison of β -AR down-regulation between lymphoblasts coincubated with isoprenaline for 4 hours and lymphocytes coincubated with isoprenaline for 30 min (Sandnes *et al.*, 1987).

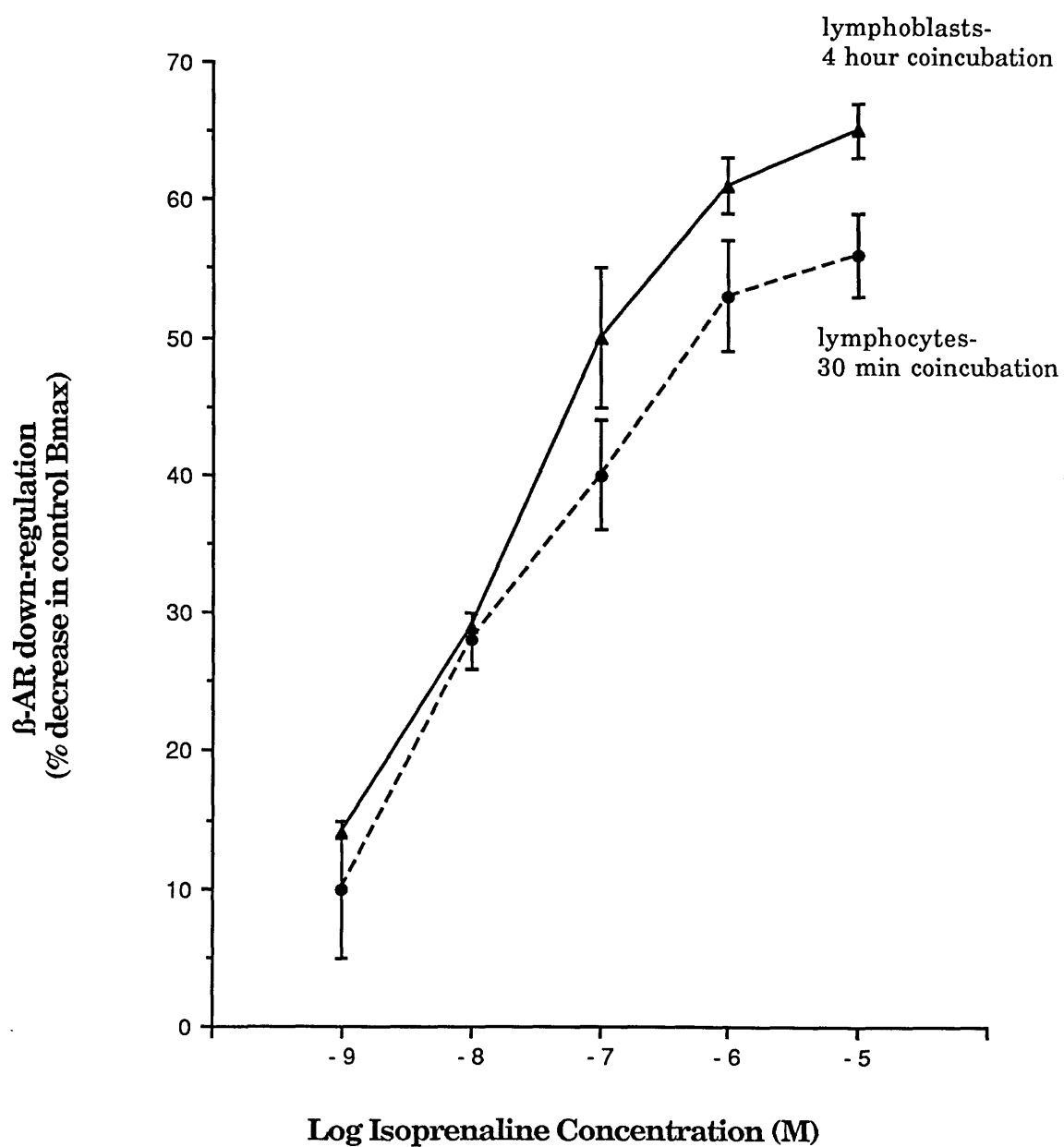
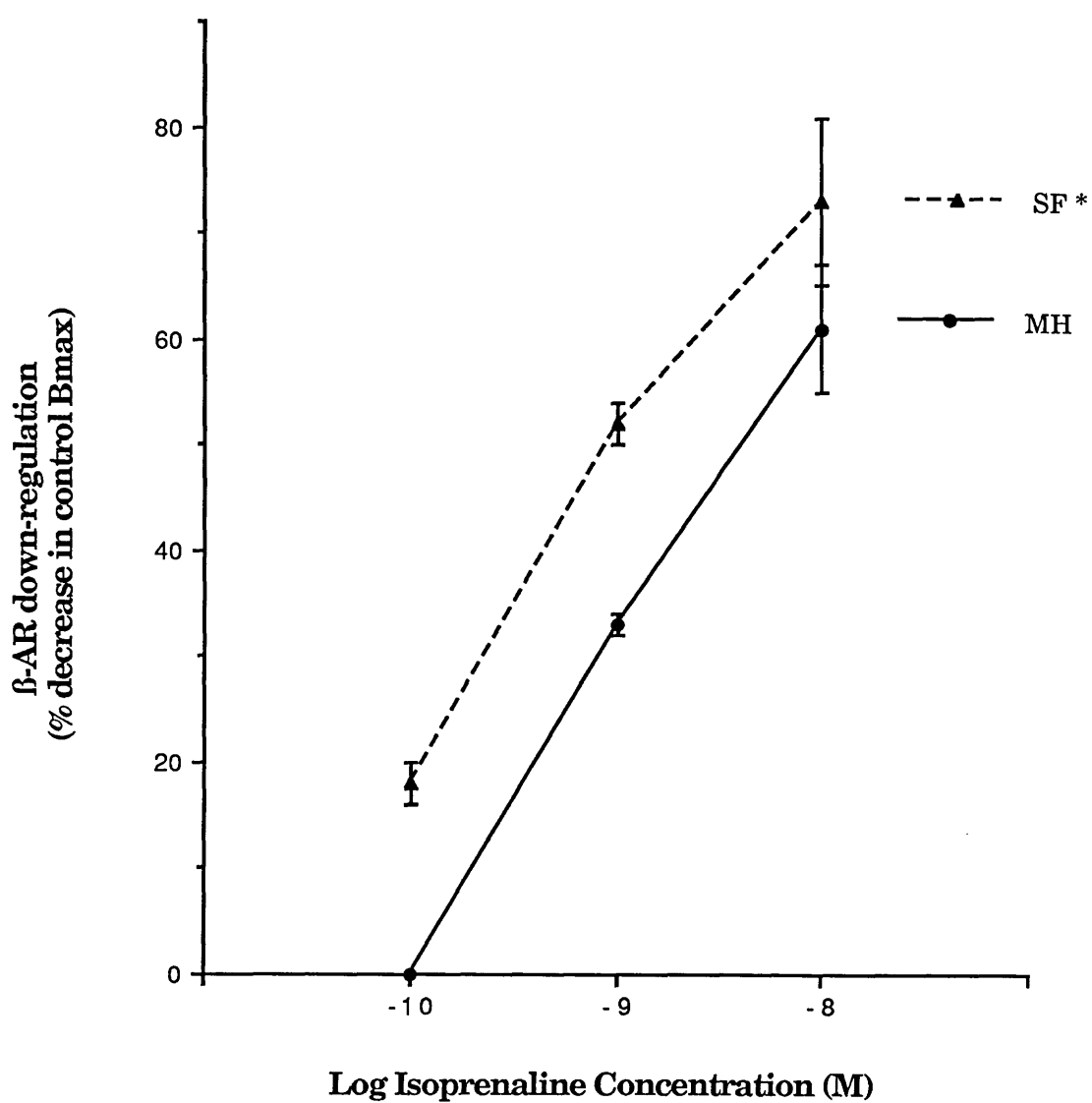


Fig. 4.4

Comparison of β -AR down-regulation between two lymphoblast cell lines after 7 day coincubation with isoprenaline. Results are expressed as the mean $\pm$ sem of 1-3 experiments.



* $p < 0.01$ v. MH (ANOVA)

There was a significant difference in their sensitivity to 24 hour coincubation with isoprenaline when tested by analysis of variance ($p < 0.01$). This difference was not a result of the significant difference in binding capacity of the two cell lines as there was no correlation between the initial Bmax values and the extent of down-regulation.

Table 4.4 Comparison of the sensitivity to 7 day coincubation with isoprenaline of two cell lines. Results are expressed as % decrease in Bmax compared to control and are the mean $\pm$ sem of 1-3 experiments.

CELL LINE	BMAX (fmol/mg protein)	ISOPRENALINE CONCENTRATION (M)		
		10^{-10}	10^{-9}	10^{-8}
MH	5.33 $\pm$ 0.68	0	33 $\pm$ 1	61 $\pm$ 6
SF*	3.58 $\pm$ 0.28!	18 $\pm$ 2	52 $\pm$ 2	73 $\pm$ 8

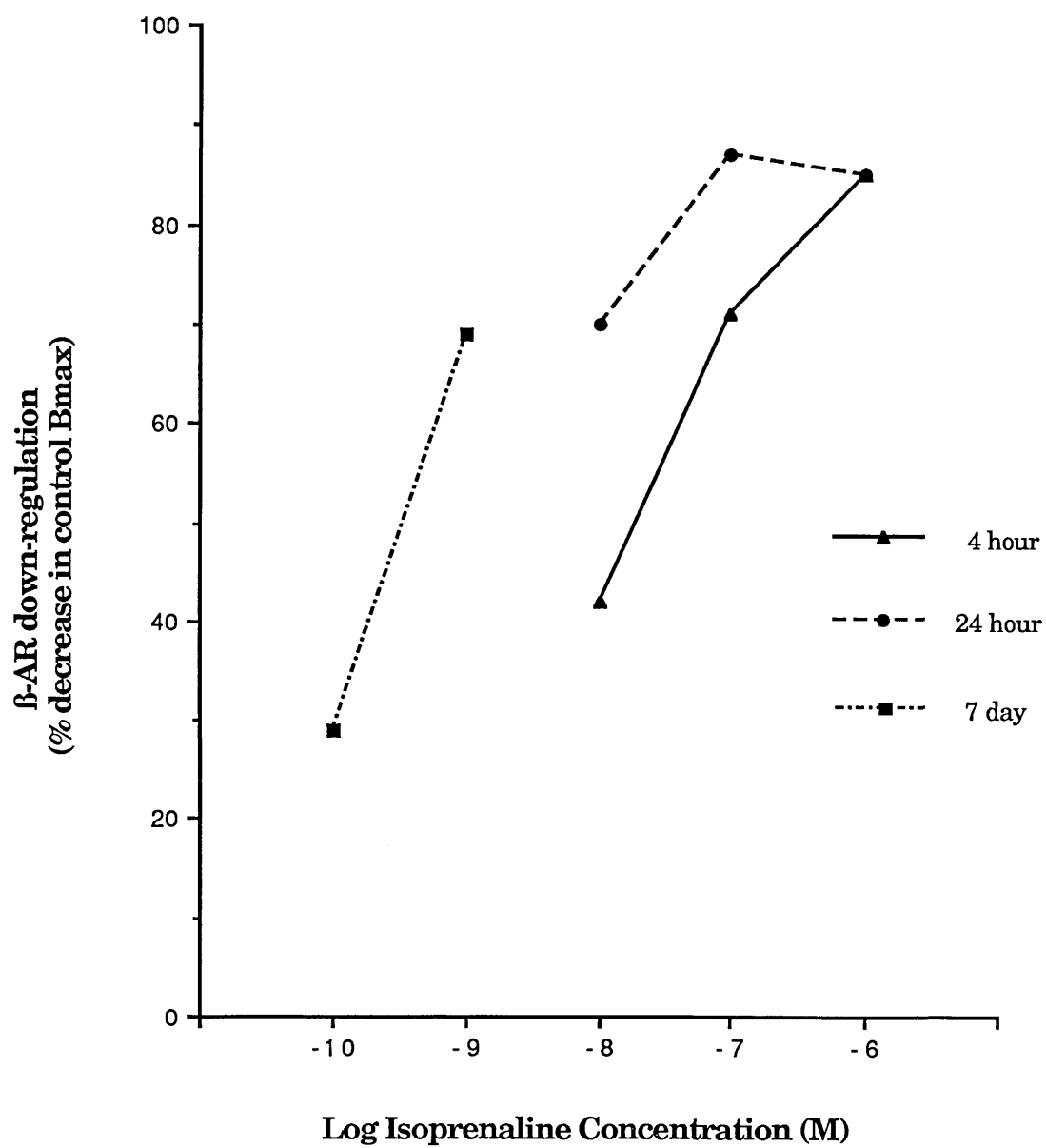
* $p < 0.01$ compared to MH (analysis of variance)

! $p < 0.01$ compared to MH Bmax value (unpaired t test)

4.3.3 Down-regulation of β -ARs on A431 cells

Coincubation of A431 cells with isoprenaline also caused down-regulation of the β -ARs which was concentration and time dependent (Fig. 4.5). A431 β -ARs were more sensitive to isoprenaline down-regulation than lymphoblast β -ARs with 4 hour coincubation with 10^{-7} M isoprenaline causing a down-regulation of 71% of the A431 β -ARs compared to 50% of lymphoblast β -ARs and 7 day coincubation with 10^{-9} M isoprenaline causing receptor losses of 69% and 52% respectively. The maximum receptor loss of 85% was achieved with 4 hour coincubation with 10^{-6} M isoprenaline and with 24 hour coincubation with 10^{-8} M isoprenaline.

Fig. 4.5 β -AR down-regulation in A431 cells coincubated with 10^{-10} - 10^{-6} M isoprenaline for 4 hours, 24 hours and 7 days.



4.3.4 Desensitization of the lymphoblast adenylyl cyclase response

Down-regulation of the lymphoblast β -AR in response to isoprenaline coincubation was accompanied by a loss of adenylyl cyclase response. All the experiments showed similar extents of desensitization of adenylyl cyclase response when either 10^{-7} or 10^{-5} M isoprenaline was used to stimulate adenylyl cyclase, or when either 10^{-7} or 10^{-6} M PGE_1 was used. The results for 10^{-5} M isoprenaline and 10^{-7} M PGE_1 will be described. The desensitization of the β -AR-coupled adenylyl cyclase response by 4 hour coincubation with isoprenaline was concentration dependent (Fig 4.6). There was no loss of response with 10^{-9} M isoprenaline, but there was a 55% reduction in cAMP accumulation with 10^{-8} M isoprenaline and a 74% reduction with 10^{-7} M isoprenaline. The adenylyl cyclase response to PGE_1 and forskolin was not reduced by coincubation with 10^{-9} and 10^{-8} M isoprenaline (Fig. 4.7A), so at these concentrations the desensitization was homologous. In contrast, 10^{-7} M isoprenaline caused heterologous desensitization in that there was an approximately 45% loss of response to both PGE_1 and forskolin (Fig. 4.7A). 24 hour coincubation with isoprenaline also caused a concentration dependent loss of adenylyl cyclase activity (Fig 4.6). 10^{-9} M isoprenaline caused a 36% loss of β -AR coupled adenylyl cyclase stimulation. PGE_1 - and forskolin-stimulated cAMP accumulation showed a 50% increase (Fig. 4.7B). Coincubation with 10^{-7} M isoprenaline resulted in a 78% loss of isoprenaline stimulated adenylyl cyclase response and a 35% loss of PGE_1 and forskolin stimulated adenylyl cyclase response (Fig. 4.7B).

Desensitization of the β -AR-coupled adenylyl cyclase response was also time dependent (Fig 4.8A). After coincubation with 10^{-9} M isoprenaline for 4 hours there was no reduction in cAMP accumulation, whereas after 24 hours there was a 36% loss of β -AR stimulated cAMP accumulation and after 7 days this reduction was increased to 84%. The response to forskolin was not affected by coincubation with 10^{-9} M isoprenaline at any of the time points. The response to PGE_1 was unaffected by 4 hour incubation, but 24 hour and 7 day coincubation both caused a 50% increase in cAMP accumulation. The results obtained using 10^{-2} M NaF to stimulate adenylyl cyclase directly were generally inconsistent.

Fig. 4.6 Concentration dependent desensitization of β -AR-stimulated cAMP accumulation in lymphoblasts after 4 hour and 24 hour coincubation with isoprenaline.

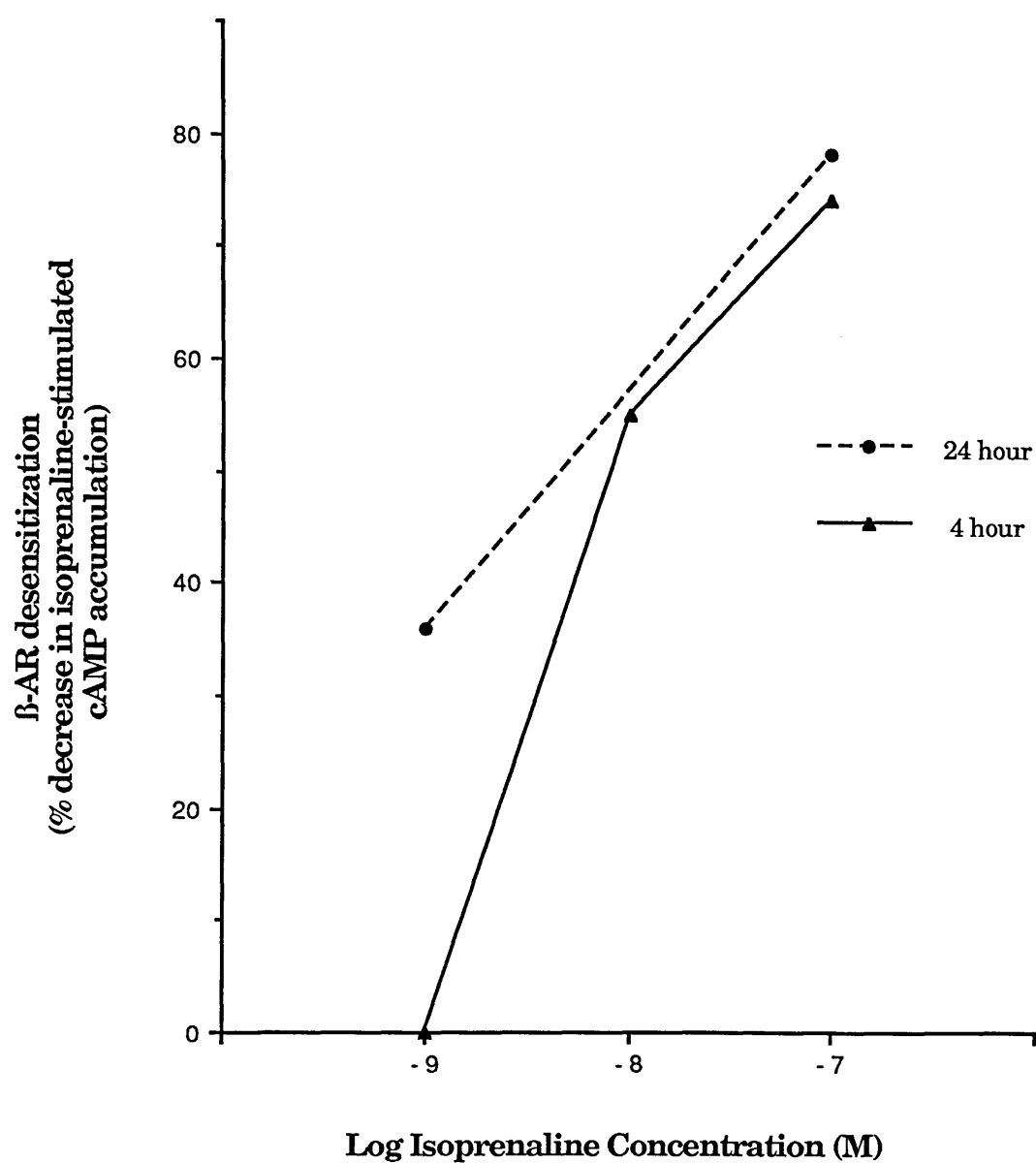


Fig. 4.7 Agonist-stimulated cAMP accumulation in lymphoblasts after 4 hour (A) and 24 hour (B) coincubation with 10^{-9} - 10^{-7} M isoprenaline.

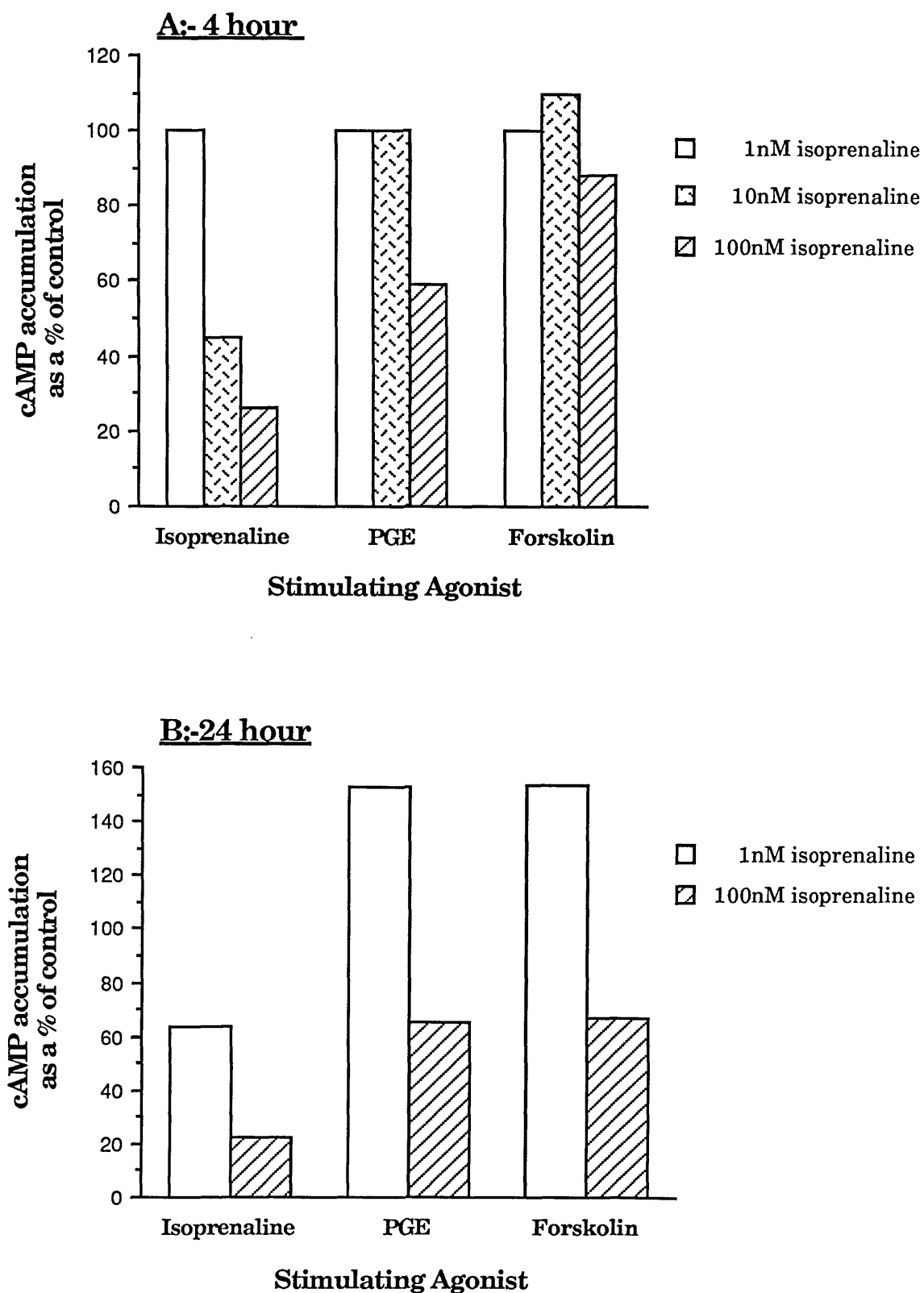
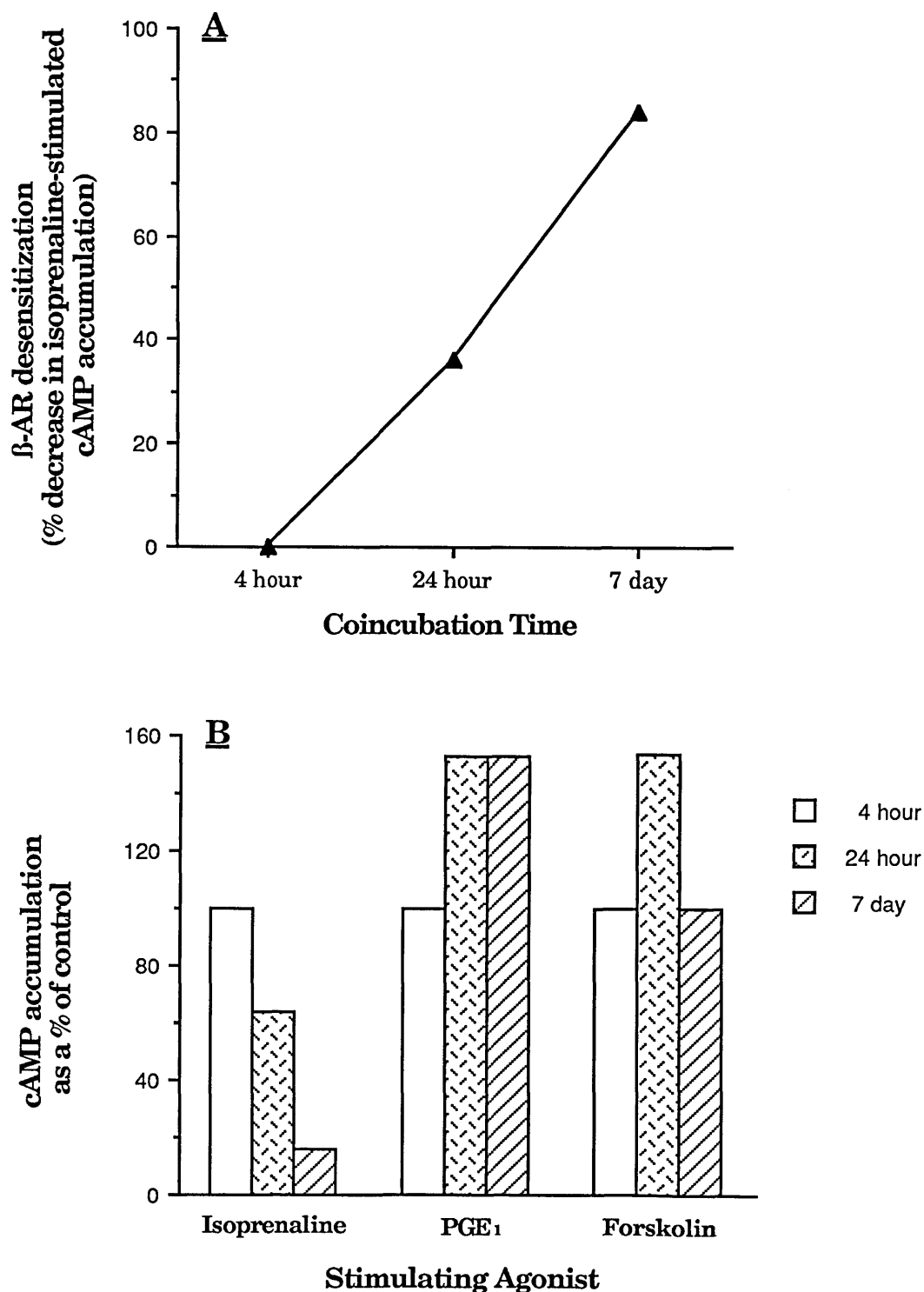


Fig. 4.8

A Time dependent desensitization of β -AR- stimulated cAMP accumulation in lymphoblasts after coincubation with 10^{-9} M isoprenaline.

B Agonist-stimulated cAMP accumulation in lymphoblasts after coincubation with 10^{-9} M isoprenaline for 4 hours, 24 hours and 7 days.



4.4 Discussion

Down-regulation of the β -AR in human lymphocytes has been widely reported both *in vivo* and *in vitro* (see Chapter One). These results show that the lymphoblast undergoes similar agonist-induced modulation of receptor number. The use of the lymphoblast however, allowed the effects of chronic incubations with low agonist concentrations to be studied, which more closely mimicked the *in vivo* situation.

When cells were incubated with isoprenaline and/or ascorbic acid for 7 days with a daily replacement of most of the medium, the cell growth in three out of five cell lines was inhibited to such an extent that there were not enough cells for an assay at the end of the 7 day period. This was thought to be due to the fact that the cells may release growth factors into the medium and by replacing most of the medium daily, these factors were lost.

Another possibility was that as the concentrations of the isoprenaline and the ascorbic acid were kept at 10^{-9} M and 10^{-3} M respectively by the daily replacement, either the isoprenaline or the ascorbic acid was toxic to the cells. As both the cells treated with ascorbic acid alone and those treated with ascorbic acid and isoprenaline did not grow, it was clear that if the latter argument was correct, then it was the ascorbic acid that was causing the toxicity. When the cells were incubated with 10^{-3} M ascorbic acid for 4 or 24 hours, no effect on cell growth was observed, so the toxicity depended on the duration of exposure to the ascorbic acid.

When the cell density was measured on days 3 and 7 of a 7 day incubation in the presence or absence of a range of concentrations of ascorbic acid, the toxicity of 10^{-3} M ascorbic acid had reduced the rate of cell growth by day 3 and caused cell death by day 7. 10^{-4} M ascorbic acid had little effect on cell growth. As 7 day isoprenaline incubation in the absence of ascorbic acid did not result in any β -AR down-regulation, 10^{-4} M ascorbic acid was used in all further incubations. As changing the medium too often was detrimental to cell growth, but only a single medium change could cause an underestimation of the

extent of down-regulation if the isoprenaline concentration was not maintained, as occurred with one cell line with a down-regulation of 33% with a single medium change compared to 60% with daily medium change, it was decided that a protocol of two changes of medium should be adopted for 7 day isoprenaline coincubations.

There was a possibility that the extent of down-regulation observed in 7 day coincubations was mainly a result of the first 24 hours or so of coincubation and that from that point on the isoprenaline concentration was much lower than the starting concentration due to loss of isoprenaline by oxidation and metabolism by the cells. If this were the case then it would have been expected that either there would be a recovery of the lost β -ARs by recycling sequestered receptors or *de novo* synthesis, in which case the extent of down-regulation would be expected to be less after 7 day coincubation than after 24 hour coincubation, or that there would be no recovery of lost β -ARs even though the isoprenaline concentration was reduced. As 7 day coincubation did in fact result in a greater extent of receptor loss than 24 hour coincubation, even when the medium was changed only once during the 7 days, then any reduction in isoprenaline concentration due to oxidation or cell metabolism was not allowing recovery of the receptors. However, as a significant proportion of the β -AR down-regulation due to 24 hour coincubation with 10^{-9} M isoprenaline was lost when a 24 hour washout period was used before assaying, it was clear that recovery of lost receptors did occur when cells with down-regulated β -ARs were placed in an isoprenaline-free medium for 24 hours. It could therefore be assumed that the extent of down-regulation in 7 day coincubations with isoprenaline resulted from the duration of the exposure to isoprenaline.

Neither the stage of the cell cycle nor the number of weeks in culture since transformation had any effect on β -AR down-regulation. As it was important to be able to harvest enough cells to have sufficient tissue for the binding assay, coincubations were run so that the cultures would be confluent when harvested, but not overgrown as a lack of nutrients or a high acidity of the medium could have had a detrimental effect on the cells.

Coincubation of lymphoblasts with isoprenaline caused a time and concentration dependent decrease in β -AR number, whilst having no effect on the binding affinity. These results were in agreement with those of Sandnes *et al.* (1987) for intact lymphocytes. Isoprenaline concentrations as low as 10^{-9} M, when coincubated with lymphoblasts for 7 days, caused a down-regulation of more than 50% of the β -ARs. Repeated experiments with each cell line showed a high degree of individual reproducibility, but there were significant differences in the extent of down-regulation between certain cell lines. There was no correlation between the binding capacity of the cell lines and their extent of down-regulation in response to isoprenaline.

The β -ARs on A431 cells also underwent time and concentration dependent down-regulation in response to coincubation with isoprenaline.

The functional relevance of the down-regulation of lymphoblast β -ARs was demonstrated by means of the adenylyl cyclase assay. The results indicated that the reduction of β -AR number following isoprenaline coincubation was accompanied by a decrease in receptor function as defined by adenylyl cyclase activity. The extent and manner of desensitization was concentration and time dependent since low concentrations of isoprenaline caused homologous desensitization as the β -AR stimulated adenylyl cyclase activity alone was reduced, whereas higher concentrations of isoprenaline (10^{-8} M or greater) or longer durations of coincubation caused heterologous desensitization in that the responses to stimulation by PGE_1 and forskolin were also decreased.

It was determined from these results that further investigation into β -AR down-regulation and desensitization should employ 24 hour coincubation with 10^{-9} M isoprenaline as these conditions resulted in a loss of about ⁴⁵~~50~~% of the β -ARs accompanied by a loss of about 35% of the β -AR-stimulated cAMP accumulation with no loss in PGE_1 - or forskolin-stimulated cAMP accumulation.

CHAPTER FIVE

THE LYMPHOBLAST β -AR IN BIPOLAR DEPRESSED PATIENTS

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5.1 Introduction

The lymphocyte β -AR has been studied in various diseases as a peripheral model of central noradrenergic function. β -AR dysfunction has been implicated in unipolar and bipolar depression and so the lymphocyte β -AR model has been used extensively to investigate β -AR binding parameters (B_{max} and K_d) and functional response (adenylyl cyclase activity) in depression (Chapter One).

The lymphocyte β -AR model does however have a number of limitations. One problem is due to the fact that the lymphocyte β -AR is sensitive to modulation by endogenous catecholamines. Physiological increases in plasma catecholamine levels have been shown to cause down-regulation of the lymphocyte β -AR and desensitization of β -AR-coupled adenylyl cyclase (Fraser *et al.*, 1981; Feldman *et al.*, 1983). β -AR antagonists cause an increase in receptor number *in vivo* (Aarons *et al.*, 1980; Fraser *et al.*, 1981) which also suggests that down-regulation of lymphocyte β -ARs occurs at physiological catecholamine concentrations.

Plasma catecholamine levels have been reported to be elevated in depression (Cooper *et al.*, 1985) and to be higher in BP patients than UP patients (Buckholtz *et al.*, 1988), thus it is difficult to know whether a reduced β -AR density in depressed patients is due to down-regulation in response to high plasma catecholamines or an intrinsic difference in β -AR capacity. Elevated plasma catecholamine levels have also been reported in situations of psychomotor agitation (Mann *et al.*, 1985) which further complicates interpretation of results.

As patients are likely to be taking antidepressants or lithium it is necessary to include a drug-free period before obtaining blood samples for lymphocyte isolation (unless *ex vivo* effects of these drugs are under investigation) as these drugs can effect β -AR number and adenylyl cyclase response (see Chapters Six & Seven).

There is also an interaction between corticosteroids and β -ARs (see below) and as cortisol secretion shows a diurnal rhythm and alterations in cortisol

secretion have been observed in depressed patients (Gibbons & McHugh, 1962; Halbreich *et al.*, 1985), plasma cortisol levels at the time of blood sampling also need to be taken into account.

A further limitation of the lymphocyte model is the short survival time *in vitro* of these cells, preventing the effects of chronic *in vitro* treatment with β -agonists or antidepressants being investigated.

The use of the lymphoblast β -AR model avoids the problems of modulation by endogenous catecholamines, drugs or hormones as the cells are grown in culture under controlled conditions for several weeks after transformation before biochemical assays are carried out. This model also allows the effects of chronic coincubations to be investigated. State-dependent effects will not be seen, so differences in β -AR number, function or regulation will be due to intrinsic differences in the lymphoblasts from depressed patients. As bipolar depression shows the strongest hereditary trait of the affective disorders (McGuffin & Katz, 1989), patients with bipolar depression were selected for this study.

Having validated the lymphoblast β -AR as a useful *in vitro* model of β -AR function and regulation, lymphoblast cell lines were established from patients with bipolar depression and age and sex matched controls. In order to investigate whether the lymphoblast β -AR was abnormal in bipolar depression, β -AR number was measured using the radioligand binding assay; β -AR function was investigated by assaying basal and stimulated levels of cAMP; and β -AR regulation was assessed in terms of down-regulation in response to isoprenaline.

Plasma cortisol levels show a daily circadian rhythm, with low cortisol levels for most of the day but high levels in the afternoon (Halbreich *et al.*, 1985). Administration of glucocorticoids at 10 p.m. suppresses the plasma cortisol peak the following day.

Abnormal functioning of the hypothalamic-pituitary-adrenal (HPA) axis has been implicated in depression in some patients (Carroll & Mendels, 1976;

Holsboer, 1988). The dexamethasone suppression test (DST) has been investigated in depression (Carroll *et al.*, 1981), and has shown a certain amount of discrimination between endogenous and nonendogenous depression. 1mg of dexamethasone, a synthetic glucocorticoid, is administered orally late at night and blood samples taken the following morning, afternoon and night. Plasma cortisol is determined and subjects can be divided into two groups, those in whom the plasma cortisol peak is suppressed to below 5 μ g/dl - suppressors - and those in whom the plasma cortisol peak is unaffected - nonsuppressors. A higher proportion of subjects with endogenous depression are nonsuppressors compared to healthy volunteers (Carroll *et al.*, 1981). The DST has also been used as a predictor of short-term treatment response, with depressed patients who are nonsuppressors being more likely to respond to antidepressant treatment than those who are suppressors, though its ability to predict response is limited (Arana *et al.*, 1985).

The physiological implications of nonsuppression are not known, but various studies have investigated the effects of glucocorticoids on the β -AR and the associated adenylyl cyclase system. Lymphocyte cells taken from high-dose corticosteroid therapy patients have been shown to have an increased catecholamine-stimulated adenylyl cyclase activity (Parker *et al.*, 1973). Desensitization of β -AR-coupled cAMP stimulation in lymphocytes from asthmatic subjects has been demonstrated (Logsdon *et al.*, 1972; Lee *et al.*, 1977). In Logsdon's study, steroid therapy was associated with a response to isoprenaline that was similar to that seen in nonasthmatic control subjects.

In normal subjects 24 hour treatment with glucocorticoids can directly increase β -AR number in both lymphocytes and granulocytes, though 4 hour treatment caused a loss of β -ARs in lymphocytes (Davies & Lefkowitz, 1980). Hydrocortisone has been shown to increase [^{3} H]-DHA specific binding on intact human lymphocytes when [^{3} H]-DHA binding was measured in the absence and presence of hydrocortisone (Cotecchia & de Blasi, 1984).

Chronic administration of hydrocortisone to rats resulted in a 70% increase in β -AR number in lung membranes and a reversal of the β -AR loss seen after

adrenalectomy (Mano *et al.*, 1979). In contrast to this study, adrenalectomy has been shown to result in an increase in β -ARs density and catecholamine-stimulated adenylyl cyclase activity in rat liver membranes whilst cortisone reversed this ~~decrease~~^{increase} (Wolfe *et al.*, 1976).

In vitro coincubation of lymphocytes from healthy controls and from asthmatic patients with hydrocortisone or cortisol caused an increase in the cAMP levels of the cells and potentiated the effect of adenylate cyclase-stimulating agents such as isoprenaline, histamine and PGE₁ (Logsdon *et al.*, 1972; Parker *et al.*, 1973; Lee *et al.*, 1977; Marone *et al.*, 1980). These effects were seen within 30 min coincubation with μ M concentrations of hydrocortisone. In asthmatic patients it has been demonstrated that coincubation with cortisol can restore the sensitivity of the lymphocyte β -AR to isoprenaline after desensitization (Lee *et al.*, 1977). *In vitro* dexamethasone treatment was also shown to increase the adenylyl cyclase response to catecholamines in lymphocytes (Lee & Reed, 1977).

Brostrom *et al.*, (1974) observed that glucocorticoid-treated C6 glioma cells had an increased basal and both NaF- and noradrenaline-stimulated adenylyl cyclase activity. In a study using dexamethasone-treated astrocytoma cells, Foster & Harden (1980) demonstrated an increase both in β -AR number and isoprenaline-stimulated adenylyl cyclase activity. Glucocorticoid-induced increase in β -ARs synthesis has also been observed in cultured human lung cells (Fraser & Venter, 1980). Hydrocortisone *in vitro* has also been shown to resensitize human neutrophils' adenylyl cyclase response that had been previously desensitized by isoprenaline (Davies & Lefkowitz, 1983).

In order to further investigate the interaction of glucocorticoids and β -ARs, the effect of hydrocortisone on the bipolar and control lymphoblast cell lines was investigated in terms of β -AR number and regulation. Parker *et al.* (1973) pointed out that it was uncertain whether the effects of glucocorticoids in short incubation experiments *in vitro* can explain their adenylyl cyclase potentiation *in vivo* which seems to evolve more slowly, allowing the possibility of a different mechanism of action such as the induction of protein synthesis. During high dose corticosteroid therapy the unbound hydrocortisone concentrations in plasma

reaches up to $1\mu\text{M}$. Furthermore, Fraser and Venter's study (1980) observed maximum increases in β -AR synthesis with $0.1\mu\text{M}$ hydrocortisone, within 24 hours. With these considerations in mind, a hydrocortisone coincubation time of 24 hours was chosen, at a concentration of $0.1\mu\text{M}$.

5.2 Methods

5.2.1 Patient and control selection

Bipolar depressed patients were recruited at two centres, the department of Psychiatry, St. Mary's Hospital and the University of Wales School of Medicine. Blood samples from seven bipolar patients attending outpatient clinics at St. Mary's Hospital were kindly taken by Dr. Baldwin and lymphoblast cell lines were established from these samples according to the protocol described in Chapter Two. Eight lymphoblast cell lines established from bipolar patients recruited in Wales were provided by Dr. Whatley at the Institute of Psychiatry. All patients were diagnosed as having bipolar depression using the revised Diagnostical and Statistical Manual of Mental Disorders III (DSMIII-R). Of the eight cell lines from the Institute of Psychiatry, six had at least two first-degree relatives with unipolar or bipolar affective disorder. In all there were 10 female bipolar subjects and 5 male.

Control subjects were recruited from healthy volunteers from various departments of St. Mary's Hospital and Medical School and were matched to patients for age and sex. A questionnaire was used to determine that the controls were healthy, not under any current medical treatment and that neither they nor their families had any history of affective disorders.

A coding system for the control and bipolar cell lines was used to ensure confidentiality. Matched cell lines were numbered 1-15, followed by a zero for control cell lines and the digit one for bipolar cell lines. A further digit indicated whether the cell line had been established from the first sample received or if repeated samples from the same subject were used. In other words, 402 and 412

are a matched pair of control and bipolar cell lines respectively, that were established from the second blood sample obtained from the subjects.

5.2.2 Basal β -adrenoceptor binding and down-regulation in response to isoprenaline

13 bipolar and matched control cell lines were coincubated for 24 hours with 1nM isoprenaline and/or 0.1mM ascorbic acid. The cells were then harvested and the radioligand binding assay carried out using [^{125} I]-CYP as described in Chapter Two. Basal β -AR number was determined from the β -AR binding capacity of cells coincubated with ascorbic acid alone.

5.2.3 Adenylyl cyclase activity and desensitization in response to isoprenaline

6 bipolar and control cell lines were coincubated as described in 5.2.2, and then the cells were harvested, washed and resuspended in BSS. The cell suspensions were incubated for 10 min either in BSS alone or with the addition of 10^{-7} and 10^{-5} M isoprenaline, 10^{-7} and 10^{-6} M PGE₁, 10^{-4} M forskolin and 10^{-2} M NaF to stimulate the adenylyl cyclase. The adenylyl cyclase assay was then carried out as described in Chapter Two.

5.2.4 The effect of hydrocortisone on β -adrenoceptor binding and down-regulation in response to isoprenaline

6 bipolar and control cell lines were coincubated with 100nM hydrocortisone, 1nM isoprenaline and/or 0.1mM ascorbic acid for 24 hours and then harvested. The radioligand binding assay was then carried as previously described.

5.3 Results

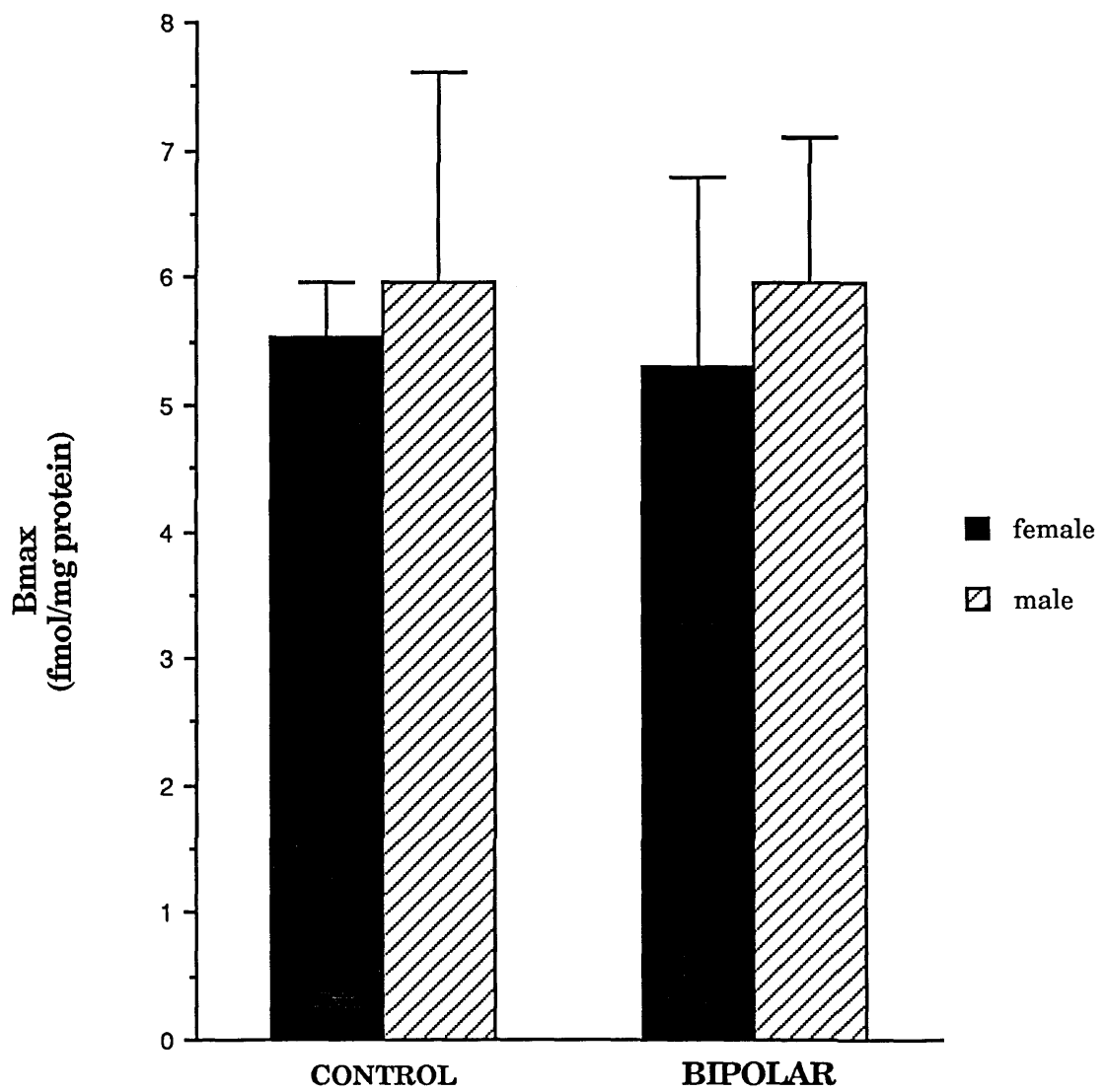
5.3.1 Patient and control data

Transformation of the lymphocytes was successfully achieved in 5 out of the 7 blood samples from bipolar patients recruited in London. The transforming lymphocytes from 2 samples were infected by contaminating bacteria before being established and frozen down. All control cell lines were eventually established, although some required repeated blood samples to be taken due to contamination. One control cell line (1503) and one patient cell line (711) had β -AR densities so low that it was very difficult to accurately determine the basal β -AR binding capacity or affinity, and isoprenaline down-regulation could not be measured. Coincidentally the control cell line was matched to a bipolar cell line (1511) from a subject of the same sex and age as that of 701, the matched control to 711. Therefore the bipolar cell line 1511 was rematched to the control cell line 701. Of the remaining bipolar cell lines 7 were from female subjects and 5 from male. The mean age of the bipolar subjects was 42.6 ± 4.4 (range 23-65; median 44) and that of the ~~(was controls)~~ controls 41.7 ± 4.4 (range 22-63; median 46).

5.3.2 Basal β -adrenoceptor binding and down-regulation in response to isoprenaline

Results for binding capacity and down-regulation were obtained from only 10 control cell lines compared to 12 bipolar cell lines due to technical difficulties. There was no difference between the basal β -AR density on lymphoblasts derived from bipolar subjects or controls when tested by Student's unpaired t-test. The mean basal Bmax value for bipolar cell lines was 5.57 ± 0.95 fmol/mg protein (n=12, coefficient of variation 17%) and that for control cell lines was 5.71 ± 0.65 fmol/mg protein (n=10, coefficient of variation 12%) (Fig. 5.1 and Table 5.1).

Fig. 5.1 β -AR binding capacity (Bmax) in lymphoblast cell lines derived from bipolar depressed patients and matched controls, subgrouped by gender. Results are expressed as mean $\pm$ sem.



From a scattergram of the Bmax values for all the cell lines (Fig. 5.2) it could be seen that the control Bmax values were distributed quite closely round the mean, with 5/10 points being within the standard error. The bipolar Bmax values showed a greater extent of variation, with only 2/12 points being within the standard error. All the bipolar values were within the 95% probability limits for the control group.

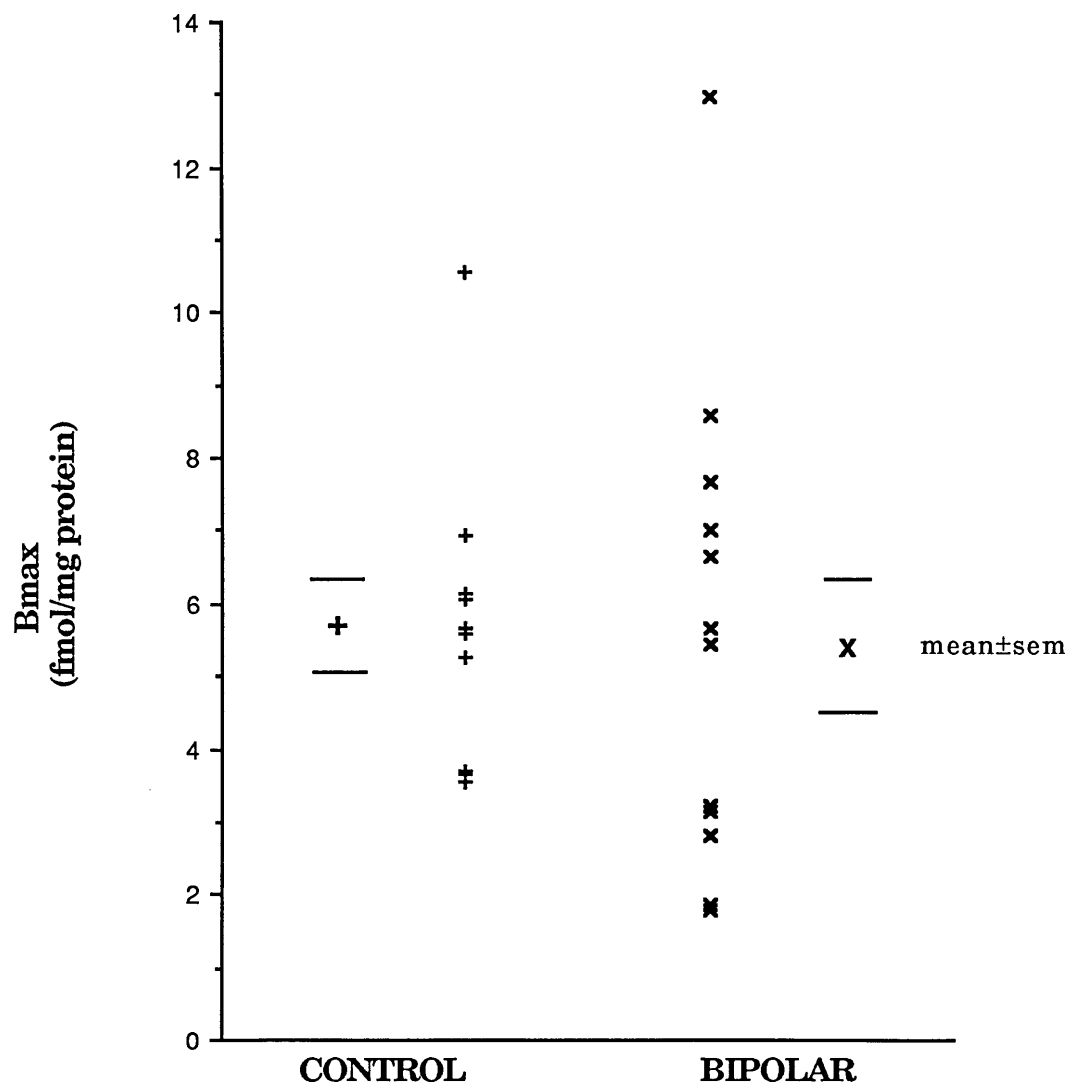
Both the bipolar and the control group had one cell line with a much greater β -AR density than the other cell lines. In the control group the outlying value was from a cell line derived from male subject (1301) and in the bipolar group the outlier was from a female cell line (1511). If these two values (10.55 and 12.98 fmol/mg protein for 1301 and 1511 respectively) were excluded, the mean control Bmax value became 5.17 ± 0.42 fmol/mg protein (coefficient of variation 8%) and the mean bipolar Bmax value was 4.90 ± 0.73 fmol/mg protein (coefficient of variation 15 %).

When the data from the two groups were separated according to the gender of the subject, there were still no differences between either bipolars and controls or males and females when tested by a two-way analysis of variance (Fig. 5.1 and Table 5.1).

Table 5.1 β -AR binding capacity (Bmax) in lymphoblast cell lines derived from bipolar depressed patients and matched controls. Results are expressed in fmol/mg protein as mean $\pm$ sem.

Cell line	CONTROL		BIPOLAR	
	n	Bmax	n	Bmax
female	6	5.53 $\pm$ 0.44	7	5.30 $\pm$ 1.48
male	4	5.97 $\pm$ 1.64	5	5.96 $\pm$ 1.13
total	10	5.71 $\pm$ 0.65	12	5.57 $\pm$ 0.95

Fig. 5.2 β -AR binding capacity (B_{max}) in individual lymphoblast cell lines derived from bipolar depressed patients and matched controls.



There was no correlation between the age of the subject at the time of blood sampling and the binding capacity for either the individual groups or the bipolars and controls combined ($r < 0.35$).

There was also no difference in [^{125} I]-CYP binding affinity between bipolar and control cell lines either overall or when male and female cell lines were examined individually by a two-way analysis of variance (Table 5.2). There was no correlation between binding affinity and age of subject at time of sampling.

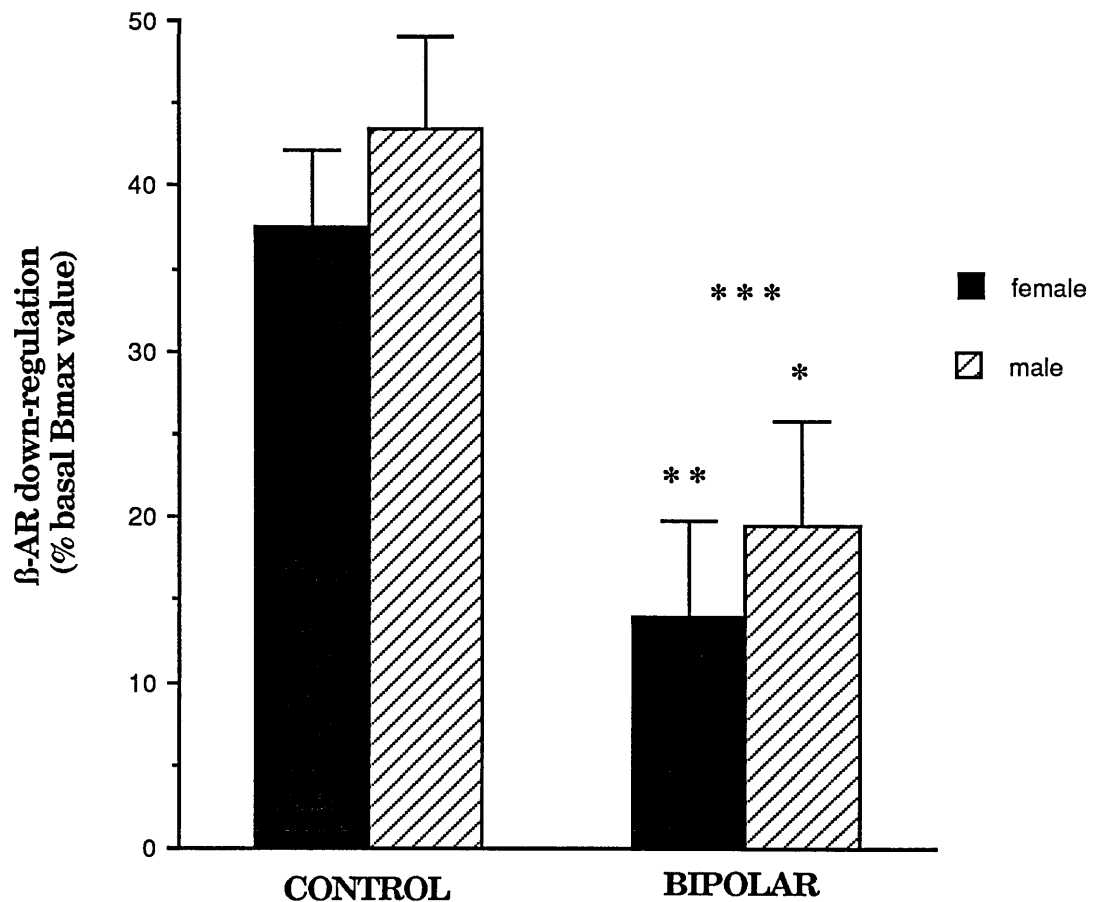
Table 5.2 β -AR binding affinity (Kd) in lymphoblast cell lines derived from bipolar depressed patients and matched controls. Results are expressed in pM as mean $\pm$ sem.

Cell line	CONTROL		BIPOLAR	
	n	Kd	n	Kd
female	6	18.8 $\pm$ 2.2	7	18.2 $\pm$ 1.5
male	4	20.0 $\pm$ 3.6	5	16.3 $\pm$ 2.7
total	10	19.3 $\pm$ 1.8	12	17.2 $\pm$ 1.5

When control cell lines were coincubated with 10^{-9} M isoprenaline for 24 hours, the extent of β -AR down-regulation was 39.9 ± 3.5 % ($n=10$, coefficient of variation 9%) (Table 5.3; Fig 5.3). In contrast, bipolar cell lines similarly treated showed a down-regulation of their β -ARs of only 20.2 ± 5.3 % ($n=12$, coefficient of variation 26%). This difference was significant when tested by Student's unpaired t-test ($p < 0.01$).

In all but one case the bipolar cell line responded to isoprenaline coincubation with a lesser degree of down-regulation than did its matched control cell line. The one bipolar cell line that showed a greater down-regulation than its control was 1511, which had a 60.6% loss of its β -ARs compared to a 41.5% loss in its control, 701. 1511 was also the cell line in the bipolar group with a particularly high binding capacity. When this cell line was excluded the mean

Fig. 5.3 β -AR down-regulation in response to 24 hour coincubation with 10^{-9} M isoprenaline in lymphoblast cell lines derived from bipolar depressed patients and matched controls, subgrouped by gender. Results are expressed as a % of basal Bmax value (ascorbic acid alone) and as mean $\pm$ sem.



* $p < 0.05$ v. male control
 ** $p < 0.01$ v. female control
 *** $p < 0.001$ bipolar v. control

down-regulation in the bipolar group became $16.5 \pm 4.1\%$ ($n=11$, coefficient of variation 25%). The exclusion of 1511 from the bipolar down-regulation data was justified statistically by carrying out an analysis of variance on the data with and without this cell line ($F(11,12)=2.79$, $p<0.05$). When the 1511 cell line was excluded the statistical difference between the control and bipolar groups was greater ($p<0.001$).

Table 5.3 β -AR down-regulation in response to 24 hour coincubation with 10^{-9} M isoprenaline in lymphoblast cell lines derived from bipolar depressed patients and matched controls. Results are expressed as a % of basal Bmax value (ascorbic acid alone) and as mean $\pm$ sem.

Cell line	CONTROL		BIPOLAR	
	n	% down-regulation	n	% down-regulation
female	6	37.5 ± 4.6	6	$13.9 \pm 5.8^{**}$
male	4	43.5 ± 5.6	5	$19.4 \pm 6.4^{*}$
total	10	39.9 ± 3.5	11	$16.5 \pm 4.1^{***}$

* $p < 0.05$ compared to male control cell lines

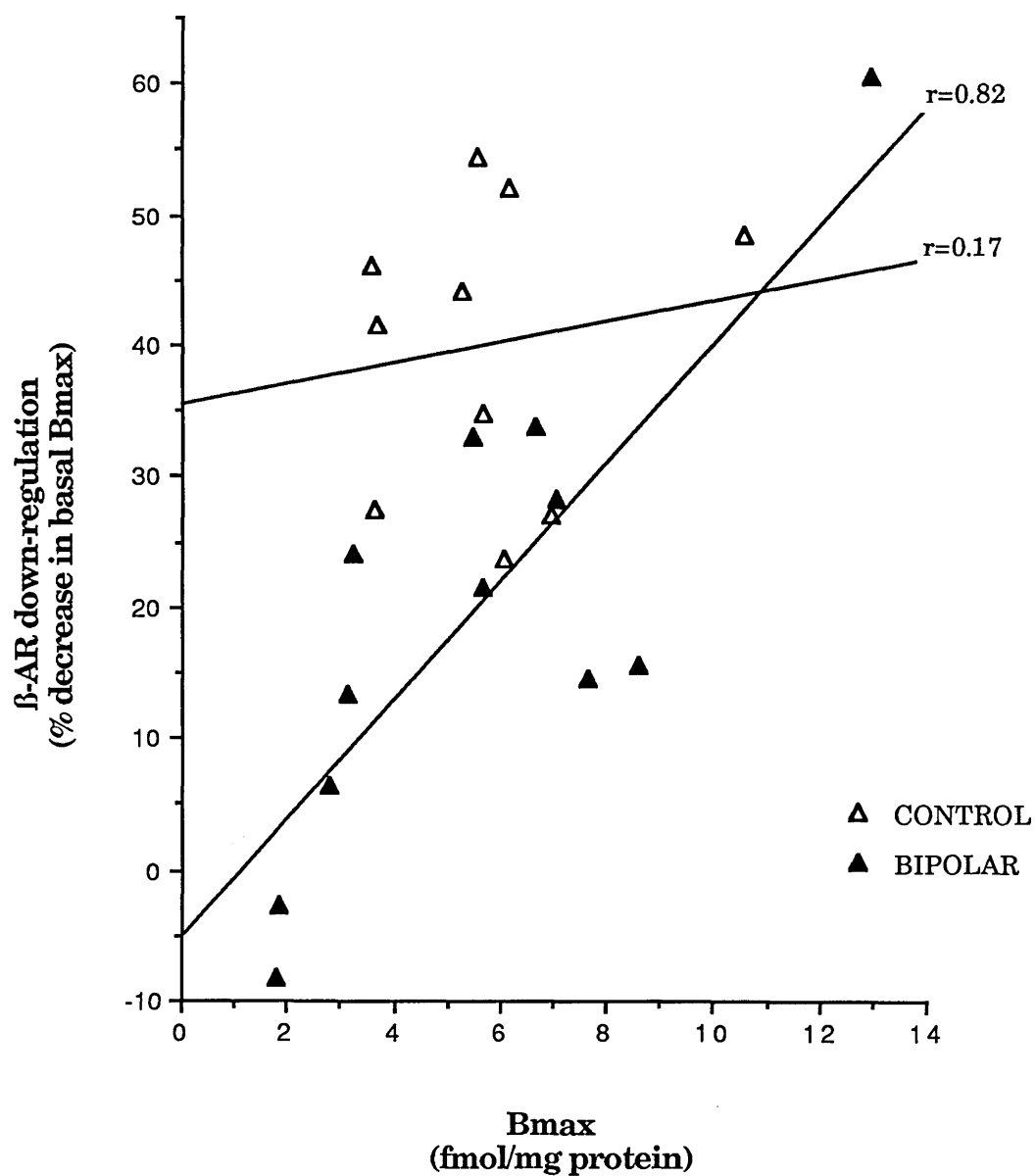
** $p < 0.01$ compared to female control cell lines

*** $p < 0.001$ compared to total control cell lines

All tested by unpaired Student's t-test, excluding cell line 1511.

The down-regulation data was also examined in terms of the effect of gender. There was no difference between cell lines derived from male or female subjects in either the control or the bipolar group (Table 5.3). In male cell lines the difference between control and bipolar cell lines was maintained, though at a lower significance level ($p<0.05$) as there were only four male control cell lines and five male patient cell lines. In female cell lines the difference in the extent of down-regulation between control and bipolar cell lines was no longer significant. However, if the 1511 cell line was excluded, this difference became significant ($p<0.01$).

Fig. 5.4 % β -AR down-regulation against basal β -AR binding for lymphoblast cell lines derived from bipolar depressed patients and matched controls.



When these data were examined by two-way analysis of variance with repeated measures, subject status had a significant effect on the extent of down-regulation ($p < 0.001$) whereas the gender of the subject did not, nor was there any significant interaction between subject status and gender.

As the greatest extent of down-regulation was seen in the cell line with the highest β -AR density, a scattergram was plotted of % down-regulation against β -AR density for all the cell lines (Fig. 5.4). When the data from both control and bipolar cell lines were examined there was a significant correlation between the extent of down-regulation and the binding capacity ($r = 0.56$, $p < 0.01$). This correlation was not apparent when the control group was examined alone ($r = 0.17$), but in the bipolar group there was a significant correlation ($r = 0.82$, $p = 0.001$). When the 1511 cell line was excluded the correlation decreased, but was still significant ($r = 0.63$, $p < 0.05$).

5.3.3 Adenylyl cyclase activity and desensitization in response to isoprenaline

The results of the investigation into basal and stimulated cAMP accumulation in control and bipolar cell lines are shown in Table 5.4 and Fig 5.5. The basal levels of cAMP in both control and bipolar cell lines showed a high degree of variability. For control cell lines the mean basal cAMP level was 4.3 ± 1.7 pmol/mg protein, with a coefficient of variation of 41% ($n = 6$). In bipolar cell lines the mean basal level of cAMP was higher at 8.9 ± 2.6 pmol/mg protein, with a coefficient of variation of 29% ($n = 6$). This difference was not significant when tested by Student's unpaired t-test. On an individual basis, three bipolar cell lines had higher basal cAMP levels than their matched controls and three had lower values.

Isoprenaline-stimulated cAMP accumulation was also highly variable. In control cell lines 10^{-7} M isoprenaline caused a significant increase in cAMP accumulation over basal of 2.5 ± 0.7 pmol/mg protein, that is, a 58% increase (Student's paired t-test, $p < 0.05$). In bipolar cell lines the increase in cAMP accumulation in response to 10^{-7} M isoprenaline was 6.1 ± 3.2 pmol/mg protein,

which was an increase of 69%, but this increase was not significant due to the high degree of variability. The coefficients of variation were 45% and 52% respectively. 10^{-5} M isoprenaline caused only a slightly increased extent of cAMP accumulation compared to 10^{-7} M isoprenaline, with a 64% increase in cAMP over basal in control cell lines and a 78% increase in bipolar cell lines. There was no correlation between the extent of isoprenaline-stimulated cAMP accumulation and the β -AR binding capacity.

Table 5.4 Basal and stimulated cAMP accumulation in lymphoblast cell lines from control and bipolar subjects. Results are expressed as the mean $\pm$ sem (n=6) in pmol cAMP/mg protein and the % increase over basal.

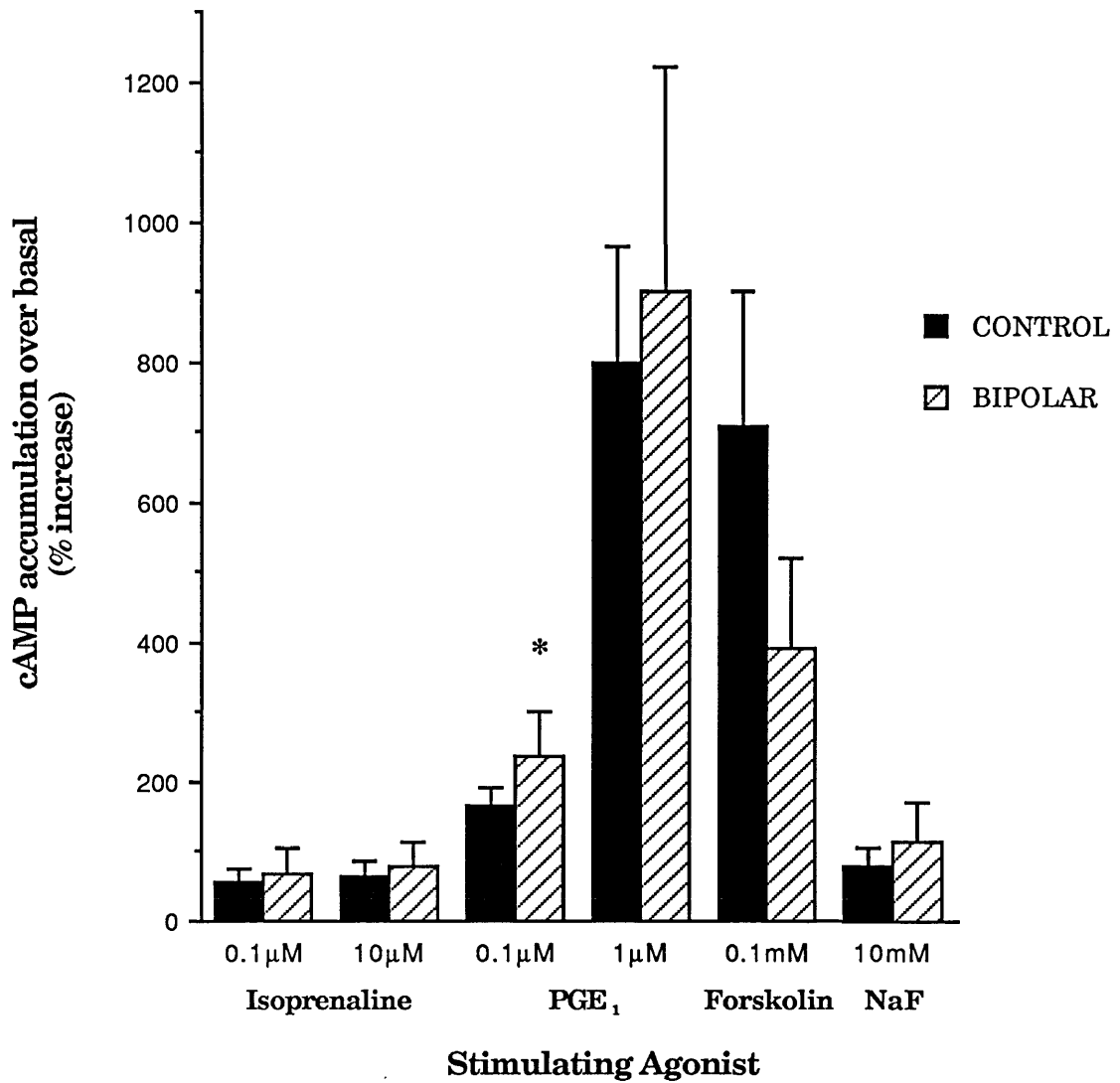
Agonist	[M]	cAMP accumulation over basal	
		CONTROL	BIPOLAR
-	(basal)	(4.3 $\pm$ 1.7)	(8.9 $\pm$ 2.6)
Isoprenaline	10^{-7}	2.5 $\pm$ 0.7 (58%)	6.1 $\pm$ 3.2 (69%)
	10^{-5}	2.7 $\pm$ 1.0 (64%)	7.0 $\pm$ 3.0 (78%)
PGE ₁	10^{-7}	7.1 $\pm$ 1.2 (166%)	20.9 $\pm$ 6.0 (236%)*
	10^{-6}	34.0 $\pm$ 7.2 (799%)	79.8 $\pm$ 28.4 (900%)
Forskolin	10^{-4}	30.2 $\pm$ 8.2 (709%)	34.9 $\pm$ 11.3 (393%)
NaF	10^{-2}	3.4 $\pm$ 1.1 (79%)	10.0 $\pm$ 5.0 (112%)

* p < 0.05 compared to control cell lines (unpaired t-test)

PGE₁ at both 10^{-7} and 10^{-6} M and 10^{-2} M NaF also caused more adenylyl cyclase stimulation in bipolar cell lines than in control cell lines, whereas 10^{-4} M forskolin resulted in a greater increase in cAMP accumulation in control cell lines than bipolar cell lines.

None of these differences between bipolar and control cell lines were significant when tested by Students t-test except for the response to 10^{-7} M PGE₁ (p<0.05).

Fig. 5.5 Basal and stimulated cAMP accumulation in lymphoblast cell lines derived from bipolar depressed patients and matched controls. Results are expressed as the % increase over basal cAMP and as the mean $\pm$ sem of 6 experiments.



* $p < 0.05$ v. control cell lines

The percentage change in agonist-stimulated cAMP accumulation due to isoprenaline coincubation was calculated by subtracting the agonist-stimulated cAMP accumulation over basal for lymphoblasts coincubated with ascorbic acid ([AgSt cAMP]_{AA}) from that for lymphoblasts coincubated with isoprenaline ([AgSt cAMP]_I), dividing the result by [AgSt cAMP]_{AA} and expressing this as a percentage. This is described by the following formulae:-

AgSt cAMP = agonist-stimulated cAMP accumulation

= absolute [cAMP] in the - basal [cAMP]
presence of agonist

[AgSt cAMP]_{AA} = amount of agonist-stimulated cAMP accumulation over basal in cells coincubated with ascorbic acid

[AgSt cAMP]_I = amount of agonist-stimulated cAMP accumulation over basal in cells coincubated with isoprenaline

$$\% \text{ change in cAMP accumulation} = \frac{[\text{AgSt cAMP}]_I - [\text{AgSt cAMP}]_{AA}}{[\text{AgSt cAMP}]_{AA}} \times 100$$

24 hour coincubation with 10⁻⁹M isoprenaline caused desensitization of the adenylyl cyclase response to 10⁻⁷M isoprenaline in four out of six control cell lines and all six bipolar cell lines. The response to 10⁻⁵M isoprenaline was less reliable as it was attenuated in only three of the six control cell lines and four of the six bipolar cell lines, with the remaining cell lines having an increased cAMP accumulation by varying amounts. The desensitization of the isoprenaline-stimulated adenylyl cyclase response was greater than 100% in several cell lines. This was a result of the cAMP accumulation in response to isoprenaline being less than the basal level. As a loss of more than 100% of the response was not possible in real terms, in those cases where the amount of cAMP was less than basal, the desensitization was taken to be 100% (i.e. % change in cAMP accumulation after 24 hour coincubation with 10⁻⁹M isoprenaline was -100%).

The % change in isoprenaline-stimulated adenylyl cyclase response for control and bipolar cell lines is shown in Table 5.5 and Fig.5.6. There was no difference between control or bipolar cell lines in the extent of desensitization of

β -AR-coupled adenylyl cyclase response. There was also no correlation between the extent of desensitization of β -AR coupled adenylyl cyclase response (using 10^{-7} M isoprenaline) and the extent of down-regulation of β -ARs in the same cell lines ($r=0.03$) (Fig. 5.7).

Table 5.5 The effect of 24 hour coincubation with 10^{-9} M isoprenaline on isoprenaline-stimulated adenylyl cyclase response in lymphoblast cell lines from control and bipolar subjects. The results are expressed as a % of the response to coincubation with ascorbic acid alone.

Cell type	CONTROL		BIPOLAR	
[Isoprenaline] M	10^{-7}	10^{-5}	10^{-7}	10^{-5}
Cell line				
101, 111	-100	-100	-100	- 86
501, 511	- 21	163	- 68	328
601, 611	-100	- 71	- 36	- 31
1001, 1011	-100	34	- 72	- 38
1201, 1211	- 17	- 28	-100	-100
1301, 1311	975*	229	- 15	6
mean $\pm$ sem	-68 $\pm$ 20	38 $\pm$ 54	-65 $\pm$ 14	13 $\pm$ 65
% change in β -AR number	-35 $\pm$ 6		-14 $\pm$ 6**	

* value excluded

** $p < 0.01$ compared to control

The response to 10^{-7} M PGE₁ (Table 5.6) was attenuated in one of the six control cell lines and four out of the six bipolar cell lines, whilst that to 10^{-6} M PGE₁ was attenuated in two control and three bipolar cell lines. However, when the mean of the results of the six control and six bipolar cell lines was calculated, overall there was no desensitization of the PGE₁-stimulated adenylyl cyclase response in either group, nor was there any difference between the two groups.

Fig. 5.6 **A** % change in β -AR-stimulated cAMP accumulation due to 24 hour coincubation with 10^{-9} M isoprenaline in lymphoblast cell lines derived from control and bipolar subjects. The results are expressed as the mean $\pm$ sem of 6 experiments.

B % change in β -AR number in the same cell lines under the same conditions.

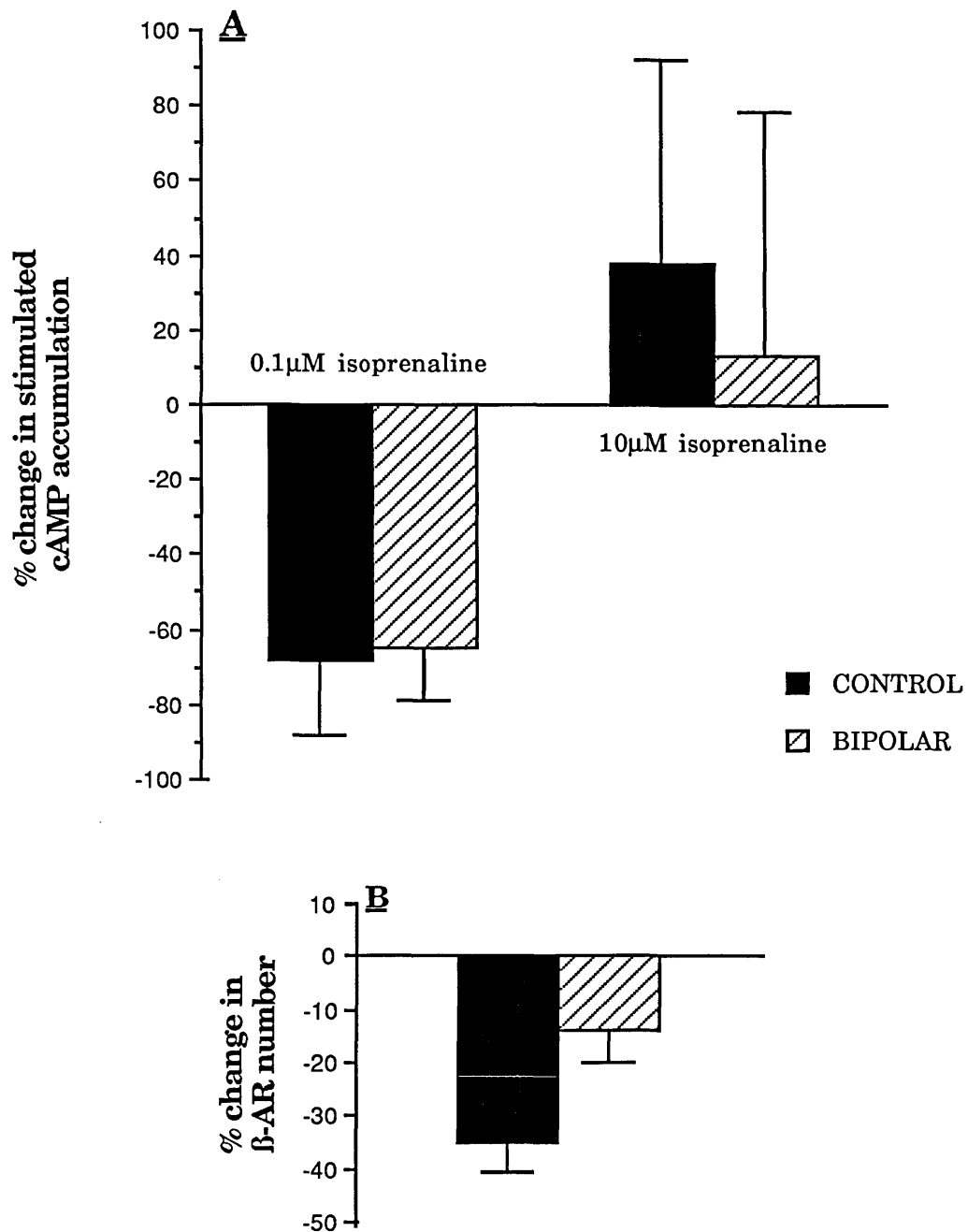
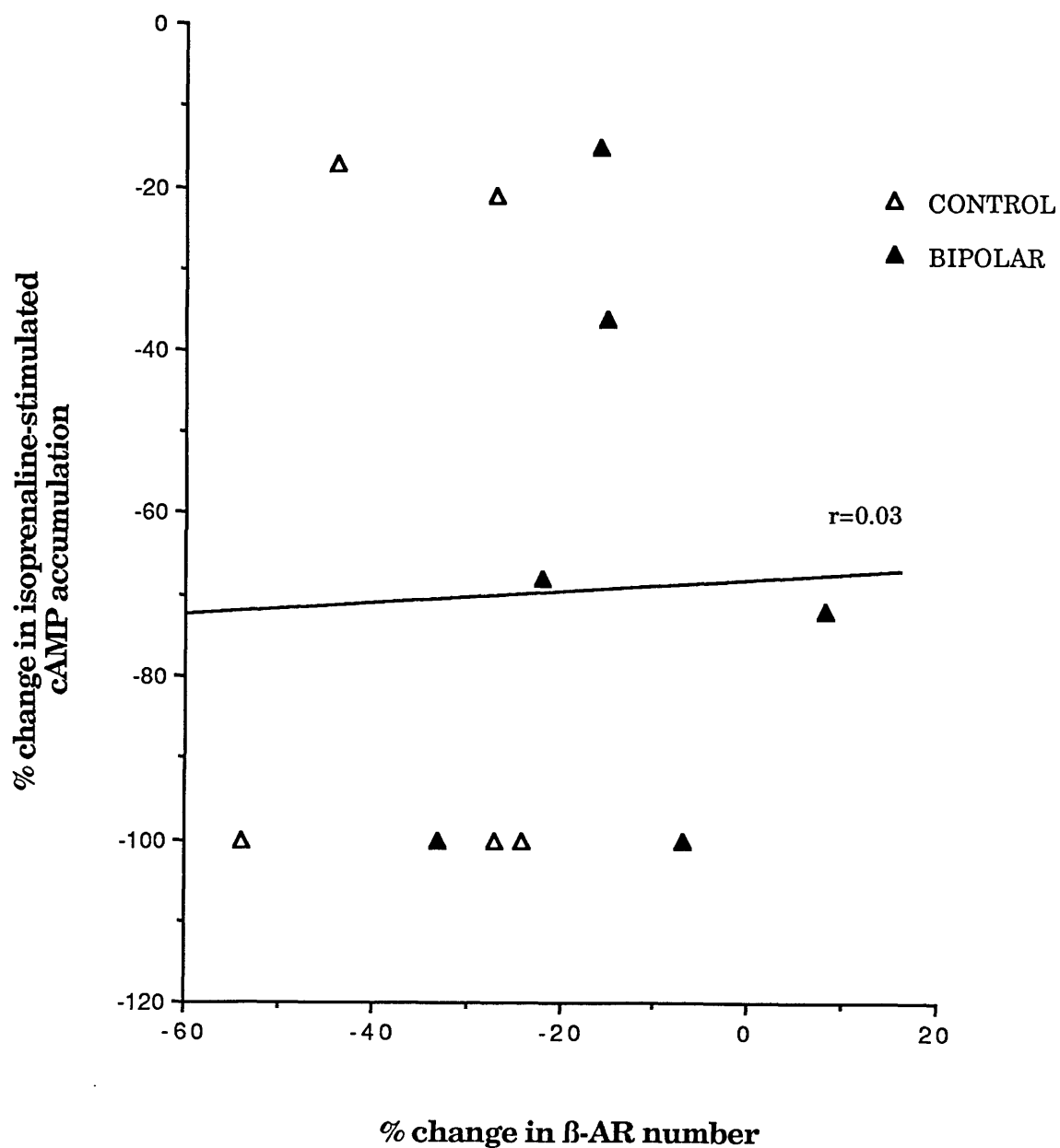


Fig. 5.7 % change in β -AR binding capacity against % change in isoprenaline-stimulated cAMP accumulation for lymphoblasts coincubated with 10^{-9} M isoprenaline for 24 hours.



Forskolin-stimulated cAMP accumulation (Table 5.6) was reduced in one control cell line and four bipolar cell lines, with no overall desensitization in the control group, but a 33% desensitization in the bipolar group. NaF-stimulated adenylyl cyclase response (Table 5.6) was decreased in half the control cell lines and all the bipolar cell lines. Overall there was an increase in the response to NaF after isoprenaline coincubation in control cell lines and a decrease in bipolar cell lines.

Table 5.6 The effect of 24 hour coincubation with 10^{-9} M isoprenaline on the adenylyl cyclase response to isoprenaline, PGE_1 , forskolin and NaF in lymphoblast cell lines from control and bipolar subjects. The results are expressed as mean $\pm$ sem (n=6), as a % of the response to coincubation with ascorbic acid alone.

Agonist	[M]	% change in agonist stimulated cAMP accumulation after 24h isoprenaline	
		CONTROL	BIPOLAR
Isoprenaline	10^{-7}	-68 $\pm$ 20	-65 $\pm$ 14
	10^{-5}	38 $\pm$ 54	13 $\pm$ 65
PGE_1	10^{-7}	127 $\pm$ 76	13 $\pm$ 20
	10^{-6}	76 $\pm$ 72	34 $\pm$ 60
Forskolin	10^{-4}	75 $\pm$ 50	-33 $\pm$ 30
NaF	10^{-2}	100 $\pm$ 516	-92 $\pm$ 5

It can be seen from Table 5.6 that desensitization of adenylyl cyclase by isoprenaline was homologous in control cell lines as the responses to PGE_1 , forskolin and NaF were not decreased. In bipolar cell lines cAMP accumulation in response to both forskolin and NaF was reduced, although that in response to PGE_1 was unaffected. In the case of forskolin, the amount of cAMP accumulation after isoprenaline coincubation was not significantly different to that after coincubation with ascorbic acid alone when tested by Student's unpaired t-test.

The variability of the effects of isoprenaline coincubation on adenylyl cyclase activity is clear from Table 5.6 as the standard errors are very high. The effect of isoprenaline coincubation on cAMP accumulation in control cell lines in response to 10^{-5} M isoprenaline varied from a 229% increase in cAMP accumulation to a complete loss of activity. In bipolar cell lines the range was from a 328% increase in accumulation to a 100% loss of stimulated cAMP accumulation. The high standard errors were a result of the high variability of the cAMP assay itself being compounded in the calculation of % change in cAMP accumulation after isoprenaline coincubation (see page 152).

5.3.4 The effect of hydrocortisone on β -adrenoceptor binding and down-regulation in response to isoprenaline

24 hour coincubation with 100nM hydrocortisone had no effect on the basal β -AR binding or affinity in either control or bipolar cell lines (Table 5.7). As before there was no significant difference between the binding capacity in control or bipolar cell lines. However, when cell lines were coincubated with hydrocortisone, the binding capacity in bipolar cell lines was slightly lower than that in control cell lines and this difference was almost significant when tested by an unpaired t-test ($p=0.068$). There was no significant interaction between subject status and the effect of hydrocortisone on B_{max} values (two-way analysis of variance).

24 hour coincubation of control cell lines with 100nM hydrocortisone in addition to 1nM isoprenaline resulted in the same mean extent of down-regulation as did isoprenaline alone (Table 5.7). The loss of β -ARs was increased in three control cell lines, unchanged in one cell line, and decreased in two cell lines.

24 hour coincubation of bipolar cell lines with hydrocortisone and isoprenaline resulted in a greater loss of β -ARs than with isoprenaline alone (Table 5.7; Fig 5.8). The extent of down-regulation was increased in four bipolar cell lines, but decreased in one cell line. This difference was not significant when

tested by Student's paired t-test but was almost significant when tested with an unpaired t-test ($p=0.053$).

After hydrocortisone coincubation there was no longer any difference between the extent of down-regulation in control and bipolar cell lines. There was no significant interaction between the subject status and the effect of hydrocortisone on isoprenaline down-regulation (two-way analysis of variance).

Table 5.7 The effect of coincubation with 100nM hydrocortisone on β -AR binding (B_{max}), affinity (K_d) and down-regulation in response to isoprenaline in lymphoblasts from control and bipolar cell lines. Results are expressed as mean $\pm$ sem ($n=6$).

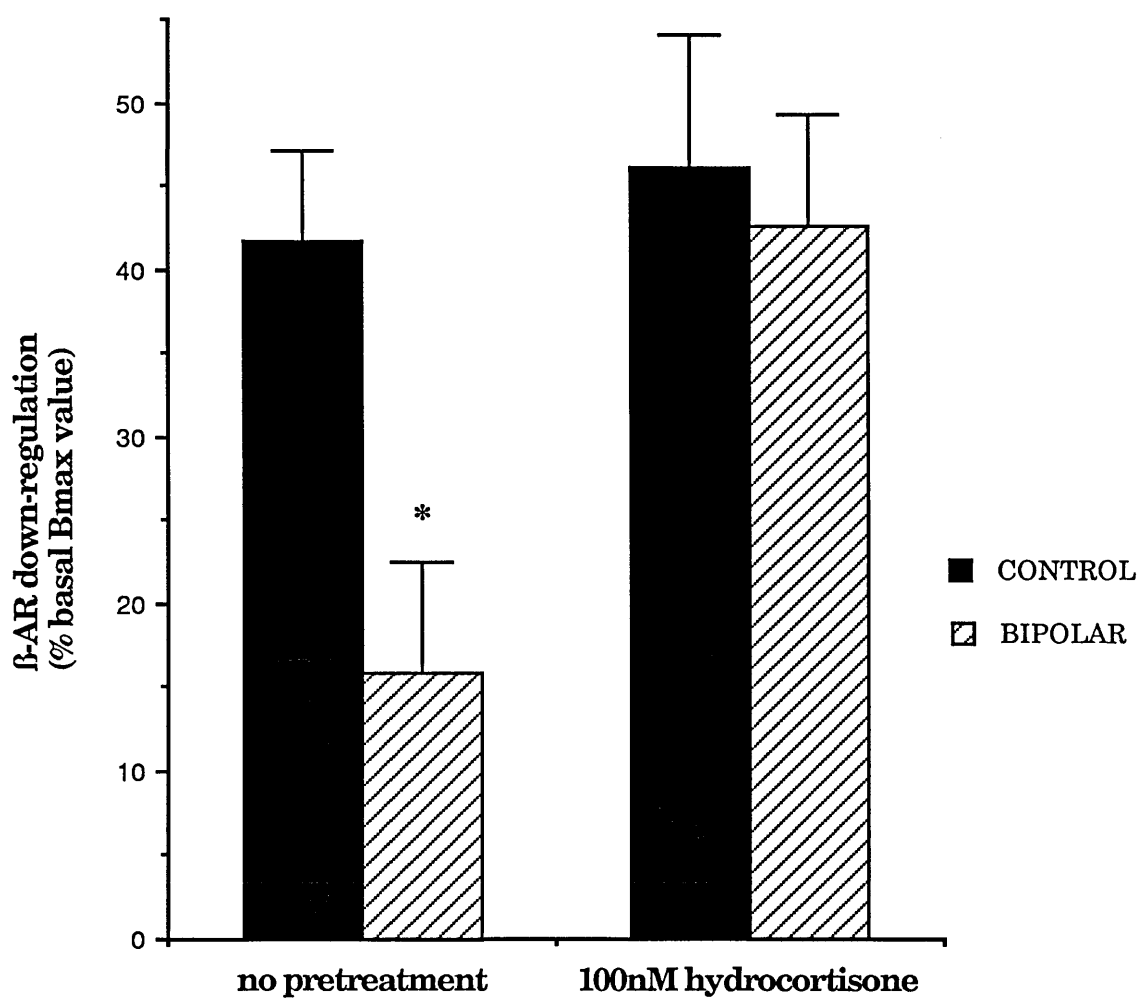
	CONTROL	BIPOLAR
<u>B_{max}</u> (fmol/mg protein)		
no pretreatment	6.76 $\pm$ 0.79	5.49 $\pm$ 1.10
100nM hydrocortisone	6.79 $\pm$ 0.49	4.99 $\pm$ 0.75*
<u>K_d</u> (pM)		
no pretreatment	19.6 $\pm$ 2.3	18.2 $\pm$ 3.1
100nM hydrocortisone	17.0 $\pm$ 4.0	16.9 $\pm$ 3.3
<u>Down-regulation</u> (%)		
no pretreatment	41.7 $\pm$ 5.4	15.9 $\pm$ 6.6!
100nM hydrocortisone	46.2 $\pm$ 7.9	42.7 $\pm$ 10.4

* $p = 0.068$ compared to control (unpaired t-test)

! $p = 0.053$ compared to ^{no pretreatment} control (unpaired t-test)

$p < 0.05$ compared to control (unpaired t-test)

Fig. 5.8 Effect of 24 hour coincubation with 100nM hydrocortisone on isoprenaline-induced β -AR down-regulation in lymphoblast from control and bipolar cell lines. Results are expressed as mean $\pm$ sem of 6 experiments.



* $p < 0.05$ v. control

5.4 Discussion

There was no difference in the β -AR binding capacity or affinity between lymphoblasts derived from subjects with bipolar depression and those derived from matched controls.

A comparison between the results of this study and the two previous studies which investigated the lymphoblast β -AR in bipolar depression, is shown in Table 5.8.

Table 5.8 Comparison between the Bmax values for lymphoblast β -ARs in this study and those of Wright *et al.* (1984) and Berrettini *et al.* (1987). Results are expressed as the mean $\pm$ sem in fmol/mg protein with the number of cell lines in parentheses.

	CONTROL	BIPOLAR
This study	5.71 $\pm$ 0.66 (10)	5.57 $\pm$ 0.95 (12)
Wright <i>et al.</i> (1984)	5.63 $\pm$ 0.73 (7)* 6.83 $\pm$ 0.63 (9)	3.91 $\pm$ 0.91 (5)
Berrettini <i>et al.</i> (1987)	3.74 $\pm$ 1.43 (14)	5.40 $\pm$ 4.05 (17)

* non-affected relatives

The results are in agreement with those of Berrettini *et al.* (1987) who investigated 17 bipolar and 14 control cell lines. Wright *et al.* (1984), however, reported lower β -AR binding capacity (less than 3.0 fmol/mg protein) in four out of six cell lines (from three out of five families) from bipolar subjects. Three of these cell lines had binding capacities that were significantly lower than those of cell lines from non-affected individuals from the same family, from the combined non-affected controls and from the combined non-psychiatric controls. The binding capacity of one of the four cell lines was not significantly different to its familial control. In our study there were three bipolar cell lines with Bmax values

of less than 3.0 fmol/mg protein, whereas the lowest binding capacity in the control group was 3.55 fmol/mg protein.

The mean β -AR binding capacity for affected patients in Wright's study was 3.59 ± 0.81 fmol/mg protein ($n=6$), whereas for non-affected relatives the B_{max} was 5.63 ± 0.59 fmol/mg protein ($n=7$) and for non-psychiatric controls the mean B_{max} was 6.83 ± 0.63 fmol/mg protein ($n=9$). On examination of the data it appeared that one of the group of affected patients had not in fact been diagnosed as bipolar but as depressed. If this subject was omitted, the mean B_{max} for the manic-depressive group became 3.91 ± 0.91 fmol/mg protein and there was no longer a significant difference between the binding capacity in bipolar cell lines and the cell lines derived from non-affected relatives. There was no significant difference between the binding capacity in the bipolar group of Wright's study and that of the bipolar group in the present study.

Berrettini's control group had a mean B_{max} of 3.74 ± 1.43 fmol/mg protein and the bipolar group had a B_{max} of 5.40 ± 4.05 fmol/mg protein. The large standard error in Berrettini's bipolar group indicates that there must have been a number of cell lines with particularly high or low β -AR densities in that study also, as was clear from the published scattergram, but that was also true of the control group.

In a similar way to the above studies examining the lymphoblast β -AR in bipolar depression, studies which have investigated the lymphocyte β -AR in depression have been inconclusive regarding basic differences in B_{max} . Five out of ten such studies demonstrated reduced β -AR densities in depressed patients compared to control subjects with two studies finding an increase in β -AR density and three finding no patient-control differences (see Chapter One). As plasma catecholamine levels have been shown to be elevated in depression (Chapter One), the lymphocyte β -AR may be down-regulated in depressed patients in response to the elevated circulating levels of catecholamines.

From this investigation, it would seem that although lymphoblast cell lines derived from bipolar subjects do not have lower β -AR binding capacities when the

mean of the bipolar group is compared to that of the control group, there are some bipolar subjects whose lymphoblast cell lines do have particularly low β -AR densities. It would be interesting to investigate these cell lines further, especially as to whether other members of the family, both affected and non-affected, had similarly low receptor densities. Two cell lines in the study, one from a female bipolar subject and one from a male, were in fact derived from members of the same family, but their Bmax values, 3.23 and 7.03 fmol/mg protein respectively, were not similar. In contrast to the results of Magliozzi *et al.* (1989), who found a decreased Bmax in UP patients that was only seen in females, in our study there was no effect of gender on binding capacity in patients or controls.

The extent of down-regulation in response to 24 hour coincubation with 10^{-9} M isoprenaline was much less in the bipolar cell lines than in the control cell lines. Three cell lines responded to isoprenaline coincubation with less than 10% loss of their β -ARs. These three cell lines were also those with Bmax values below 3.0 fmol/mg protein. The small loss of receptors was unlikely simply to be due to the low number of receptors available as the bipolar group as a whole did not have lower β -AR density than the control group and there was no correlation between the receptor density and the extent of down-regulation in the control group. Neither is the lower mean % down-regulation due solely to the lack of down-regulation in those three cell lines since omission of these values resulted in mean % down-regulation of 23.1 ± 2.9 % (n=8), which remained significantly different to the extent of down-regulation in control cell lines (39.9 ± 3.5 %).

However, as there was a correlation between the Bmax values and % down-regulation in the bipolar group itself, it is possible that in cell lines derived from bipolar subjects there is a relationship between the receptor density of a cell line and its response to isoprenaline coincubation. This would be unlikely to be a cause and effect relationship as low receptor capacity does not automatically in result in low receptor down-regulation as explained above (no correlation in control cell lines). It is more likely that whatever factor(s) determine(s) the receptor density, presumably a gene, but not necessarily that which encodes the β -AR itself, also determines the regulation of the β -ARs.

What is unclear from these results is whether the reduced extent of down-regulation in the bipolar cell lines is due to a reduced sensitivity to isoprenaline or to an inability to respond to isoprenaline with down-regulation. In the case of the former hypothesis, increasing the concentration of isoprenaline should increase the extent of down-regulation, so producing in effect a parallel shift in the dose response curve to isoprenaline. However, the latter hypothesis might predict that increased concentrations of isoprenaline would not increase the extent of down-regulation in bipolar cell lines, or if it did, that the maximum down-regulation would be less than in control cell lines. It would be interesting to investigate this further by constructing dose response curves for isoprenaline down-regulation in bipolar cell and matched control cell lines. This would perhaps give a clearer insight into the mechanism involved in the reduced response in bipolar β -ARs of isoprenaline-induced down-regulation.

There were no significant differences between bipolar and control cell lines in either their basal cAMP levels or their isoprenaline-stimulated cAMP accumulation. This could be largely accounted for by the lack of consistency of the cAMP assay, due partly to technical difficulties of the assay caused by the low adenylyl cyclase activity in lymphoblasts and partly to the compound effect of the variations for each individual assay carried out to try to find a difference in the extent of agonist-stimulated cAMP accumulation, with and without isoprenaline coincubation, in control and bipolar cell lines (see page 152).

Although it is very important to support differences in β -AR regulation with functional differences as measured by the adenylyl cyclase response, this was not possible in our study. It is appropriate, therefore, to consider the finding of other studies that investigated the β -AR functional response in depressed patients.

As detailed in the first chapter, the majority of studies on lymphocyte preparations demonstrated reduced β -AR functional response in depressed patients compared to controls (five out of seven studies). Two studies (Pandey *et al.*, 1979; Ebstein *et al.*, 1988) compared isoprenaline-stimulated cAMP levels in unipolar and bipolar patients and found that they were reduced in both unipolar and bipolar subjects compared to controls and that there were no

significant differences between the two groups. The similar findings of the two studies were obtained even though different methods of measuring the isoprenaline-stimulated adenylyl cyclase response; Pandey *et al.* measured the rate of conversion of [^3H]-adenine to [^3H]-cAMP and Ebstein *et al.* used the protein binding assay to determine the cAMP level of samples. The most recent study (Halper *et al.*, 1988) demonstrated both lower basal cAMP levels and diminished isoprenaline-stimulated cAMP accumulation across an isoprenaline concentration range of 10^{-10} - 10^{-5} M in lymphocytes from depressed subjects compared to controls. As the same group had previously reported (Mann *et al.*, 1985) that lymphocytes from depressed patients did not differ from those of controls in terms of binding capacity and affinity to [^{125}I]-CYP despite having a diminished adenylyl cyclase response to 10^{-5}M isoprenaline, they concluded that the reduced β -AR-coupled cAMP response was due to a reduced responsiveness or uncoupling of the receptors rather than a reduced number of receptor sites.

One explanation for the desensitization of the β -AR-coupled cAMP response in lymphocytes from depressed patients is that it is due to elevated levels of endogenous catecholamines (see Chapter One). This would also explain the lower basal cAMP levels found in lymphocytes of depressed patients (Halper *et al.*, 1988; Ebstein *et al.*, 1988), assuming that basal cAMP is a result of residual effects of *in vivo* stimulation by hormones (as opposed to intrinsic adenylyl cyclase activity), as in this case the basal cAMP levels would also be lowered in depressed patients as a result of elevated levels of circulating catecholamines.

This hypothesis of elevated plasma catecholamine levels in depression causing desensitization of β -AR-coupled adenylyl cyclase has to be balanced with the results of this study suggesting that desensitization would be reduced in depressed patients, so increased plasma catecholamines should result in a greater cAMP response in patients than in controls. It may be that the circulating catecholamine levels are high enough to overcome the lower sensitivity of the β -AR-adenylyl cyclase complex. If the blunted adenylyl cyclase response in lymphocytes from depressed patients is indeed due to desensitization caused by the elevated circulating catecholamine levels, then that would explain why reduced basal and isoprenaline-stimulated adenylyl cyclase levels were not observed in

lymphoblasts from bipolar subjects in the present study as the lymphoblasts were grown in culture for several months before biochemical assay so the levels of circulating catecholamines (or other hormones) at the time of blood sampling are irrelevant.

Another explanation of the diminished β -AR-coupled adenylyl cyclase response in lymphocytes from depressed patients is that it is an intrinsic defect in the β -AR-adenylyl cyclase complex. Although this does not explain our findings as regards the comparison of cAMP levels in lymphoblasts from bipolar and control subjects, as one would have anticipated a lower β -AR-stimulated cAMP response in bipolar cell lines, it provides a possible explanation of the reduced extent of β -AR down-regulation in response to isoprenaline coincubation. Down-regulation of β -ARs occurs as a homeostatic mechanism to prevent the production of excessive amounts of cAMP in response to prolonged occupation of the receptor by isoprenaline. If a defect in the β -AR-adenylyl cyclase complex results in a reduced production of cAMP in response to a particular concentration of isoprenaline in bipolar subjects, then the extent of down-regulation required to damp down the cAMP response will be less.

This hypothesis is supported by the results of Moylan *et al.* (1982), who found that raising the intracellular levels of cAMP (or its analogue) in rat astrocytoma cells by prolonged treatment with the cAMP analogue, dibutyl cAMP, plus IBMX, a phosphodiesterase inhibitor, or with cholera toxin with or without IBMX, could cause down-regulation of β -ARs. In HC-1 hepatoma cells, that lack adenylyl cyclase activity, cholera toxin treatment did not affect β -AR number, whereas treatment with dibutyl cAMP plus IBMX caused a considerable loss of β -ARs. According to this theory, if the bipolar cell lines were to be coincubated with a higher concentration of isoprenaline, then the extent of down-regulation would increase as a shift in the adenylyl cyclase response curve to isoprenaline was seen in lymphocytes from depressed patients compared to healthy controls (Halper *et al.*, 1988). It could also be predicted from their adenylyl cyclase response curve that the maximum extent of down-regulation would be lower in bipolar cell lines than control cell lines as they reported that the maximum isoprenaline-stimulated cAMP levels were lower in depressed

patients than in controls. Construction of dose response curves for isoprenaline down-regulation in bipolar cell and matched control cell lines, as mentioned previously, would be a test of this hypothesis.

There have, however, been reports that do not support such an hypothesis. Yamashita *et al.* (1987) concluded from their investigation into isoprenaline desensitization in rat reticulocytes that it was likely that it was agonist binding to β -ARs, rather than an increase in cAMP level, that was responsible for the enhancement of dibutyryl cAMP-or phorbol ester-induced desensitization. With the use of the *cyc⁻* variant of the S49 lymphoma cell line, it has also been demonstrated that homologous desensitization occurs even in the absence of a functional Gs protein, indicating that homologous desensitization is not cAMP-mediated (Green & Clark, 1981; Green *et al.*, 1981).

In contrast, other groups (Shear *et al.*, 1975; Su *et al.*, 1980; Mahan *et al.*, 1985) have shown that in S49 *cyc⁻* and *unc* variant cells agonist-induced down-regulation does not occur or is blunted despite the fact that these cells apparently exhibit the desensitization/ sequestration process. This suggests that the down-regulation process in S49 cells does indeed require a functional Gs protein, although activation of adenylyl cyclase is not required as HC-1 hepatoma cells that possess the β -AR and Gs but no functional catalytic unit still undergo receptor down-regulation in response to isoprenaline (Su *et al.*, 1980). Hence, although cAMP can itself cause down-regulation of β -ARs, it is not necessary for the process of agonist-induced β -AR down-regulation. Hadcock & Malbon (1988) have suggested from their investigation into mRNA levels in DDT₁ MF-2 hamster cells, that there are two components of β -AR down-regulation, firstly, a cAMP-mediated reduction in β -AR mRNA levels, and secondly, modulation of receptor mRNA levels by the β -AR agonist itself.

Hydrocortisone incubation had no effect on the β -AR density in either control or bipolar cell lines. This is in accordance with the results of Pandey *et al.* (1987) who found that there was no correlation between plasma cortisol levels and lymphocyte β -AR binding capacity but was in contrast to the finding of glucocorticoid-induced increase in β -AR number by other groups (see page 138).

However there was a slight differential effect between the two groups as after hydrocortisone treatment the binding capacity in bipolar cell lines was lower than that in control cell lines, although this difference did not reach significance.

As results for the effect of hydrocortisone were obtained from only five cell lines in the bipolar group, the extension of the study to include more cell lines is needed to clarify whether there is indeed a differential effect of hydrocortisone on lymphoblast β -ARs from bipolar subjects compared to controls. The concentration of hydrocortisone used in this study may have been too low to see a significant effect on β -AR binding, so it would be worthwhile to repeat this study using a higher concentration of hydrocortisone. In fact, initial trials were carried out in the laboratory using 1 μ M hydrocortisone, but this concentration was found to obliterate all β -AR binding.

It would also be useful to know whether the bipolar subjects were DST suppressors or nonsuppressors and whether there was a correlation between cortisol suppression and β -AR binding capacity and between cortisol suppression and any change in β -AR density due to coincubation with hydrocortisone. Halper *et al.* (1988) found that 12/16 of the depressed patients were nonsuppressors and these patients had lower basal and stimulated lymphocyte cAMP levels, although this difference was not significant, so it might be expected that lymphoblast cell lines from bipolar depressives that were nonsuppressors would likewise have lower basal and stimulated cAMP levels than those from suppressors and presumably would also have lower Bmax values. If, however, the reduced basal and stimulated lymphocyte cAMP levels in depressed patients that were nonsuppressors were a result of abnormalities in the HPA axis, then no difference in the aforementioned parameters would be seen in cultured lymphoblast cells.

When hydrocortisone was coincubated simultaneously with isoprenaline, there was no change in the extent of β -AR down-regulation in control cell lines, whereas in bipolar cell lines the extent of down-regulation was increased from 16% hydrocortisone to 43%. This increase was not significant as the standard errors were high due to the small number of cell lines studied (n=5 for the bipolar group), but four out of the five cell lines had greater losses of β -ARs when

coincubated with both isoprenaline and hydrocortisone than with isoprenaline alone.

There was no significant difference in the extent of down-regulation in response to isoprenaline and hydrocortisone combined between control and bipolar cell lines. This was in contrast to the significantly lesser extent of down-regulation in bipolar cell lines compared to control cell lines when coincubated with isoprenaline alone.

Corticosteroids have been shown to attenuate the extent of isoprenaline-induced desensitization in neutrophils, coincubated with isoprenaline and/or cortisone for 3 hours, by diminishing isoprenaline-induced β -AR uncoupling without altering the isoprenaline-induced β -AR down-regulation (Davies & Lefkowitz, 1983), although Marone *et al.* (1980) found no effect on isoprenaline desensitization when lymphocytes were coincubated with 10^{-5} M hydrocortisone and isoprenaline for 90 min. Therefore, one would perhaps have anticipated that hydrocortisone would have no effect on isoprenaline down-regulation. Hydrocortisone itself caused a slight, but non-significant, loss of β -ARs in bipolar cell lines which may account partly for the increased down-regulation in bipolar cell lines with combined isoprenaline and hydrocortisone incubation.

As detailed in the introduction, several studies have demonstrated an increase in catecholamine-stimulated cAMP levels in lymphocytes, C6 glioma cells and astrocytoma cells either obtained from high dose corticosteroid-therapy patients or treated *in vitro* with glucocorticoids. A possible explanation for the reduced isoprenaline down-regulation in bipolar cell lines was described above (page 165). If there were indeed a defect in the β -AR-adenylyl cyclase complex resulting in a reduced production of cAMP in response to a particular concentration of isoprenaline in bipolar subjects, and so resulting in a decreased extent of down-regulation, then an increase in isoprenaline-stimulated cAMP levels by hydrocortisone would result in an increase in the extent of down-regulation. Marone *et al.* (1980) determined that the potentiation of adenylyl cyclase-stimulating compounds by hydrocortisone was not due to inhibition of cAMP phosphodiesterase, increased receptor numbers or the prevention of β -AR

agonist-induced desensitization, and they suggested the possibility of a direct effect on the adenylyl cyclase system, such as increased receptor-cyclase coupling.

Again our data needs to be extended to verify the findings and it would also be useful to examine the basal and stimulated cAMP levels in lymphoblasts coincubated with hydrocortisone alone and hydrocortisone and isoprenaline combined.

CHAPTER SIX

THE EFFECT OF LITHIUM CHLORIDE ON β -ADRENOCEPTOR REGULATION IN LYMPHOBLASTS

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6.1 Introduction

The psychotropic action of lithium salts was discovered by Cade in 1949. Lithium is effective clinically for the treatment of acute mania (American Psychiatric Association, 1975) and for prophylaxis in bipolar depression (Schou, 1976; Prien *et al.*, 1984). It is also used both in the prophylaxis of unipolar depressive disorder and in conjunction with antidepressant drugs for the treatment of resistant depressive illness (De Montigny *et al.*, 1983; Heninger *et al.*, 1983).

Lithium is the major drug therapy in use to treat bipolar depression (Rosenthal & Goodwin, 1982) but despite widespread acceptance of its efficacy, there is little agreement as to the mechanism of action of lithium. Several groups have failed to find a change in β -AR binding in the rat brain following chronic lithium administration (Maggi & Enna, 1980; Schultz *et al.*, 1981), but Rosenblatt *et al.* (1979) noted a small but significant decrease in β -AR receptor binding after three week administration of lithium. Regionally variable effects on noradrenaline concentrations have been seen when rats were dosed with lithium carbonate (1mmol/kg/day, i.p.) for 12 days. Cortical noradrenaline showed a marked increase, while mid-brain and hypothalamic levels were decreased (Ahluwalia & Singhal, 1980).

There are at present several theories of the biochemical action of lithium. One theory is that lithium inhibits adenylyl cyclase function (Ebstein *et al.*, 1976; Belmaker, 1981). Lithium is reported to inhibit the formation of cAMP in a number of different tissues, and in response to a number of different stimuli. In humans, lithium reduces the activity of β -AR-coupled adenylyl cyclase in the plasma in response to subcutaneous adrenaline (Belmaker, 1981). In the rat, lithium has been shown to inhibit the cAMP response to noradrenaline in hypothalamic brain slices and cerebral cortical membrane preparations (Palmer *et al.*, 1972; Newman & Belmaker, 1987). In rat brain homogenates and crude synaptosome preparations, Walker (1974) demonstrated that basal and noradrenaline-stimulated adenylyl cyclase activity in the cortex and hippocampus was inhibited by lithium, whereas that in the cerebellum was unaffected.

Ebstein *et al.* (1987) investigated the mechanism of lithium inhibition of forskolin-stimulated adenylyl cyclase in human platelets. Forskolin was used to activate the adenylyl cyclase directly, distal to the receptor. They found that *in vitro* lithium chloride inhibited the forskolin-stimulated adenylyl cyclase activity by competing with magnesium ions for a site on the catalytic sub-unit. Three weeks of lithium treatment *in vivo* in manic patients resulted in an increase in the adenylyl cyclase affinity for lithium.

Another theory of the biochemical action of lithium is that it inhibits phosphatidylinositol (PI) turnover (Berridge *et al.*, 1982). The net effect of inhibiting inositol phosphate metabolism is that lithium suppresses the recycling of inositol by inhibiting the hydrolysis of intermediate inositol phosphates and this leads to a depletion of inositol (Allison *et al.*, 1976; Berridge *et al.*, 1989). The brain is particularly sensitive to the inhibition of inositol phosphate metabolism as the blood-brain barrier is relatively impermeable to inositol (Barkai, 1981) so the brain must produce its own inositol via lithium-sensitive inositol phosphate metabolism (Berridge *et al.*, 1982). Peripheral cells are protected from the effects of lithium by access to plasma inositol and by resynthesis of inositol from glucose. This resynthesis pathway may not be available in the brain as the enzyme required for the conversion of glucose-6-phosphate to *myo*-inositol-1-phosphate is restricted to the cerebral vasculature (Wong *et al.*, 1987). These hypotheses may explain the neural effects of lithium on the basis that it inhibits signal transduction indirectly by reducing the supply of the precursor lipid required to generate second messengers such as inositol 1,4,5-triphosphate ($\text{Ins}(1,4,5)\text{P}_3$) and diacylglycerol (DAG) (Berridge *et al.*, 1982). However, Downes & Stone (1986) have shown that, in rat parotid acinar cells, the levels of phosphatidyl-inositol 4,5-bisphosphate ($\text{PtIns}(4,5)\text{P}_2$), and presumably $\text{Ins}(1,4,5)\text{P}_3$ and DAG are maintained even in conditions of inositol depletion. $\text{Ins}(1,4,5)\text{P}_3$ acts by releasing calcium from internal stores (Berridge, 1987) whereas DAG stimulates protein kinase C (PKC) (Nishizuka, 1984 & 1988).

The inhibitory action of lithium is uncompetitive (Gee *et al.*, 1988) which means that it binds solely to the enzyme-substrate complex. This introduces a positive feedback loop into the inhibitory action of lithium in that an increase in

substrate concentration enhances the inhibition by providing more enzyme-substrate complex. Lithium therefore selectively affects those cells whose receptors are being actively stimulated. When receptors are functioning within their normal operation range, there is a low rate of inositol cycling and lithium has a small effect. When receptors become hyperactive, the rate of hydrolysis of the intermediate inositol phosphates increases, and lithium becomes increasingly effective (Berridge *et al.*, 1989). Consequently, Berridge proposed that the PI cycle is overactive in bipolar depression due to hyperactivity of phosphoinositide-dependent receptors.

As there have been few studies into the effects of lithium *in vitro* on human tissue and on the β -AR in particular, an investigation into the effect of coincubation of lymphoblast cells with lithium was undertaken. The lowest proposed minimum plasma concentration for effective prophylaxis is 0.6mmol/l (Coppin *et al.*, 1983). This minimum was defined as the value 12 hours after the last oral dose in patients at steady state and represented trough values in patients on a twice daily regime. Peak values would therefore be in the range of 1-2 mmol/l and values above 2.5mmol/l are usually associated with serious side-effects (Wood & Goodwin, 1987). With this in mind, a lithium concentration of 1 mM in the cell medium was selected. Lithium is administered chronically in the prophylactic treatment of bipolar depression and in manic or depressed patients the therapeutic action of lithium treatment is only apparent after a week of treatment (Schou, 1976) so seven day coincubations were employed. As the down-regulation of the lymphoblast β -ARs in response to isoprenaline was seen to be less in the bipolar cell lines than in control cell lines, and it has been suggested that lithium selectively effects cells whose receptors are being actively stimulated, the effect of lithium pretreatment on isoprenaline down-regulation was investigated in both groups as well as its effect on basal binding capacity.

6.2 Methods

Lymphoblast cell lines from 12 bipolar cell lines and 12 matched control cell lines were coincubated with 1mM lithium chloride for 7 days. On day 6, 1nM isoprenaline and/or 0.1mM ascorbic acid were added to the cells. The cells were harvested 24 hours later and the β -AR binding assay was carried out using [^{125} I]-CYP as described previously.

6.3 Results

6.3.1 β -Adrenoceptor binding capacity and affinity

Table 6.1 and Fig 6.1 show the results of 7 day coincubation of the lymphoblast cell lines with 1 mM lithium chloride. In control cell lines lithium pretreatment resulted in a slight reduction in the β -AR binding capacity to 3.89 ± 0.47 fmol/mg protein compared to 5.71 ± 0.65 fmol/mg protein for cells without lithium pretreatment. This change was not significant when tested by a Student's paired t test ($p=0.087$), but when an unpaired t-test was used the significance increased ($p<0.05$). There was no change in binding capacity in bipolar cell lines.

After lithium coincubation the binding capacity in control cell lines was lower than that in bipolar cell lines although this difference was not significant ($p=0.082$, Student's unpaired t-test), whereas without lithium coincubation there was no difference in binding capacity between the two groups. There was no effect of either lithium treatment or the status of the subject on the Bmax, nor was there any interaction between subject status and the effect of lithium treatment on Bmax (two-way analysis of variance).

Table 6.1 The effect of 7 day coincubation with 1mM lithium chloride on β -AR binding capacity (Bmax) in lymphoblast cell lines derived from bipolar depressives and matched controls. Results are expressed in fmol/mg protein as mean $\pm$ sem.

Treatment	CONTROL		BIPOLAR	
	n	Bmax	n	Bmax
NO LITHIUM	10	5.71 $\pm$ 0.65	12	5.57 $\pm$ 0.95
1mM LITHIUM	11	3.89 $\pm$ 0.47*	10	5.69 $\pm$ 0.88

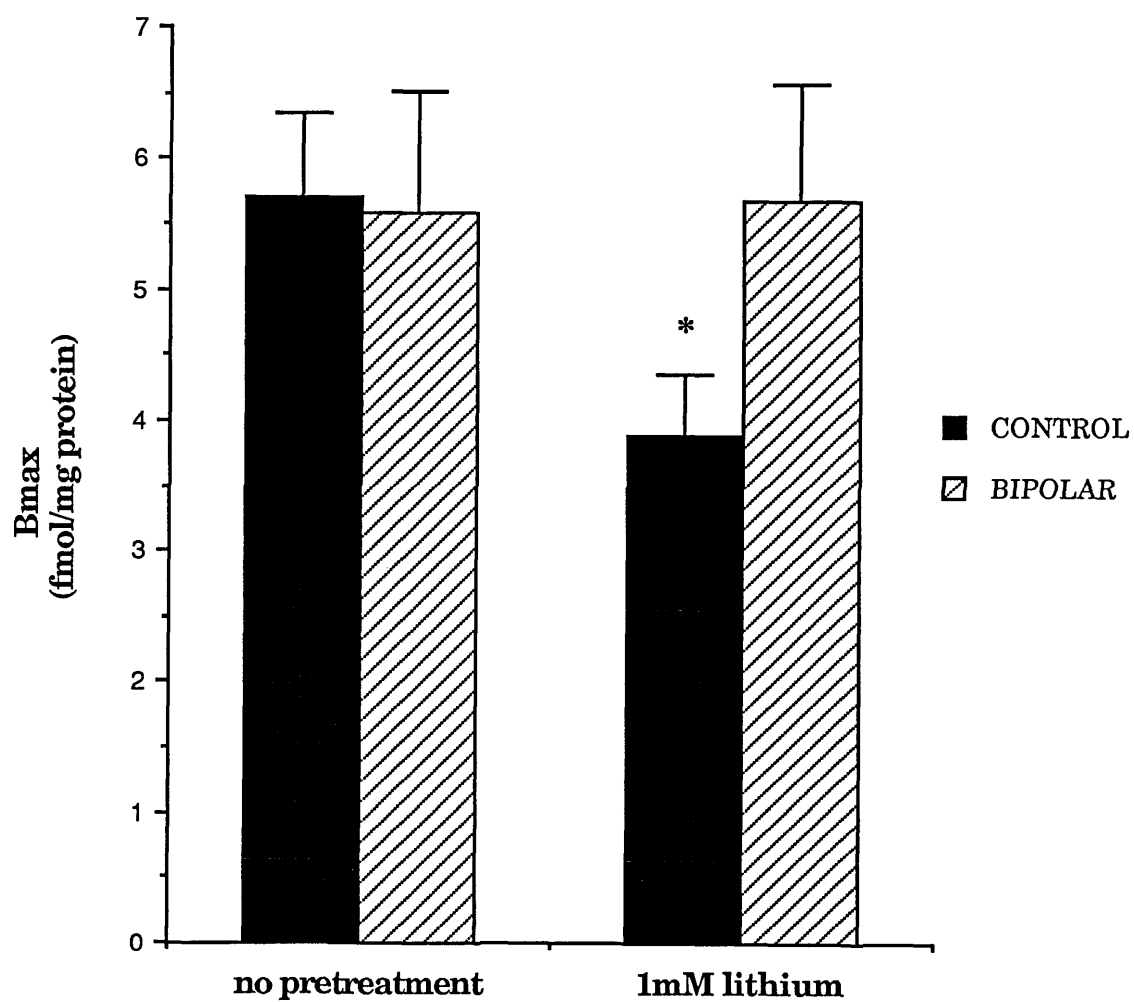
* p < 0.05 compared to no lithium (unpaired t-test)

Table 6.2 The effect of 7 day coincubation with 1mM lithium chloride on β -AR binding affinity (Kd) in lymphoblast cell lines derived from bipolar depressives and matched controls. Results are expressed in fmol/mg protein as mean $\pm$ sem.

Treatment	CONTROL		BIPOLAR	
	n	Kd	n	Kd
NO LITHIUM	10	19.3 $\pm$ 1.8	12	17.2 $\pm$ 1.5
1mM LITHIUM	11	14.8 $\pm$ 2.1	10	16.9 $\pm$ 1.4

Table 6.2 shows the results of lithium coincubation on the β -AR binding affinity. 7 day coincubation with lithium caused a slight increase in the affinity of the β -AR for [^{125} I]-CYP in control cells from a Kd of 19.3 $\pm$ 1.8 pM without lithium treatment to a Kd of 14.8 $\pm$ 2.1 pM in lithium treated cells, however there was no significant effect of treatment when the results were analysed by two-way analysis of variance and no significant interaction between treatment and binding affinity. There was no change in binding affinity in bipolar cell lines and there was no significant difference between the binding affinity in control cell lines to that in bipolar cell lines either with or without lithium treatment.

Fig. 6.1 Effect of 7 day coincubation with 1mM lithium chloride on β -AR binding capacity in lymphoblasts from control and bipolar cell lines. Results are expressed as mean $\pm$ sem of 10-12 experiments.



* $p < 0.05$ v. no pretreatment

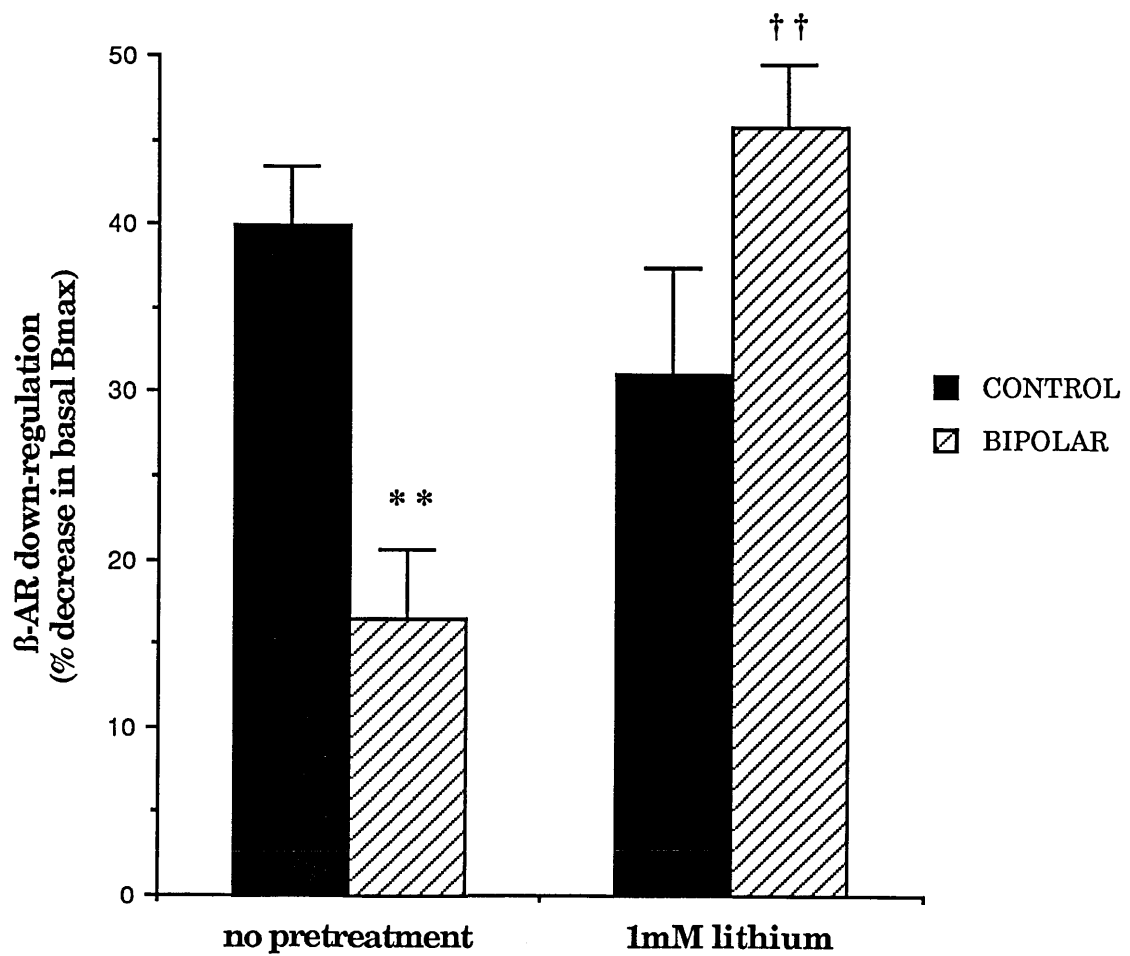
6.3.2 β -Adrenoceptor down-regulation in response to isoprenaline

Table 6.3 and Fig. 6.2 show the results for the down-regulation of β -ARs in response to isoprenaline with and without lithium pretreatment. There was a slight decrease in the extent of down-regulation in control cell lines after 7 day lithium coincubation from 39.9 ± 3.5 % loss of receptors in response to isoprenaline without lithium to 31.0 ± 6.3 % receptor loss with lithium. This was not quite significant when tested by a Student's paired t-test ($p=0.063$).

In contrast, in bipolar cell lines there was a marked increase in the down-regulation in response to isoprenaline from 16.5 ± 4.1 % without lithium to 45.8 ± 3.8 % with 7 day coincubation with 1mM lithium. This increase was highly significant when tested by a paired t-test ($p<0.001$). After lithium pretreatment the extent of down-regulation in bipolar cell lines was greater than that in control cell lines after lithium pretreatment but not significantly ($p=0.065$, unpaired t-test). The extent of down-regulation in lithium-pretreated bipolar cell lines was not significantly different to that in control cell lines without lithium pretreatment.

There was a highly significant interaction between the effect of lithium coincubation and the subject status on isoprenaline down-regulation (analysis of variance, $p=0.0001$). In other words, lithium had a differential effect on isoprenaline-induced down-regulation in control and bipolar cell lines.

Fig. 6.2 Effect of 7 day coincubation with 1mM lithium chloride on isoprenaline-induced β -AR down-regulation in lymphoblasts from control and bipolar cell lines. Results are expressed as mean $\pm$ sem of 10-12 experiments.



** $p < 0.001$ v. control
 †† $p < 0.001$ v. no pretreatment

Table 6.3 The effect of 7 day coincubation with 1mM lithium chloride on β -AR down-regulation in response to 24 hour coincubation with 1nM isoprenaline in lymphoblast cell lines derived from bipolar depressives and matched controls. Results are expressed as % of basal Bmax value (ascorbic acid alone) and as mean $\pm$ sem.

Treatment	CONTROL		BIPOLAR***	
	n	% down-regulation	n	% down-regulation
NO LITHIUM	10	39.9 $\pm$ 3.5	11	16.5 $\pm$ 4.1**
1mM LITHIUM	11	31.0 $\pm$ 6.3	10	45.8 $\pm$ 3.8!!

** p < 0.001 compared to control (unpaired t-test)

!! p < 0.001 compared to no lithium (paired t-test)

*** p < 0.0001 interaction between subject status and lithium treatment
(analysis of variance)

6.4 Discussion

Coincubation of lymphoblast cells with lithium chloride for seven days had no significant effect on β -AR binding capacity in either control or bipolar cell lines, although there was a trend towards a reduction in Bmax in control cell lines. This is in agreement with those groups who found no change in β -AR binding in rat brain following administration of lithium (page 173).

Although adrenergically stimulated adenylyl cyclase has been shown to be inhibited by lithium, this does not necessarily have to be a result of receptor loss. In fact, as both GppNHp-induced and forskolin-induced adenylyl cyclase activation have also been shown to be reduced (Newman & Belmaker, 1987), a reduction in β -AR number would not account for this loss of activity.

Adrenergic agonists and GppNHp are clearly acting through GTP-binding proteins to stimulate adenylyl cyclase (Chapter One). As there is there is a high-affinity binding site for forskolin in the rat brain thought to be associated with the

complex of the catalytic unit and the activated G-proteins (Seamon & Daly, 1986), therefore the forskolin-stimulated adenylyl cyclase activation may also be mediated through an effect on G-proteins. In other words, the attenuation of stimulated adenylyl cyclase activity by lithium may be via a direct effect on the G-proteins.

Such a hypothesis was suggested by Avissar *et al.* (1988), who demonstrated that lithium blocked both adrenergic and cholinergic agonist-induced increases in [³H]-GTP binding in rat cerebral cortex both *in vitro* and *ex vivo*. Lithium also abolished guanine nucleotide modulation of agonist binding. In a further study, this group have shown that lithium exerts its effects by competing with magnesium ions on low affinity Mg²⁺ binding sites on the α -subunit of G proteins, which are essential for guanine nucleotide exchange on these proteins (Avissar *et al.*, 1991).

These findings may also suggest that altered G protein function is of pathophysiological importance in BP disorder. Results providing support for this proposal have been reported by the same group who found hyperactive function of G proteins in mononuclear leukocytes of patients with mania. They demonstrated that isoprenaline-and carbamylcholine-induced increases in GppNHp binding capacity were twofold to threefold higher in leukocytes from untreated manic patients compared to healthy volunteers or lithium-treated, euthymic BP patients (Schreiber *et al.*, 1991).

With the results from these studies in mind, it would be useful to investigate whether adenylyl cyclase stimulation is reduced by coincubation with lithium in our lymphoblast β -AR model, and whether any effect was differential with respect to control and bipolar cell lines.

The effect of lithium coincubation on down-regulation in response to isoprenaline was very interesting, as lithium pretreatment seemed to restore the impaired down-regulation response previously seen in bipolar cell lines, so that it became equivalent to that seen in control cell lines without lithium pretreatment. The difference in the extent of isoprenaline down-regulation between control and bipolar cell lines was reversed by lithium pretreatment in that now the bipolar cell lines showed a greater extent of down-regulation than control cell lines.

It would be useful to know if there were any corresponding changes in adenylyl cyclase desensitization. Direct inhibition of adrenergically activated adenylyl cyclase function by lithium would result in a reduced production of cAMP in response to a particular concentration of isoprenaline. According to the hypothesis put forward in the previous chapter, that the extent of β -AR down-regulation in response to isoprenaline is related to amount of isoprenaline-stimulated cAMP accumulation, a reduction in the amount of cAMP produced in response to isoprenaline by lithium would cause a decreased extent of down-regulation. This would perhaps account for the reduction in isoprenaline down-regulation seen in control cell lines after lithium coincubation but could not explain the increase in isoprenaline down-regulation in bipolar cell lines.

Lithium also inhibits PI turnover leading to a reduction in the production of the second messengers $\text{Ins}(1,4,5)\text{P}_3$ and DAG (page 174). Although β -ARs are not coupled to stimulated polyphosphoinositide turnover, there are reciprocal interactions between the inositol lipid pathway and other second messenger pathways (Berridge, 1987), in particular the cyclic nucleotides (Nishizuka, 1984; 1986). It has been shown, for example, that the production of cAMP in the pineal by β -ARs is enhanced by the activation of α_1 -ARs and that this enhancement appears to be mediated through the DAG/PKC pathway because phorbol esters augmented the accumulation of cAMP by β -ARs in this tissue (Sugden *et al.*, 1985). Another example of such interactions is that isoprenaline-stimulated cAMP accumulation in intact human astrocytoma cells has been shown to be inhibited by carbachol, a muscarinic receptor agonist, via activation of phosphodiesterase (Tanner *et al.*, 1986). The possibility that lithium could exert its effects on the adrenergic system through a nonadrenergic mechanism (i.e. via inhibition of PI turnover) has been suggested by Schultz *et al.* (1981) from their investigations of Purkinje cell firing rates in chronic lithium-treated rats with and without pretreatment with 6-hydroxydopamine (6-OHDA) to remove endogenous cerebellar NA.

DAG stimulates PKC which has been implicated in the phosphorylation step of the process of β -AR desensitization (Yamashita *et al.*, 1986 & 1987), and Toews *et al.* (1987) demonstrated that in human astrocytoma cells PKC activation

can itself directly cause β -AR desensitization. A reduction in the stimulation of PKC by DAG could therefore also result in a decrease in β -AR desensitization and presumably down-regulation. However, Toews *et al.* (1987) concluded from their work that involved exposing astrocytoma cells to a PKC activator and/or to isoprenaline, that activation of PKC is probably not the mechanism of agonist-induced desensitization. In another study, Yamashita *et al.* (1988) demonstrated that acute activation of PKC caused a 50% decrease in β -AR-stimulated adenylyl cyclase activity due to a reduction in the V_{max} of the adenylyl cyclase system, whilst having no effect on β -AR number.

Other studies have also found that treatment of cells with phorbol esters, which activate PKC, can lead to β -AR desensitization (Sibley *et al.*, 1984; Kelleher *et al.*, 1984; Kassis *et al.*, 1985; Johnson *et al.*, 1986) which Kelleher's group, who used turkey erythrocytes, suggested was due to phosphorylation of the β -AR and therefore its uncoupling from Gs. In contrast, Kassis *et al.* demonstrated that in rat glioma C6 cells phorbol esters did not alter the activity of the receptors, but caused their internalization into a light membrane fraction devoid of Gs.

Caution must be taken in the interpretation of these and the following results as it has been suggested that the interaction between the inositol lipid and the adenylyl cyclase pathways may depend upon the cell types (Nishizuka, 1986). In cells such as platelets, neutrophils and lymphocytes, the signal-induced breakdown of inositol phospholipids and the subsequent events eventually leading to cellular responses are all profoundly blocked by cAMP. Conversely, in avian erythrocytes and C6 cells, PKC inhibits and desensitizes the adenylyl cyclase system, whereas in pinealocytes and S49 cells, PKC greatly potentiates cAMP production (Nishizuka, 1986). It is also important to note that the turkey erythrocyte β -AR does not become internalized during desensitization and that the result of β -AR phosphorylation in turkey erythrocytes and β -AR internalization rat C6 cells is in both cases an inability of the β -ARs to couple to Gs to activate adenylyl cyclase. However, in several studies phorbol esters caused an increase in β -AR-stimulated adenylyl cyclase activity (Bell *et al.*, 1985; Johnson *et al.*, 1986; Summers *et al.*, 1988). All the above studies employed acute incubation with phorbol esters (5-90 minutes).

The outcome of either increased or decreased adenylyl cyclase activity seems to depend on the exact conditions of the phorbol ester treatment (duration and concentration) and subsequent assay (concentration of hormone used to stimulate adenylyl cyclase) (Johnson *et al.*, 1986), and whether intact or broken cell preparations are used (Johnson & Toews, 1990) as well as the type of cell employed. Little information was given in these studies about the effect of PKC activation on the β -AR density. Kelleher *et al.* (1984) observed no change in β -AR number after 30 min phorbol ester treatment. Kassis *et al.* (1985) similarly did not see any change in β -AR number after 60 min however they did observe a β -AR loss of about 25% after prolonged (24 hour) phorbol ester treatment. This loss was much lower and less extensive than that induced by isoprenaline in the same cells (turkey erythrocytes).

It would seem likely that the acute effects on cAMP accumulation are not due to a change in receptor density but to the effects of PKC on adenylyl cyclase itself as Summers *et al.* (1988) have shown that the addition of purified PKC to pituitary membrane preparations causes an increase in cAMP synthesis and that PKC induces an apparent increase and stabilization of adenylyl cyclase activity in these preparations. They suggested that PKC may regulate cAMP production by altering an equilibrium between high and low activity states of adenylyl cyclase.

Another suggestion for the mechanism of interaction between PKC and cAMP production is that PKC may facilitate the interaction between Gs and adenylyl cyclase (Bell *et al.*, 1985). The interaction between the different pathways is reciprocal in that it has been shown that cAMP will inhibit the hydrolysis of $\text{PtIns}(4,5)\text{P}_2$ resulting in a marked reduction in both DAG and $\text{I}(1,4,5)\text{P}_3$ formation (Watson *et al.*, 1984; Nakashima *et al.*, 1986).

A possible hypothesis from consideration of the above studies is that if lithium is inhibiting PI turnover so causing a reduction in the production of DAG, this would result in a decrease in the activity of PKC and may therefore result in a decrease in isoprenaline-induced adenylyl cyclase desensitization and thus an increase in cAMP accumulation. This is the reverse both of what would be predicted from the down-regulation data, and of the aforementioned results of

other groups demonstrating lithium inhibition of cAMP accumulation. However, it must be remembered that as most of these studies investigated the acute effects of phorbol ester treatment, the relevance to the present study using 24 hour coincubation with isoprenaline and 7 day coincubation with lithium, must be questioned. Furthermore, the effect of lithium on cAMP accumulation may differ depending whether or not the β -AR is occupied by an agonist. In the situation of agonist occupancy by isoprenaline it may be that lithium causes a decrease in desensitization, and the resulting increase in isoprenaline-induced cAMP accumulation acts to increase receptor down-regulation in order to reduce adenylyl cyclase activity. It is interesting to note that phorbol ester- and isoprenaline-induced β -AR desensitization involve distinct mechanisms (Kassis *et al.*, 1985).

As mentioned previously, inhibition of PI turnover by lithium also leads to a decrease in the production of $\text{Ins}(1,4,5)\text{P}_3$ and therefore a reduction in the amount of calcium released from internal stores (Berridge, 1987). As calcium stimulates cAMP phosphodiesterase which breaks down cAMP (Tanner *et al.*, 1986), then a lowering of the free calcium levels will tend to increase cAMP levels in the cell. This in turn may lead to an increase in β -AR down-regulation. If PI turnover is selectively inhibited in bipolar patients as hypothesised by Berridge *et al.* (1982; 1989), then this increase in β -AR down-regulation would be expected to be seen only in the bipolar cell lines, which is in fact what was observed.

Another consequence of the inhibition of PI turnover by lithium may be an accumulation of PI. Incorporation of PI into turkey erythrocytes has been shown to inhibit isoprenaline-stimulated cAMP accumulation in a linear, dose-dependent manner whilst other phospholipids had little effect (McOsker *et al.*, 1983). This inhibition of cAMP accumulation was not accompanied by a decrease in either β -AR capacity or affinity, but the relative percentage of high affinity binding (a measure of coupled receptors) was decreased in PI-modified membranes.

McOsker's work suggests that it would be useful to investigate whether lithium coincubation affects the proportion of PI in lymphoblast cell membranes and if any effect was differential in respect to control and bipolar cell lines.

Furthermore, if specific [^{125} I]-CYP binding in control and bipolar lymphoblast cell lines was determined using a β -AR agonist to define non-specific binding, the effect of lithium coincubation on the proportions of high and low affinity binding sites could be examined to investigate whether lithium affects the coupling of β -ARs to Gs. There is evidence that the K_L/K_H ratio is increased by antidepressant therapy (Buckholtz *et al.*, 1988) which is opposite to the effect that would have been predicted from McOsker's study in turkey erythrocytes for lithium-induced PI accumulation.

According to the proposal of Berridge *et al.* (1982) that the therapeutic action of lithium in bipolar depression is due to a preferential depletion of cellular inositol levels and polyphosphoinositides in hyperactive pathways, it would be expected that there should be an increased rate of PI turnover in lymphoblasts from bipolar patients than from controls. In fact, Banks *et al.* (1990) demonstrated that there was significantly less incorporation of [^{3} H]-inositol into the phosphoinositides of lymphoblast cell lines derived from bipolar subjects than in those derived from control subjects, which would seem to indicate that either rate of PI turnover is less in bipolar patients or that the rate at which PtdI(4,5)P₂ is converted to inositol(1,4,5)P₃ is greater than the rate at which inositol is incorporated into phosphatidylinositols in bipolar patients. Banks *et al.* suggested that a smaller phosphoinositide pool size in bipolar subjects would result in a smaller signal being generated for each agonist event and/or a more rapid depletion of phosphoinositide pools on repeated stimulation. This, together with the inhibitory actions of lithium, may overcome any homeostatic mechanisms able to maintain Ins(1,4,5)P₃ and DAG and eventually bring about a reduction in signalling capacity. This hypothesis assumes that hyperactive pathways in bipolar depressed patients exist which are primarily responsible for the illness and that there is a defect in the amount or synthesis of phosphoinositides.

One problem with interpreting the results from Banks' study are that inositol incorporation into the phosphoinositides rather than PI turnover was measured. It would be interesting to investigate inositol turnover in our cell lines, to see if the rate of turnover is greater in the bipolar cell lines and whether lithium decreases the turnover rate.

CHAPTER SEVEN

THE EFFECT OF DESIPRAMINE ON β -ADRENOCEPTOR REGULATION IN LYMPHOBLASTS

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7.1 Introduction

Desipramine (DMI) is a secondary tricyclic antidepressant commonly used to treat depressed patients. Acute administration of DMI causes a decrease in NA turnover in rat brain (Bareggi *et al.*, 1978) due to inhibition of NA reuptake (Carlsson & Lindqvist, 1978) which activates a negative feedback mechanism that results in a reduction in NA neuronal impulse flow, and so a reduction in turnover (Nyback *et al.*, 1975). This inhibition of noradrenergic activation by NA appears to be mediated by α_2 -adrenergic autoreceptors (Cedarbaum & Aghajanian, 1977).

As the onset of the therapeutic action of DMI does not occur until after at least one week of treatment (Oswald *et al.*, 1972), the physiological and biochemical effects of acute DMI administration may not be directly relevant to its mechanism of antidepressant action. Chronic DMI administration has been shown to increase NA turnover (Bareggi *et al.*, 1978; Sugrue, 1980) and to depress the NA-neuron firing rate (McMillen *et al.*, 1980; Schultz *et al.*, 1981). Both acute and chronic DMI administration can enhance the amount of NA released and metabolised per neuronal impulse (McMillen *et al.*, 1980). Long-term DMI treatment causes a decrease in both NA- and isoprenaline-stimulated cAMP accumulation in rat brain slices, whilst acute treatment has no effect (Ventulani & Sulser, 1975; Frazer *et al.*, 1978; Wolfe *et al.*, 1978). This effect is unlikely to be due to a direct action of DMI on the adenylyl cyclase system because no change was observed in the activity of NA-stimulated adenylyl cyclase in the rat diaphragm, a tissue lacking sympathetic innervation (Frazer *et al.*, 1978).

Long-term DMI administration also causes a decrease in β -AR density in rat brain slices taken from the cerebral cortex (Sarai, 1978; Wolfe *et al.*, 1978; Minneman *et al.*, 1979a; Schweitzer *et al.*, 1979; Maggi *et al.*, 1980; Sellinger-Barnette *et al.*, 1980; Koshikawa *et al.*, 1987); hippocampus (Maggi *et al.*, 1980); cerebellum (Maggi *et al.*, 1980; Schultz *et al.*, 1981); and whole brain (Bannerjee *et al.*, 1977). In the cat chronic administration of imipramine (which is metabolized to DMI) was shown to decrease binding in cerebral cortex membrane preparations (Briley *et al.*, 1982). DMI administration to rats has been shown to selectively down-regulate β_1 -ARs in the brain with β_2 -ARs being relatively

unaffected (Minneman *et al.*, 1979a; Beer *et al.*, 1987; Stahl *et al.*, 1987; Heal *et al.*, 1989). In the light of this it may seem suprising that significant down-regulation has been observed in the rat cerebellum which has predominantly β_2 -ARs, though it was suggested by Schultz *et al.* (1981) that the radioligand, [^{3} H]-DHA, employed in their study and in that of Maggi *et al.* (1980), selectively binds to β_1 -ARs so that the observed down-regulation was due to loss of the β_1 -type receptors which comprise about 15% of the β -AR population in the rat cerebellum. β -AR down-regulation in the cerebellum in response to DMI was not seen by Beer and Stahl's group, who used a non-selective β -AR antagonist, [^{125} I]-iodopindolol, as the radioligand (Beer *et al.*, 1987; Stahl *et al.*, 1987).

The incubation of cortical slices from chronic desipramine-treated rats with guanine nucleotides does not restore β -AR binding as it does in acute isoprenaline-treated cortical slices (Wolfe *et al.*, 1978). Acute *in vitro* incubation of cortical slices with isoprenaline following *in vivo* desipramine treatment leads to a further decrease in the density of binding sites (Dibner & Molinoff, 1979). Hence, it would seem that receptor down-regulation by chronic desipramine and acute isoprenaline are mediated by different mechanisms. However, an *in vitro* study investigating the effect of acute coincubation of rat cortical slices with either tricyclic antidepressants and/or isoprenaline, showed that β -AR binding was decreased by both treatments individually and that the effects were not additive, suggesting that a similar mechanism was involved in receptor down-regulation due to acute *in vitro* coincubation with either antidepressants or isoprenaline (U'Pritchard & Enna, 1979).

Investigations have also been carried out into the *in vitro* effects of DMI on cells in culture. Honnegger *et al.* (1986) exposed three types of human cells - skin fibroblasts, lung fibroblasts and macrophages - to 2 and 5 μ M DMI for 12 days. They found that chronic DMI exposure induced a decrease in β -AR binding in all three tissue types, with no effect on binding affinity. Rat glioma C6 cells, cultured in the presence of DMI showed a time and concentration dependent decrease in β -AR number (Fishman & Finberg, 1987). Coincubations with DMI for two days at concentrations above 50 μ M were toxic to the cells. Seven day exposure to 10 μ M DMI caused a 43% loss of β -ARs and concentrations above

10 μ M were toxic after seven days. β -AR down-regulation in response to 3 hour coincubation with 1 μ M isoprenaline was not affected by 48 hour pretreatment with 50 μ M DMI. They concluded from their study that the mechanism of β -AR down-regulation by DMI did not involve a direct interaction with the receptors.

Manji *et al.* (1991) similarly found that five day coincubation of C6 cells with 10 μ M DMI resulted in a 35% reduction in binding capacity that was accompanied by a 38% loss in isoprenaline-stimulated cAMP accumulation. The reduction in Bmax was found to be accounted for mainly by a decrease in the high affinity form of the receptor. DMI also produced a significant reduction in the dissociation constant for the agonist in the high affinity state (K_H) and a significant increase in the K_L/K_H ratio. This ratio reflects the extent to which agonists stabilize the high affinity ternary complex of agonist, receptor and Gs, and has been demonstrated to strongly correlate with agonist-induced adenylyl cyclase activation (Kent *et al.*, 1980). In other words, the K_L/K_H ratio is thought to reflect two properties of the same coupling phenomenon, namely, the formation of the high affinity complex and stimulation of adenylyl cyclase. β -AR desensitization is usually accompanied by a decrease in the K_L/K_H ratio (Kent *et al.*, 1980), not an increase as occurred in this study. The authors suggested that DMI decreases the affinity of guanine nucleotides for Gs, thereby preventing the breakdown of the high affinity complex and subsequent activation of adenylyl cyclase.

As no previous study has investigated DMI exposure in cultured cells from bipolar depressive patients, the effects of chronic coincubation of control and bipolar lymphoblast cell lines with DMI and subsequent isoprenaline down-regulation, were examined.

7.2 Methods

Lymphoblast cell lines from 6 bipolar cell lines and 6 matched control cell lines were coincubated with 10 μ M DMI for 7 or 14 days. On day 6 or 13, 1nM isoprenaline and/or 0.1mM ascorbic acid were added to the cells. The cells were harvested 24 hours later and the β -AR binding assay was carried out using [¹²⁵I]-CYP as described previously.

7.3 Results

7.3.1 β -Adrenoceptor binding and affinity

Coincubation of lymphoblast cell lines with 10 μ M DMI for 7 and 14 days had no significant effect on the mean β -AR binding capacity in either control or bipolar cell lines when tested by analysis of variance (Table 7.1 and Fig. 7.1).

Individually, as shown by Fig. 7.2, one control cell line showed about a 50% loss of β -ARs after both 7 and 14 day DMI coincubations and two further control cell lines had receptor losses of 40 and 60 % after 14 day coincubation. In bipolar cell lines 7 day DMI coincubation caused decreases of 60, 30 and 20 % of the binding capacity in three cell lines, whilst 14 day coincubation resulted in 50, 40 and 20 % receptor loss also in three cell lines though not the same three.

Table 7.1 The effect of 7 and 14 day coincubations with 10 μ M DMI on β -AR binding capacity (Bmax) of lymphoblast cell lines derived from bipolar depressed patients and matched controls. Results are expressed in fmol/mg protein as mean $\pm$ sem.

Treatment	CONTROL		BIPOLAR	
	n	Bmax	n	Bmax
NO DMI	6	6.76 $\pm$ 0.79	6	5.49 $\pm$ 1.10
10 μ M DMI				
7 day	5	5.93 $\pm$ 0.89	5	4.51 $\pm$ 0.37
14 day	6	5.01 $\pm$ 0.62	6	4.69 $\pm$ 0.97

Fig. 7.1 Effect of 7 and 14 day coincubation with 10 μ M DMI on β -AR binding capacity in lymphoblasts from control and bipolar cell lines. Results are expressed as mean $\pm$ sem of 5-6 experiments.

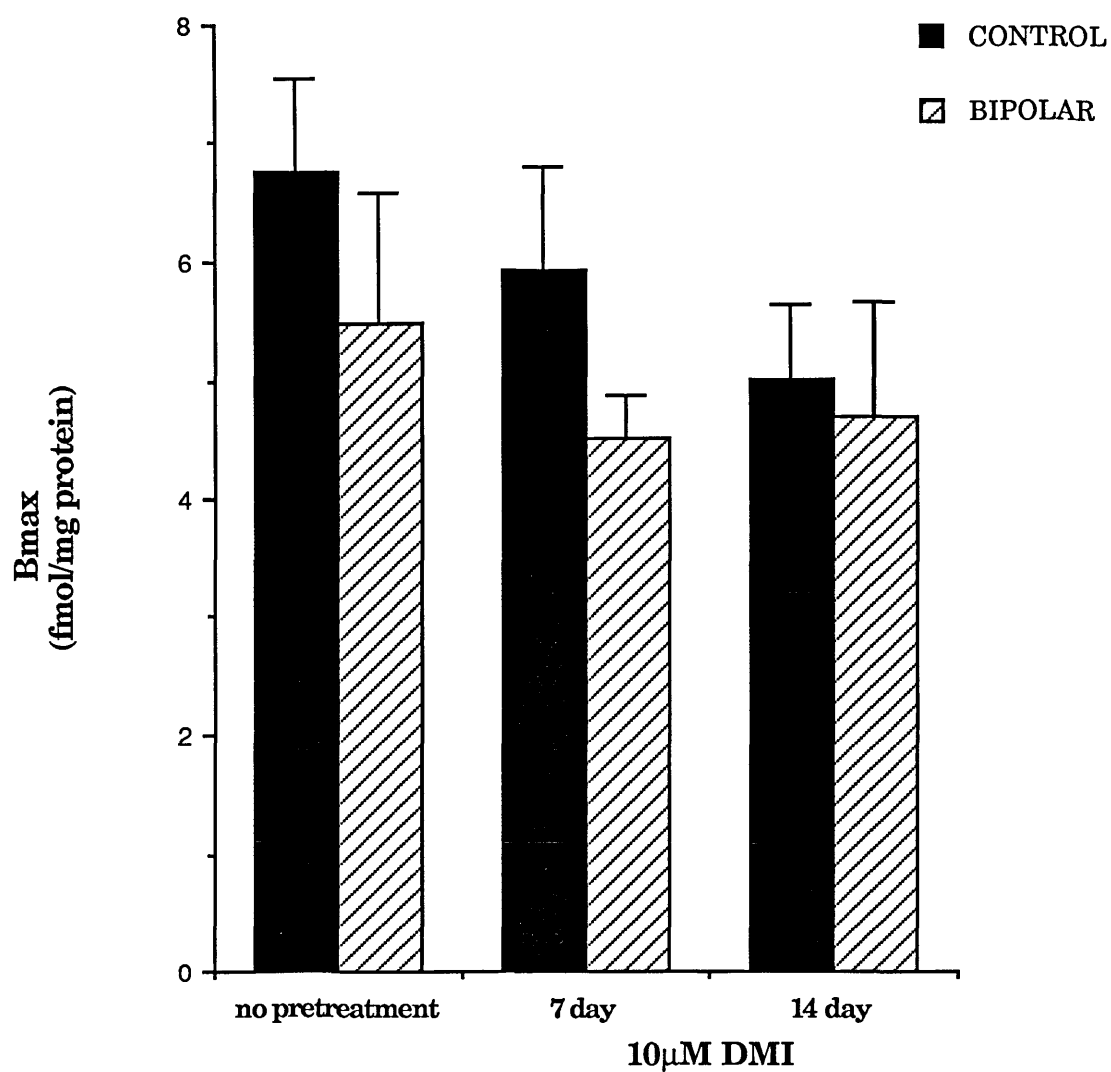


Fig. 7.2 Effect of 7 and 14 day coincubation with 10 μ M DMI on β -AR binding capacity in individual lymphoblast cell lines from controls and bipolar depressed patients.

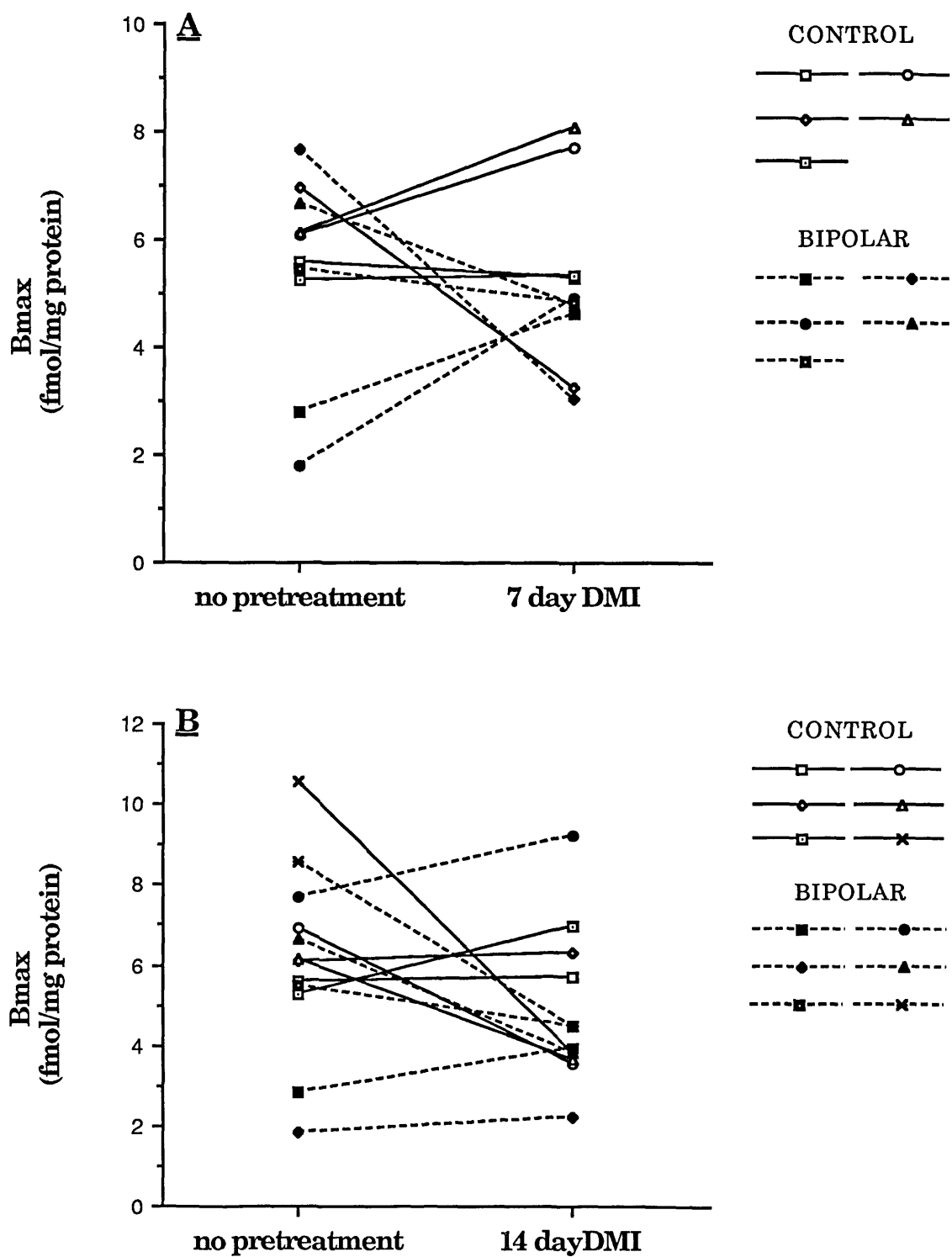
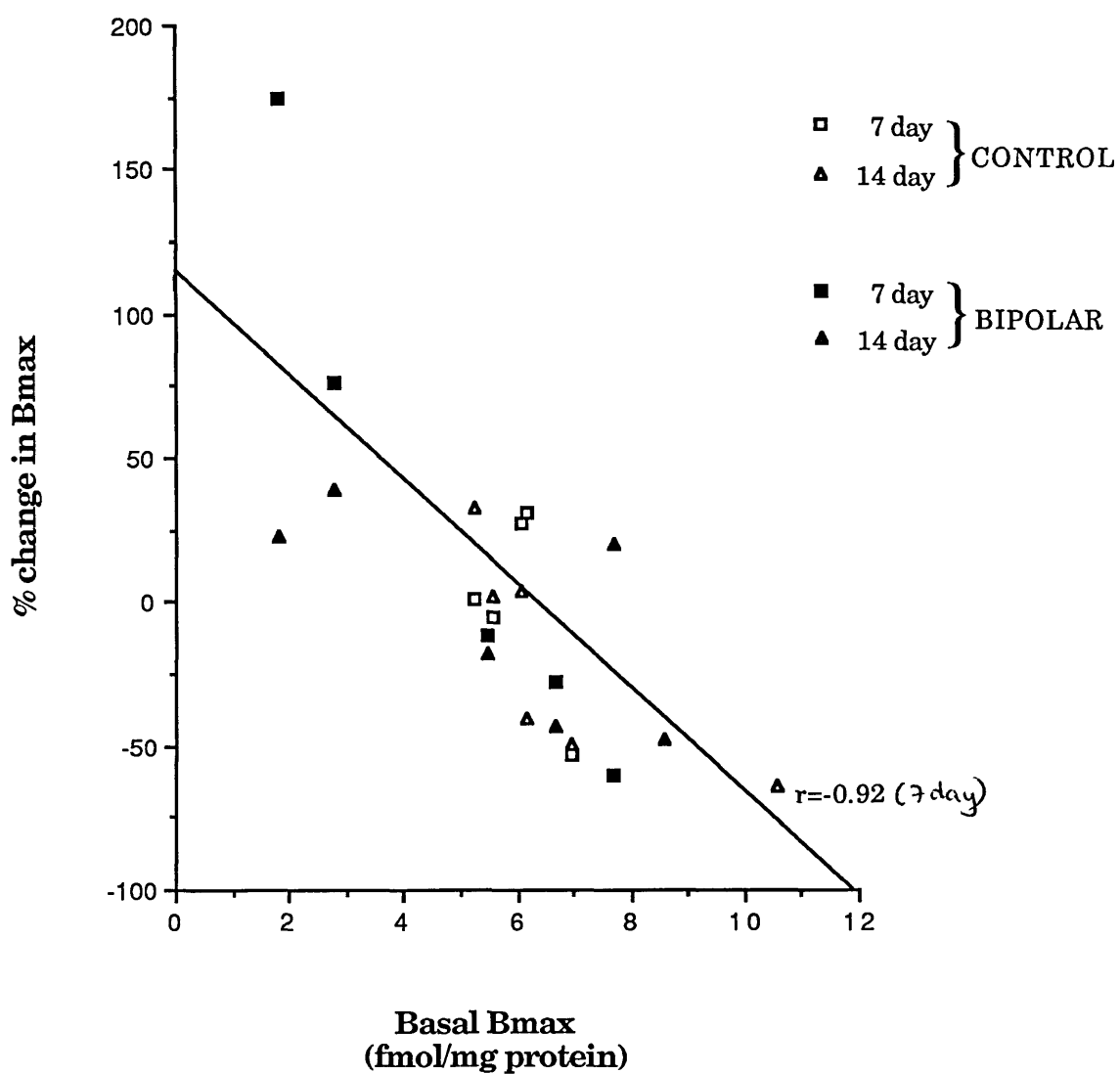


Fig. 7.3 % change in lymphoblast β -AR binding capacity after 7 and 14 day
coincubation with $10\mu\text{M}$ DMI against basal binding capacity for
control and bipolar cell lines.



For both 7 and 14 day DMI coincubation there was a negative correlation between the initial Bmax values and the % change in Bmax, with higher initial Bmax values resulting in greater receptor loss and low initial Bmax values resulting in an increase in receptor number (7 day, $r = -0.92$, $p < 0.001$; 14 day, $r = -0.73$, $p < 0.01$). If the control and bipolar groups were examined separately the correlation was only significant in the bipolar group with 7 day coincubation ($r = -0.96$, $p < 0.01$) (Fig 7.3).

In control cell lines there was a significant increase in binding affinity for both 7 and 14 day DMI coincubations ($p < 0.05$, paired Student's t test). There was no change in affinity in bipolar cell lines (Table 7.2).

Table 7.2 The effect of 7 and 14 day coincubations with $10\mu\text{M}$ DMI on β -AR binding affinity (K_d) of lymphoblast cell lines derived from bipolar depressed patients and matched controls. Results are expressed in pM as mean $\pm$ sem.

Treatment	CONTROL		BIPOLAR	
	n	Kd	n	Kd
NO DMI	6	19.6 ± 2.4	6	18.1 ± 2.7
$10\mu\text{M}$ DMI				
7 day	5	$11.4 \pm 1.5^*$	5	14.9 ± 3.8
14 day	6	$13.8 \pm 1.2^*$	6	15.1 ± 1.4

* $p < 0.05$ compared to no DMI (paired t -test)

7.3.2 β -Adrenoceptor down-regulation in response to isoprenaline

Table 7.3 shows the effects of coincubation with $10\mu\text{M}$ DMI for 7 and 14 days on the down-regulation in response to 1nM isoprenaline. As before, in cells without any pretreatment, there was significantly less down-regulation in bipolar cell lines than in control cell lines ($p < 0.02$, Student's unpaired t -test).

Table 7.3 The effect of 7 and 14 day coincubation with 10 μ M DMI on β -AR down-regulation in response to 24 hour coincubation with 1nM isoprenaline in lymphoblast cell lines derived from bipolar depressives and matched controls. Results are expressed as % of basal Bmax value (ascorbic acid alone) and as mean $\pm$ sem.

Treatment	CONTROL		BIPOLAR	
	n	% down-regulation	n	% down-regulation
NO DMI	6	41.7 $\pm$ 5.3	6	16.2 $\pm$ 6.5**
10 μ M DMI				
7 day	5	33.8 $\pm$ 7.5	5	31.4 $\pm$ 8.9
14 day	6	26.8 $\pm$ 5.6	6	40.7 $\pm$ 7.5*

* p < 0.07 compared to patient, no DMI (paired t-test)

** p < 0.02 compared to control (unpaired t-test)

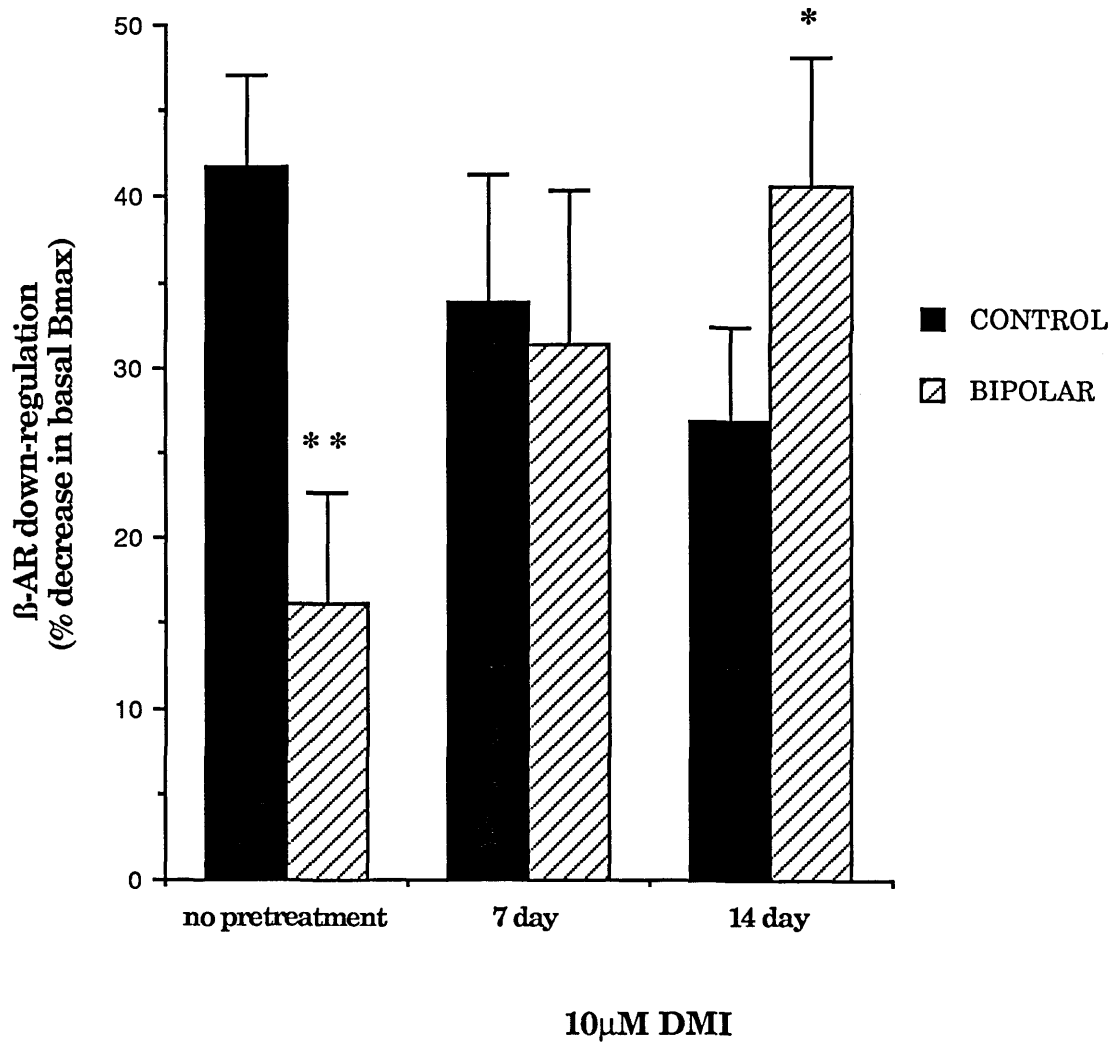
p < 0.02 interaction between treatment and group (2-way ANOVA)

After 7 days coincubation with DMI, there was a slight decrease in the loss of β -ARs in control cell lines and an increase in bipolar cell lines, however neither of these changes were significant when tested by two-way analysis of variance (Fig 7.4). After 7 days DMI pretreatment there was no longer a significant difference in down-regulation in response to isoprenaline between control and bipolar cell lines.

When the lymphoblasts were coincubated with DMI for 14 days, the extent of isoprenaline-induced down-regulation decreased further in control cell lines and increased in bipolar cell lines. Again there was no significant difference between the isoprenaline-induced down-regulation after 14 day DMI coincubation in control and bipolar cell lines. The difference in isoprenaline-induced β -AR loss in bipolar cell lines that were pretreated for 14 days with DMI compared to untreated lymphoblasts was of borderline significance (p<0.07, Student's paired t-test).

When the data was analysed by two-way analysis of variance, there was a significant interaction between the effect of 14 day DMI coincubation on isoprenaline-induced down-regulation and whether the lymphoblasts were derived from control or bipolar subjects ($p < 0.02$).

Fig. 7.4 Effect of 7 and 14 day coincubation with 10 μ M DMI on isoprenaline-induced β -AR down-regulation in lymphoblasts from control and bipolar cell lines. Results are expressed as mean $\pm$ sem of 5-6 experiments.



* p<0.07 v. bipolar, no pretreatment
 ** p<0.02 v. control
 p<0.02 interaction between treatment and group (2-way ANOVA)

7.4 Discussion

Unlike previous studies in rat brain slices and in cultured cells, no significant reduction in lymphoblast β -ARs was seen when the cells were coincubated with 10 μ M DMI for either 7 or 14 days.

The loss of β -ARs in rat brain slices after chronic DMI administration has been shown to be dependent on the presence of intact sympathetic innervation, implying that DMI exerts its effects by blockade of the neuronal NA uptake system thus causing an increase in the effective amount of NA in the synapse (Frazer *et al.*, 1978; Wolfe *et al.*, 1978; Schweitzer *et al.*, 1979). Therefore the lack of β -AR down-regulation in cultured lymphoblasts is perhaps not surprising as such cells are not innervated. However this is not sufficient as an explanation, since down-regulation in response to chronic DMI coincubation has been demonstrated in other cultured cells (Honegger *et al.*, 1986; Fishman and Finberg, 1987).

It is possible that the concentration of DMI used was too low, however this was unlikely as Honegger *et al.* (1986) demonstrated significant receptor down-regulation in response to 12 day coincubation with 2 μ M DMI in human fibroblasts and macrophages and Fishman and Finberg (1987) also saw significant receptor loss in C6 cells coincubated with 10 μ M DMI for 7 days. Based on the latter's results, concentrations above 10 μ M would probably have been toxic to the cells.

Another, more probable, explanation of the lack of DMI-induced down-regulation, is the fact that the lymphoblast β -AR is of the β_2 subtype, whereas desipramine has been shown to selectively down-regulate β_1 -ARs (Minneman *et al.*, 1979; Stahl *et al.*, 1987; Beer *et al.*, 1987). The adrenoceptors on glioma C6 cells are predominately (80-90 %) of the β_1 subtype (Homburger *et al.*, 1981) so are down-regulated by DMI (Fishman & Finberg, 1987). However, β -AR loss was seen in fibroblasts in which the β -ARs are of the β_2 -subtype (Honegger *et al.*, 1986).

A reduction in β -AR number following DMI coincubation was seen in some cell lines, regardless of whether the lymphoblasts were from control or

bipolar subjects. There was a negative correlation between the binding capacity in untreated lymphoblasts and the % change in β -AR number after 7 and 14 day coincubation with DMI, with cell lines with a high receptor density showing down-regulation and those with a low receptor density actually having an increase in β -AR number.

Although other groups did not observe any change in binding affinity with DMI treatment, this study demonstrated a significant increase in affinity for [^{125} I]-CYP in control cell lines both with 7 day and 14 day DMI coincubation. This would seem to indicate that DMI is having a direct effect on the β -AR, but as no change in affinity was seen in bipolar cell lines, this is perhaps unlikely.

It would be useful to extend the data to the remaining cell lines as it is difficult to reach any conclusions on the basis of six control and six bipolar cell lines. It would also be interesting to investigate the adenylyl cyclase response after DMI coincubation.

It has been suggested that the effect of DMI on β -AR density in C6 cells is due to its toxicity under certain culturing conditions. If conditions under which DMI had no effect on cell proliferation were employed, then no down-regulation was observed (Fowler & Brännström, 1990). Serum-free media were used in this study, which may in part account for the high toxicity. In the present study, no effect of DMI was observed on cell growth and there were no differences in the protein content of the membrane homogenates with or without DMI treatment. This would seem to be in support of Fowler and Brännström's hypothesis that β -AR loss is a toxic effect of DMI except that there were some cell lines in which DMI coincubation did cause a reduction in β -AR number, and a reduction in cell proliferation was not observed in these cell lines either. Furthermore, the toxic effects seen by Fowler and Brännström may be limited to C6 cells as an increase in cell proliferation was observed when fibroblasts were incubated with 10 μ M DMI for seven days (Honegger *et al.*, 1983).

β -AR down-regulation in response to isoprenaline in control and bipolar cell lines was differentially affected by DMI coincubation. After 7 day DMI

coincubation there was a trend towards a decrease in down-regulation in control cell lines and an increase in bipolar cell lines. This trend was clearer after 14 day coincubation, and the differential effect of DMI of a decrease in control and an increase in bipolar cell lines was significant.

These results are in contrast to those of Fishman and Finberg (1987) who found that 48 hour pretreatment with DMI had no effect on down-regulation in response to 3 hour coincubation with isoprenaline. However, as the effect of DMI is time-dependent, it is likely that 48 hours pretreatment was not long enough. Furthermore, the rat C6 cell line was used so there may be species and receptor sub-type differences.

As with lithium coincubation, DMI seems to be normalising the regulation of β -ARs in bipolar cell lines. DMI pretreatment also decreases the extent of down-regulation in response to isoprenaline in control cell lines, bringing it almost to the level seen in bipolar cell lines without DMI treatment. Again, extension of these results to the other cell lines and investigation of the adenylyl cyclase response would help to clarify the situation.

Chronic coincubation of fibroblasts with DMI has been shown to produce intracellular granulation, possibly by interfering with the recycling of plasma membrane vesicles, as the DMI accumulates in the cells (Honegger *et al.*, 1983). Uptake of DMI was independent of the amount of DMI previously accumulated in the cell, indicating that the DMI was being segregated into a cellular compartment. If this is also the case in lymphoblasts, then the accumulation of DMI in the cells would depend upon the duration of exposure to DMI. This could be an explanation of the time-dependency of the effects of DMI on isoprenaline-induced down-regulation. This clear time-dependency for the effect of DMI on β -AR regulation coincides well with the time-dependency of the development of the therapeutic effect of DMI (Oswald *et al.*, 1972; Garver & Davis, 1979).

Like lithium, DMI has effects on phospholipid metabolism due to its cationic and amphiphilic nature (Fauster *et al.*, 1983). Coincubation of human

fibroblasts with DMI for longer than 6-12 hours resulted in a dose- and exposure-dependent accumulation of phospholipids, particularly phosphatidylinositol (PI), due to a decrease in the breakdown of phospholipids in the presence of DMI, and therefore the rapid turnover of PI was inhibited by DMI (Fauster *et al.*, 1983). It has also been shown in C6 cells that the incorporation of $^{32}\text{P}_i$ into total phospholipids was increased by coincubation with DMI by a saturable process (Leli & Hauser, 1987). This increase was accompanied by a shift of the pattern of the incorporation of $^{32}\text{P}_i$ from neutral phospholipids, such as phosphatidylethanolamine (PE) and phosphatidylcholine (PC), to acidic phospholipids such as PI, phosphatidylinositol 4-phosphate and $\text{PI}(4,5)\text{P}_2$. As described in the previous chapter, an increase in the relative proportion of PI in turkey erythrocytes has been shown to inhibit isoprenaline-stimulated cAMP accumulation due to an uncoupling of β -ARs (McOskey *et al.*, 1983). It has also been demonstrated that PE and PC are required for the efficient reconstitution of the β -AR-Gs system (Schramm *et al.*, 1988), so a reduction in their relative proportions in the membrane could effect the coupling of β -AR to Gs. It would be useful to investigate whether DMI coincubation has a differential effect on the proportion of PI in lymphoblast cell membranes from control and bipolar cell lines; the DMI coincubation time necessary for the accumulation of PI; whether an increase in PI in lymphoblasts will inhibit isoprenaline-stimulated cAMP accumulation; and the extent of PI accumulation needed for this effect to be seen.

Furthermore, if specific [^{125}I]-CYP binding in control and bipolar lymphoblast cell lines was determined using a β -AR agonist to define non-specific binding, the effect of DMI coincubation on the proportions of high and low affinity binding sites could be examined to investigate whether DMI affects the coupling of β -ARs to Gs and whether any observed effect is dependent on subject status. If the K_L/K_H ratio is increased by DMI coincubation as has been observed in C6 cells coincubated with $10\mu\text{M}$ DMI for 5 days (Manji *et al.*, 1991), this may explain the increase in isoprenaline-induced down-regulation.

This work brings into question the relative validity of using animal models as compared to human cell culture models. Animal models have mainly been concerned with the effects of antidepressants on the rat brain, particularly the

cerebral cortex. Since DMI has been shown to selectively down-regulate β_1 -ARs, and this subtype is predominant in rat cerebral cortex and in the human brain, the rat brain would seem to be more suitable for such investigations than the lymphoblast β -AR which is of the β_2 -type. However, the relative β_1/β_2 selectivity of DMI only has been assessed in the rat brain and may not be relevant to the human brain. Also DMI administration does not reduce central β -AR density in all species: in the guinea-pig central β -AR binding has been shown to be unaffected by chronic DMI treatment (Hu *et al.*, 1980). Furthermore, as it is clear from the present study that DMI does have an effect on the regulation of β_2 -ARs, the selectivity of DMI may only be relevant to its direct effect on β -AR number.

Another important disadvantage of the rat brain model is that only the effect of DMI on "normal" brain tissue can be investigated. This is particularly relevant in the light of the results of this study indicating that DMI has a differential effect on lymphoblasts derived from control and bipolar depressed subjects, with isoprenaline-induced down-regulation being decreased in control cell lines and increased in bipolar cell lines.

The lymphoblast β -AR model also has limitations, not least of which its lack of innervation. It has been suggested that DMI-induced β -AR down-regulation is in response to the increase in synaptic NA due to reuptake inhibition by DMI, as chemical sympathectomy abolished DMI-induced down-regulation in rat brain (Wolfe *et al.*, 1978; Schweitzer *et al.*, 1979). However, this cannot be the only mechanism of β -AR down-regulation by DMI as DMI causes down-regulation in cultured fibroblasts, macrophages and glioma cells. The lack of innervation is in some ways an advantage though, as it allows the direct effects of DMI on the β -AR-adenylyl cyclase complex itself to be examined in isolation from any effects on NA release or reuptake.

CHAPTER EIGHT

DISCUSSION

The lymphoblast provides a useful model for investigating β -AR binding characteristics and regulation in affective disorders and the effect of antidepressants on the β -AR. This study has demonstrated that the lymphoblast β -AR has binding characteristics similar to the lymphocyte β -AR with displacement curves typical of the β_2 -AR and specific [^{125} I]-CYP binding affinity of 15-20 pM. This receptor forms part of the β -AR-G protein-adenylyl cyclase complex, the activation of which leads to cAMP production.

The lymphoblast β -AR undergoes agonist-stimulated down-regulation and desensitization in a similar manner to the lymphocyte β -AR. Coincubation with isoprenaline results in time and concentration dependent down-regulation of the β -AR and desensitization of isoprenaline-stimulated cAMP accumulation. The manner of this functional desensitization is also time and concentration dependent, as short incubations with low isoprenaline concentrations cause homologous desensitization whereas longer incubation or higher isoprenaline concentration result in heterologous desensitization. 24 hour incubation with 10^{-9} M isoprenaline resulted in a loss of about 50% of the β -ARs, which was accompanied by a 35% loss of the β -AR-coupled cAMP response, with no effect on adenylyl cyclase activity in response to prostaglandin E_1 or forskolin.

There was no difference in specific [^{125} I]-CYP binding capacity or affinity between lymphoblast cell lines derived from control subjects or patients with bipolar depression. These results were not as predicted in Chapter One based on reports of the lymphocyte β -AR in depression, but confirm the results of Berrettini *et al.* (1987). However, as in Wright's study (1984), there were some bipolar cell lines with particularly low β -AR binding capacity (< 3 fmol/mg protein). The lowest binding capacity in the BP group was 1.80 fmol/mg protein, whereas that in the control group was 3.55 fmol/mg protein. If this finding is taken together with that of Wright's group that β -AR binding capacity was significantly reduced in four out of six bipolar cell lines, it would suggest that there may be a reduction in the density of β -ARs in a sub-group of bipolar patients. This may be due to a defect in a gene, or genes, controlling either β -AR synthesis and/or degradation or receptor expression.

β -AR down-regulation in response to 24 hour coincubation with 10^{-9} M isoprenaline was significantly lower in bipolar cell lines than in control cell lines. This indicates that there is a defect in the process of down-regulation in these cell lines. Such a defect complicates the explanation as to why reduced binding capacity has been observed in lymphocytes from depressed patients, whilst in this study lymphoblast β -AR binding was no different from control. The reduced sensitivity to isoprenaline-induced β -AR down-regulation seen in lymphoblasts from BP patients suggests that increased plasma catecholamine levels in depression are unlikely to cause lymphocyte β -AR down-regulation. There are two possible explanations of this paradox. Firstly, plasma catecholamine levels may be high enough to overcome the reduced sensitivity, so lymphocyte β -AR binding would still be lower in depressed patients than controls. This is perhaps unlikely as increased plasma catecholamine levels have not been observed in all studies. A second possibility is related to the patient groups under study, as most studies of lymphocyte β -AR binding have investigated depressed patients, with diagnoses of UP affective disorder, whereas the present study investigated patients with BP affective disorder only. A recent study (Jeanningros *et al.*, 1991), in which lymphocytes from UP depressed patients had reduced β -AR density compared to controls whereas those from BP patients did not, supports this idea. The results of Buckholtz *et al.* (1988) indicated that agonist-induced down-regulation might be reduced in BP disorder as plasma NA levels were higher in BP patients than in UP patients whilst β -AR number was the same in the two groups.

The three BP cell lines with β -AR binding capacities of less than 3 fmol/mg protein showed virtually no down-regulation (< 10% receptor loss) in response to isoprenaline coincubation. As there was a correlation in bipolar cell lines between binding capacity and the extent of down-regulation, which was probably due in the main to these three cell lines plus one cell line with a particularly high β -AR density that showed a greater degree of down-regulation than its matched control, there may be a defence against down-regulation when the binding capacity is very low. This is unlikely to be the main cause of the defect in down-regulation as the correlation does not hold for control cell lines and binding capacity was not lower in the bipolar group than the control group.

As described in the first chapter, there is strong evidence that receptor-Gs coupling is crucial to β -AR down-regulation (Mahan *et al.*, 1985; Campbell *et al.*, 1991), so it may be that this coupling is abnormal in bipolar disorder. The extent of β -AR-Gs coupling can be assessed in a binding study by defining non-specific binding with a β -AR agonist and measuring the K_L/K_H ratio. Buckholtz *et al.* (1988) found that this ratio was increased in the lymphocytes of BP patients compared to UP patients in spite of the fact that plasma NA was higher in the BP group, which suggests a dissociation between agonist concentration and the K_L/K_H ratio measure of sensitivity. Such a dissociation may play a part in the reduced down-regulation seen in response to isoprenaline in bipolar cell lines. A comparison of the K_L/K_H ratio in control and BP cell lines would determine whether β -AR coupling to Gs is different in lymphoblasts derived from BP patients. Correlations between the K_L/K_H ratios before and after isoprenaline coincubation and the extent of down-regulation in response to isoprenaline in BP and control lymphoblast cell lines may help to explain the defective down-regulation observed in BP cell lines.

β -AR phosphorylation induced by nanomolar concentrations of isoprenaline has been shown to be due to PKA alone, whereas that induced by micromolar isoprenaline concentrations involves both PKA and β ARK (Hausdorff *et al.*, 1989; Lohse *et al.*, 1989; 1990). Phosphorylation by PKA is cAMP-dependent, so a defect in the β -AR-adenylyl cyclase complex in lymphoblasts from bipolar patients could be expected to result in reduced β -AR phosphorylation in response to coincubation with 10^{-9} M isoprenaline. However, these studies were carried out using short periods of coincubation in hamster fibroblasts and the human A431 cell line, and it is not known at present whether longer periods of exposure to low isoprenaline concentrations would similarly involve only PKA and whether phosphorylation of the lymphoblast β -AR is by the same mechanism. If β ARK, which is cAMP-independent, were to be involved in the phosphorylation that occurs when the receptor is exposed to nanomolar isoprenaline concentrations for 24 hours, Hausdorff's findings would suggest that part of the phosphorylation process would still be due to PKA. This implies that a reduction in isoprenaline-induced cAMP accumulation could still reduce the extent of β -AR phosphorylation and desensitization. Whether a reduction in receptor

phosphorylation could be the cause of the reduction in β -AR down-regulation is uncertain but perhaps is unlikely as β -AR down-regulation has been shown not to require phosphorylation either by PKA (Mahan *et al.*, 1985) or by β ARK (Hausdorff *et al.*, 1991). It does suggest though that desensitization may also be defective in these bipolar cells lines.

The proposal that bipolar depression is associated with a defect in β -AR down-regulation is reinforced by the findings of this study that coincubation of the lymphoblasts with lithium, an effective drug in the treatment and prophylaxis of this disorder, normalizes down-regulation in response to isoprenaline. It has been suggested that lithium has a direct effect on G proteins that causes attenuation of stimulated cyclase activity (Avissar *et al.*, 1988) and that altered G protein function may be of pathophysiological importance in BP disorder (Schreiber *et al.*, 1991). Such an effect on cAMP production would be expected to decrease cAMP-dependent phosphorylation and possibly down-regulation. This may explain the slight decrease in down-regulation observed in control cell lines, but cannot account for the increase in BP cell lines. Berridge (1982 & 1989) has hypothesized that PI turnover is selectively inhibited by lithium in BP disorder. Such inhibition would result in a decrease in calcium release from internal stores and thus a decrease in phosphodiesterase activity (Berridge, 1987), which could in turn lead to an increase in cAMP levels. The resulting increase in cAMP-dependent β -AR phosphorylation would cause an increase in desensitization and possibly down-regulation.

Another consequence of lithium inhibition of PI is a decrease in the activity of PKC. This suggests that isoprenaline-induced adenylyl cyclase desensitization would be decreased and thus cAMP accumulation would increase leading to increased cAMP-dependent β -AR phosphorylation, desensitization and down-regulation as above.

It is difficult to extrapolate from studies of the effects of lithium on animals or cultured cells that are "normal" as this study has shown that there is a differential effect of lithium on control and BP cell lines, so results from such

studies may be in the opposite direction from what would have occurred in BP patients or in cell cultures derived from them.

Coincubation with DMI has similarly been shown to have a differential effect on β -AR down-regulation in lymphoblasts. In BP cell lines, DMI caused an increase in down-regulation whereas in control cell lines it caused a decrease. This differential effect was significant after 14 days coincubation. DMI has been shown to inhibit lysosomal phospholipid degradation (Honegger *et al.*, 1983). Alterations in membrane phospholipid composition can modulate the coupling of β -ARs to adenylyl cyclase and hence the number of functional receptor sites (Fauster *et al.*, 1983). Antidepressant drug treatment, which included DMI, has been shown to increase the K_L/K_H ratio in lymphocytes from depressed patients (Buckholtz *et al.*, 1988) as has coincubation of C6 cells with DMI (Manji *et al.*, 1991). Thus increased sensitivity of the β -AR to agonists may be responsible for the increase in down-regulation in response to isoprenaline seen in BP cell lines, although this does not explain the decrease in control cell lines. Assessment of the effect of DMI coincubation on K_L/K_H ratios with and without coincubation with isoprenaline in BP and control lymphoblast cell lines may help to elucidate the mechanism whereby DMI interacts with isoprenaline-induced down-regulation in BP and control cell lines. Another possible explanation for DMI's effects on isoprenaline-induced down-regulation is suggested by the fact that rapid turnover of PI has been shown to be inhibited by DMI in fibroblasts (Fauster *et al.*, 1983) and so DMI may act through the PI system in a similar way to lithium.

The lack of a direct effect of either lithium or DMI on β -AR density in our lymphoblast model contradicts the hypothesis that β -AR down-regulation is the primary mechanism of action of antidepressants (Sulser *et al.*, 1978). However, this hypothesis has already been questioned as there are several antidepressants that do not cause β -AR down-regulation. Mianserin and nioxetine, for example, cause desensitization of the β -AR-coupled cAMP response without having any effect on β -AR number (Sugrue, 1983). This would suggest that it is important to ascertain whether lithium or DMI cause desensitization in lymphoblast cell lines and that it would be very interesting to test out atypical antidepressants such as mianserin and nioxetine in the lymphoblast model of β -AR down-regulation.

These antidepressants may also increase isoprenaline-induced down-regulation in BP cell lines. DMI has been shown to cause selective down-regulation of β_1 -ARs in the rat cerebral cortex (Minneman *et al.*, 1979a; Beer *et al.*, 1987; Stahl *et al.*, 1987). This does not however rule out the possibility that DMI has a different effect on β_2 -ARs, such as facilitation of isoprenaline-induced down-regulation, or that its effects on human β -ARs are different to those seen in rats. Interestingly, there is little evidence of antidepressant-induced decreases in β -AR density from brain *post-mortem* studies of drug-free and antidepressant-treated suicides or from studies of melatonin secretion in depression (see Chapter One) or indeed that central β -AR density in depressed, drug-free suicides is increased, which would be expected if the consequence or mechanism of therapeutic action of antidepressants is down-regulation.

Much of the evidence of antidepressant-induced β -AR down-regulation in the brain has come from studies in the rat, which, in the main, examined the cerebral cortex and in some cases, the limbic forebrain. It is of interest to note at this point that when comparisons were made between the binding characteristics of the α_2 -AR in the rat cerebral and in the human cerebral cortex and between the human platelet and the human cerebral cortex, species differences were deemed to be greater than tissue differences (Summers *et al.*, 1983). Not only is it difficult to extrapolate such effects in healthy rats to humans with affective disorders, particularly as species differences have been demonstrated (Hu *et al.*, 1980), but also the emphasis on the cerebral cortex may mean that effects in other brain areas are overlooked. Vetulani and Sulser's group examined the limbic forebrain as they felt that this area provides a key integrating system related to selective modulation of emotion and sensory mechanisms of the brain (Sulser *et al.*, 1978). In human studies, the choice of brain area is crucial as reduced β -AR density in depressed, drug-free suicides has been observed in some areas (temporal cortex and occipital cortex) but not others (hippocampus, amygdala and thalamus) (De Paermentier *et al.*, 1990; Horton, 1991).

The major questions that must be addressed in the evaluation of a peripheral model as a biochemical marker for affective disorders are as follows:- firstly, does the model represent a state or trait marker and to what extent is it

effective in diagnosis; secondly, within which sub-categories of affective disorder is the model reliable; next, how does the observed difference between healthy subjects and patients with the affective disorder arise and can this information improve our understanding of the biochemical changes associated with affective disorder; and finally, does the model reflect similar changes in the brain. As regards the model of reduced sensitivity of the lymphoblast β -AR to agonist-induced down-regulation in BP affective disorder proposed in this study, these questions can only be answered in part at present. This model represents a trait-dependent marker and as such could be useful in diagnosis, or in the identification of individuals at risk. Although it is not possible as yet to know how such a difference arises, the normalization of the isoprenaline-induced down-regulation observed after coincubation with lithium and with DMI, suggests that this model is relevant in the treatment of BP disorder and so is likely to represent biochemical differences that are important in this disorder.

The unanswered questions provide the basis of further research into this model and its relevance as a biochemical marker of affective disorder. Investigation into lymphoblast β -AR regulation in cell lines derived from UP depressed patients would demonstrate whether this model is also applicable as a marker in this sub-category. This may be particularly interesting as lithium, the drug of choice in BP disorder, had a greater effect on β -AR regulation in patient cell lines than did DMI, an antidepressant. This suggests either that reduced isoprenaline down-regulation is more likely to be a marker for BP disorder than UP disorder, or that the effect of these drugs is dependent on the affective disorder sub-category of the patient, so that lithium will have a greater effect on lymphoblasts derived from BP patients while DMI might have more effect on lymphoblasts derived from UP patients. It would also be interesting to investigate whether patients whose lymphoblast β -ARs do not respond to lithium or DMI with an increase in isoprenaline-induced down-regulation are refractory to lithium or DMI drug therapy.

Further evidence is needed to be able to conclude that this model is a consistent trait-dependent marker. It is important to investigate the families of the probands used in this study to see whether reduced isoprenaline down-

regulation in lymphoblast β -ARs is seen in other family members. If this model is a trait-dependent marker, then defective down-regulation should be present in relatives who also have affective disorder, but not in those who do not. It has been proposed by Hill *et al.* (1988) that genetic linkage studies that subtype patients according to biological markers may be more successful than those with patients subtyped according to diagnostic categories as the former might be more closely related to gene action. Furthermore, they suggested that genetic analyses should be more powerful when classifications of affective disorders are based on such biological measures or on direct observations of behaviours that more closely reflect them. According to this argument, lymphoblast β -AR down-regulation in affective disorders may provide a useful marker for linkage studies.

It is, of course, important to establish whether β -AR down-regulation is also defective in the brain of BP patients. In practice, this is extremely difficult to do. It would be possible to coincubate brain slices from depressed, drug-free suicides and from matched controls with isoprenaline, carry out a radioligand binding assay and so measure the extent of down-regulation. The brain slices would need to be washed well to remove endogenous catecholamines and hormones and to have a period of preincubation to ensure that β -AR levels had recovered from any effects of endogenous substances, although it is unlikely that resynthesis of down-regulated receptors could occur within the survival time of the tissue *in vitro*. Similarly, 24 hour isoprenaline coincubation times would not be feasible so it perhaps would be necessary first to establish whether a significant difference in isoprenaline down-regulation between patients and controls could be observed in lymphoblasts using shorter coincubation times. The effects of drug treatment could be investigated by comparing drug-free and antidepressant- or lithium-treated suicides. As BP disorder is a sub-category of affective disorder, it would take a long time to amass sufficient data from drug-free suicides with a firm diagnosis of BP disorder, so it would be more practical to extend the investigation to include UP depression.

A more feasible way of examining central β -AR number could be by positron emission topography (PET). A PET-scan following agonist challenge might reveal differences in β -AR regulation between control subjects and bipolar

patients, and between drug-free patients and antidepressant- or lithium-treated patients.

Another way to investigate central β -AR regulation might be to measure melatonin secretion after an injection of isoprenaline in patients and matched controls. Results from such a study could be difficult to interpret as isoprenaline would be expected to have many effects in such circumstances and the pathways involved from injection to effect would be numerous.

Further experiments using this model would be in four directions. Firstly, the data should be expanded to include more cell lines, more data for the effects of coincubation with DMI and hydrocortisone, and the investigation of the effects of other antidepressants, particularly those that do not cause down-regulation in the rat model, and clenbuterol which has been shown to cause β_2 -AR down-regulation in the rat cortex (Heal *et al.*, 1989). Secondly, investigation into the mechanisms involved in the reduced isoprenaline down-regulation response by examining the K_L/K_H ratio, isoprenaline down-regulation dose response (see page 164), PI turnover, and PI accumulation (see page 185). Thirdly, a more suitable and accurate assay of adenylyl cyclase activity or cAMP accumulation in lymphoblasts needs to be established in order to investigate whether changes in β -AR down-regulation are reflected by changes in desensitization of β -AR-coupled adenylyl cyclase. Finally, the investigation of Gs proteins and of the kinases, PKA, PKC and β ARK involved in desensitization, in BP disorder may reveal differences that can account for the defect in isoprenaline-induced down-regulation.

While the lymphoblast β -AR model, like others, has many limitations, it does allow precise biochemical investigation of receptor function in cells derived from patients with identified medical history and may therefore prove particularly useful in the search for biochemical dysfunction associated with affective disorders.

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