614

COMPARATIVE STUDIES ON PROTEIN KINASE C ISOTYPES

by

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This thesis is submitted in partial fulfilment
for the degree of Doctor of Philosophy
of the University of London

May 1989

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ABSTRACT

The multiplicity of protein kinase C (PKC) has been investigated by examining the biochemical properties of the purified and separated isotypes -α, -β_1 and -γ. Peptides chosen to represent sequences unique to each isotype have been synthesised and used to generate antibodies specific for PKC-α, PKC-β_1, PKC-β_2 and PKC-γ. Peptides corresponding to the carboxyl termini of the isotypes gave better titres than those representing internal sequences. These antibodies have been used to map the domain structure of the PKC molecule and to investigate the purification, separation and cellular expression of the individual isotypes.

A purification protocol has been developed which produces milligram amounts of the PKC isotypes in a highly purified state. This preparation has been further separated into three pools of activity which have been shown to contain pure PKC-α, PKC-β_1 and PKC-γ. The activation kinetics and substrate specificities of these three isotypes have been defined *in vitro*. The isotypes are similar with respect to their dependence on phospholipid, Ca^{++}, Mg^{++} and ATP , but differ in their activation by phorbol esters or diacylglycerols and in their substrate specificities. This suggests that *in vivo* they may be activated through different pathways and subsequently may activate distinct physiological effects by selective substrate phosphorylation.

ACKNOWLEDGEMENTS

I am indebted to Dr. Peter Parker, my supervisor for the opportunity to follow this course of study. He has been a constant source of encouragement and help, at both a practical and an intellectual level. I would also like to thank Dr. Peter Sugden of the British Cardiothoracic Institute for his efforts as my University supervisor. Further acknowledgement is extended to Dr. Jim Woodgett, Dr Bill Gullick and Dr. Johnathan Rothbard for their practical help and critical comments during the course of these studies. Mr. Nick Totty and Mr Rob Philp are also to be thanked for their excellent technical help in sequencing, synthesising and analysing most of the peptides used. I am also grateful to the numerous people who have collaborated with me, enabling me to complete many of the aspects of this study; they are acknowledged at appropriate places in the text. Of note are Dr. Catherine Crawford (Oxford University), Drs. Clive Morris and Enrique Rozengurt (ICRF, London) and Prof. Leila Diamond (Wistar Institute, Philadelphia).

The members of the 'Cell Regulation laboratory' at the Ludwig Institute were very patient and kind to me.

Finally I would like to thank Dr. Caroline Springer for her encouragement, critical comments and for proof reading this thesis.

TABLE OF CONTENTS

	Page
Title page	1
Abstract	2
Acknowledgements	3
Table of Contents	4
List of Tables	8
List of Figures	9
CHAPTER ONE INTRODUCTION	12
1.1 Protein phosphorylation	13
1.1.1 The control of glycogen metabolism by phosphorylation	16
1.1.2 Characteristics of protein kinases	20
1.1.2.1 Cyclic nucleotide dependent kinases	20
1.1.2.2 Calcium/calmodulin dependent kinases	21
1.1.2.3 Haem dependent kinase and dsRNA dependent kinase	23
1.1.2.4 Rhodopsin kinase	24
1.1.2.5 Constitutively active kinases	25
1.1.2.6 Tyrosine kinases	26
1.1.2.7 Oncogenic protein kinases	28
1.1.2.8 Histidine kinases	29
1.1.3 Protein kinase homology	30
1.1.4 Protein kinase substrate specificity	31
1.1.5 Conformational changes induced by phosphorylation	32
1.1.6 Integrated pathways between kinases and phosphatases	33
1.1.7 Protein kinase C	35
1.2 Protein kinase C	
1.2.1 Discovery of protein kinase C	36

1.2.2 Activation of protein kinase C by lipids and calcium 37

1.2.3 PKC and phosphatidyl inositol turnover 39

1.2.3.1 Phosphatidyl inositol turnover 39

1.2.3.2 Diacylglycerol as a second messenger 41

1.2.3.3 A bifurcating pathway 42

1.2.4 The phorbol ester receptor 42

1.2.4.1 Tumour promotion 42

1.2.4.2 The structure of biologically active phorbol esters 43

1.2.4.3 Biological effects of phorbol esters 45

1.2.4.4 Properties of the phorbol ester receptor 46

1.2.4.5 Identification of protein kinase C as the phorbol ester receptor 48

1.2.5 PKC substrates 50

1.2.6 Characteristics of PKC *in vivo* 51

1.2.6.1 PKC translocation and down regulation 52

1.2.6.2 A model for the activation of platelets 54

1.2.7 Cloning PKC 59

1.2.8 A family of kinases 60

1.3 Aims of this thesis 64

CHAPTER TWO MATERIALS AND METHODS

2.1 Materials 66

2.2 Coupling of peptides to carrier proteins

2.2.1 Coupling with gluteraldehyde 67

2.2.2 Coupling with MBS 67

2.3 Enzyme linked immuno-sorbant assay 68

2.4 Polyacrylamide gel electrophoresis 69

2.5 Immuno-protein blotting 70

2.6 Preparation of the PtdSer-acrylamide column 70

2.7 Preparation fo the threonine-Sepharose column 71

2.8 Protein kinase C assay 71

2.9 Phosphopeptide mapping 72

2.10 Identification of phosphorylated residues in peptides 73

CHAPTER THREE PRODUCTION OF PKC ISOTYPE SPECIFIC ANTIBODIES 75

3.1 Generation of antibodies 76

3.2 The domain structure of PKC 92

CHAPTER FOUR PURIFICATION AND SEPARATION OF PROTEIN KINASE C ISOTYPES

4.1 Purification of protein kinase C 97

4.1.1 Purification of PKC by phosphatidylserine-affinity chromatography 98

4.1.2 Purification of PKC by threonine-Sepharose chromatography 108

4.2 Separation of PKC-α, -β_1 and -γ 122

4.2.1 Separation by Mono Q column chromatography 122

4.2.2 Miscellaneous columns 143

4.2.3 Separation of PKC-α, -β_1 and -γ by hydroxylapatite 149

CHAPTER FIVE BIOCHEMICAL ANALYSIS OF PKC-α, -β_1 AND -γ 165

5.1 Activation of PKC-α, -β_1 and -γ 167

5.1.1 Dependence on magnesium, ATP and PtdSer 167

5.1.2 Dependence on diacylglycerols 172

5.1.3 Dependence on TPA 172

5.1.4 Dependence on calcium 176

5.1.5 Effect of arachidonic acid on the PKC isotypes 180

5.1.6 *In vivo* responses of the PKC isotypes to TPA 187

5.2 Substrate specificity of PKC-α, -β_1 and -γ using peptide

substrates 193
5.2.1 Pseudosubstrate prototope peptides 195
5.2.2 Peptides bases on PKC substrates 199
5.3 Proteinaceous substrates 207
5.3.1 Histone H1, MBP and protamine 207
5.3.2 80K 218
5.3.3 Glycogen synthase 221
5.3.4 Vinculin 224

CHAPTER SIX DISCUSSION 230
6.1 Conclusions 233
6.2 Perspectives 248

REFFERENCES 250

APPENDIX 261

ABBREVIATIONS 262

LIST OF TABLES

Table		Page
1.1	Kinases of unknown regulation	27
3.1	Reactivity of antisera to immunising peptides	78
3.2	Reactivity of antisera against native PKC polypeptides	79
4.1.1	Purification of bovine brain PKC by PtdSer-affinity chromatography	102
4.1.2	Purification of bovine brain PKC by threonine-Sepharose chromatography	113
4.2.1	Mono Q column chromatography	129
4.2.2	Separation of PKC isotypes by hydroxylapatite chromatography	155
4.2.3	Immuno-depletion of PKC from the hydroxylapatite pools	156
5.1.1	Summary of effector kinetics of PKC-α, -β_1 and -γ	168
5.1.2	Calcium dependence of the PKC isoforms	178
5.1.3	Efect of arachidonic acid pre-incubation on PKC-α, -β_1 and -γ	183
5.2.1	Prototype peptide substrate specificities for PKC-α, -β_1 and -γ	197
5.2.2	Phosphorylation of physiological substrate peptides by PKC-α, -β1 and -γ	202
5.3.1	Phosphorylation of protein substrates by PKC isotypes	211
5.3.2	The PtdSer and TPA dependent phosphorylation of GS	225

LIST OF FIGURES

Figure		Page
1.1	The control of physiological responses to extracellular signals	14
1.2	The regulation of glycogen metabolism by phosphorylation	18
1.3	The structure of TPA	44
1.4	Activation of PKC by DG and phorbol esters	55
1.5	Activation of platelets by thrombin	58
1.6	The primary structure of PKC	62
3.1	Titration of PKC antibodies against purified PKC	82
3.2	Immuno-protein blot analysis of crude bovine and rat brain extracts	84
3.3	Immuno-protein blot analysis of cell lines	85
3.4	Immuno-precipitation of PKC	88
3.5	Immuno-protein blot analysis of PKC expressed in insect cells	90
3.6	Immuno-blot analysis of PKC fragments after digestion with calpain	94
3.7	Peptides used to raise antibodies to PKC	96
4.1.1	DEAE-cellulose chromatography	100
4.1.2	Gel filtration on Sephacryl S-200	103
4.1.3	SDS-polyacrylamide electrophoresis of PKC	105
4.1.4	Binding of PKC-a and -b to the PtdSer acrylamide column	107
4.1.5	DEAE-cellulose chromatography	110
4.1.6	Phenyl-Sepharose chromatography	111
4.1.7	Sephacryl S-200 gel filtration	112
4.1.8	Threonine-Sepharose chromatography	115
4.1.9	SDS-PAGE analysis of purified PKC	116
4.1.10	Co-purification of the PKC isotypes	117

4.1.11	Threonine-Sepharose chromatography	119
4.2.1	Mono Q column chromatography	124
4.2.2	Mono Q column chromatography	127
4.2.3	Mono Q column chromatography	130
4.2.4	Mono Q column chromatography	133
4.2.5	Mono Q column chromatography at pH 7.0	135
4.2.6	Mono Q column chromatography	136
4.2.7	Mono Q column chromatography	139
4.2.8	Mono Q column chromatography	141
4.2.9	RPC-PRO column chromatography	145
4.2.10	Gel filtration chromatography on Superose 12	146
4.2.11	PtdSer-acrylamide affinity chromatography	148
4.2.12	Hydroxylapatite column chromatography	151
4.2.13	Immuno-blot analysis of PKC	152
4.2.14	Hydroxylapatite column chromatography	158
4.2.15	Hydroxylapatite column chromatography	160
4.2.16	Hydroxylapatite column chromatography	163
5.1.1	Incorporation of phosphate into histone III-S	166
5.1.2	Activator dependence of PKC isotypes	170
5.1.3	Diacylglycerol dependence of PKC isotypes	173
5.1.4	Dependence of the PKC isotypes on TPA	175
5.1.5	The dependence of the PKC isotypes on calcium	177
5.1.6	Effects of arachidonic acid on PKC isotypes	182
5.1.7	Activation of PKC by arachidonic acid and PtdSer	185
5.1.8	The response of PKC to TPA in FM516 SV3/3 cells	189
5.2.1	Peptide substrates for the PKC isotypes	204
5.3.1	Phosphorylation of histone by PKC	209
5.3.2	Phosphopeptide analysis of histone	213
5.3.3	Two dimensional phosphopeptide map of MBP	215
5.3.4	The phosphorylation of 80K by the PKC isotypes	219

5.3.5	Phosphorylation of 80K by PKC isotypes	222
5.3.6	The phosphorylation of vinculin by PKC	227
6.1	Peptide substrates for the PKC isotypes	240
6.2	Phosphorylation of GS1-10 by PKC-α	242
6.3	Peptides designed to probe the substrate requirements of PKC-α, -β_1 and -γ	244
6.4	Cellular significance of PKC isotypes	247

CHAPTER ONE

INTRODUCTION

Protein phosphorylation and dephosphorylation is a major mechanism of control of cellular processes (Krebs and Beavo, 1979; Cohen, 1982). Glycogen metabolism was the first metabolic process that was shown to be regulated in this manner and since this observation many kinases have been described (Krebs, 1985). In these pathways, the control of key enzymes has been shown to be dependent upon their state of phosphorylation and thus regulatory pathways which are mediated by alternative phosphorylation and dephosphorylation have been described. One group of regulatory processes that have been shown to be regulated by kinases are the signal transduction pathways (Cohen, 1985).

Signal transduction is the phenomenon which describes the responses of cells to hormones and growth factors (Cohen, 1985). Hormones circulate at subnanomolar concentrations in the blood and combine with specific receptors on the surfaces of cells. These receptors activate trans-membrane signalling systems which amplify the original signals to over a million fold in many cases and therefore produce rapid and significant changes in the physiological processes of responsive cells (Cohen, 1985). Amplification is achieved in two principle ways; firstly by the catalytic production of 'second messengers' which are biologically active compounds produced in the cytoplasm in response to hormonal stimulation; and secondly by the reversible phosphorylation of regulatory proteins, which control metabolic processes (Cohen, 1985). The changes in phosphorylation often occur as a result of activation of kinases or phosphatases by the second messenger compounds and represent a

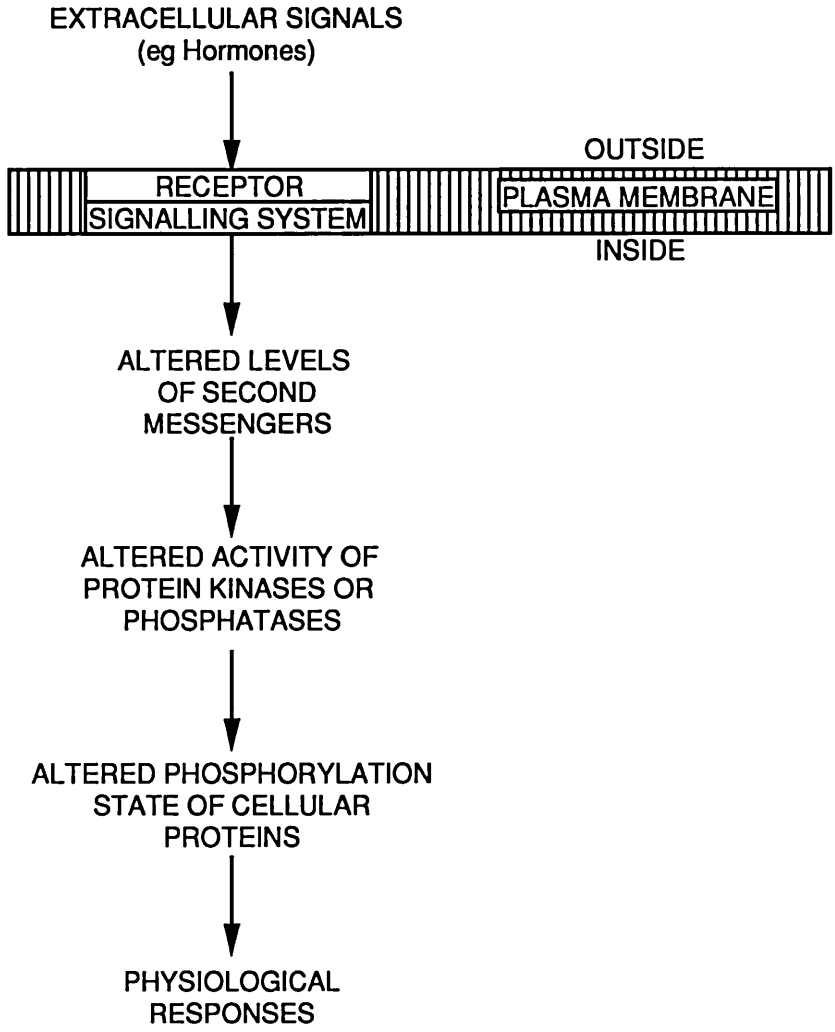
further level of amplification, one kinase or phosphatase being capable of affecting the activity of numerous metabolic enzymes (Cohen, 1985). Thus a generalised model can be proposed in which a hormone binding to its receptor activates a signalling system, which produces second messenger molecules. The second messengers activate kinases and/or phosphatases which alter the phosphorylation state of key metabolic enzymes, resulting in altered physiological states in responsive cells (Figure 1.1).

One of the kinases that has been found to be involved in signal transduction is protein kinase C (PKC). This kinase is distinguished from other kinases by its mode of regulation and occupies a pivotal role in the responses of many cell types to numerous hormones and to an important class of tumour promotors, the phorbol esters (Woodgett *et al*, 1987). PKC has recently been found to be a family of kinases with at least seven members; these are the products of six distinct genes, which have different but highly homologous polypeptide sequences (see Nishizuka, 1988). The work presented in this thesis describes one approach to investigating the reason for the multiplicity of PKC with a view to understanding why this important regulatory enzyme exists as multiple isotypes. An introduction to the discovery of kinases and the properties of some kinases is presented; this is followed by a discussion of the properties of PKC.

1.1 PROTEIN PHOSPHORYLATION

The first indications that proteins could be regulated by covalent modifications came in the 1940's from the work of Cori, Cori and Green. They were working on the enzyme glycogen phosphorylase,

Figure 1.1 The control of physiological responses to extracellular signals



(After Cohen, 1985)

which controls glycogen metabolism, by causing its degradation through the reaction:

$$\text{glycogen}_{(n\ \text{residues})} + P_i \longrightarrow \text{glycogen}_{(n-1\ \text{residues})} + \text{glucose-1-phosphate.}$$

This biochemical pathway is activated in muscle (and to a lesser extent in the liver) in response to signals which anticipate the need for muscular activity. In 1943, Cori and Green found that phosphorylase existed in two forms which they called phosphorylase *a* (which was essentially constitutively active) and phosphorylase *b* (which required high concentrations of 5'-AMP for activity). They also found that phosphorylase *a* could be converted to phosphorylase *b* enzymatically *in vitro* and subsequently the enzyme responsible for the interconversion was partially purified (Cori and Cori, 1945). The interconversion of these two forms of phosphorylase could be demonstrated *in vivo*, showing that this mechanism was physiologically relevant and not an *in vitro* artefact (Cori, 1945).

In 1955, work from two laboratories described enzymes that could convert on the one hand phosphorylase *a* to *b* and on the other phospohorylase *b* to *a*. Sutherland and Wosilait (1955) demonstrated that treatment of liver slices *in vitro* with epinephrine caused the proportion of phosphorylase *a* in these tissues to increase and that the increase in the *a* type activity was accompanied by an increased amount of phosphate in the phosphorylase isolated from treated tissues. They also demonstrated that conversion of phosphorylase *a* to *b in vitro* was accompanied by a reduction in the phosphate content of phosphorylase. At the same time, Fisher and Krebs (1955) demonstrated at about the same time that addition of metal ions to muscle extracts drove the conversion of phosphorylase *b* to *a* and

that the reaction was dependent on ATP. Thus it was shown that there was more phosphate associated with phosphorylase *a* than phosphorylase *b* and that the interconversion of the two forms involved the addition or removal of phosphate prosthetic groups. The reaction was controlled by enzymes that were dependent on Mg^{++} and ATP.

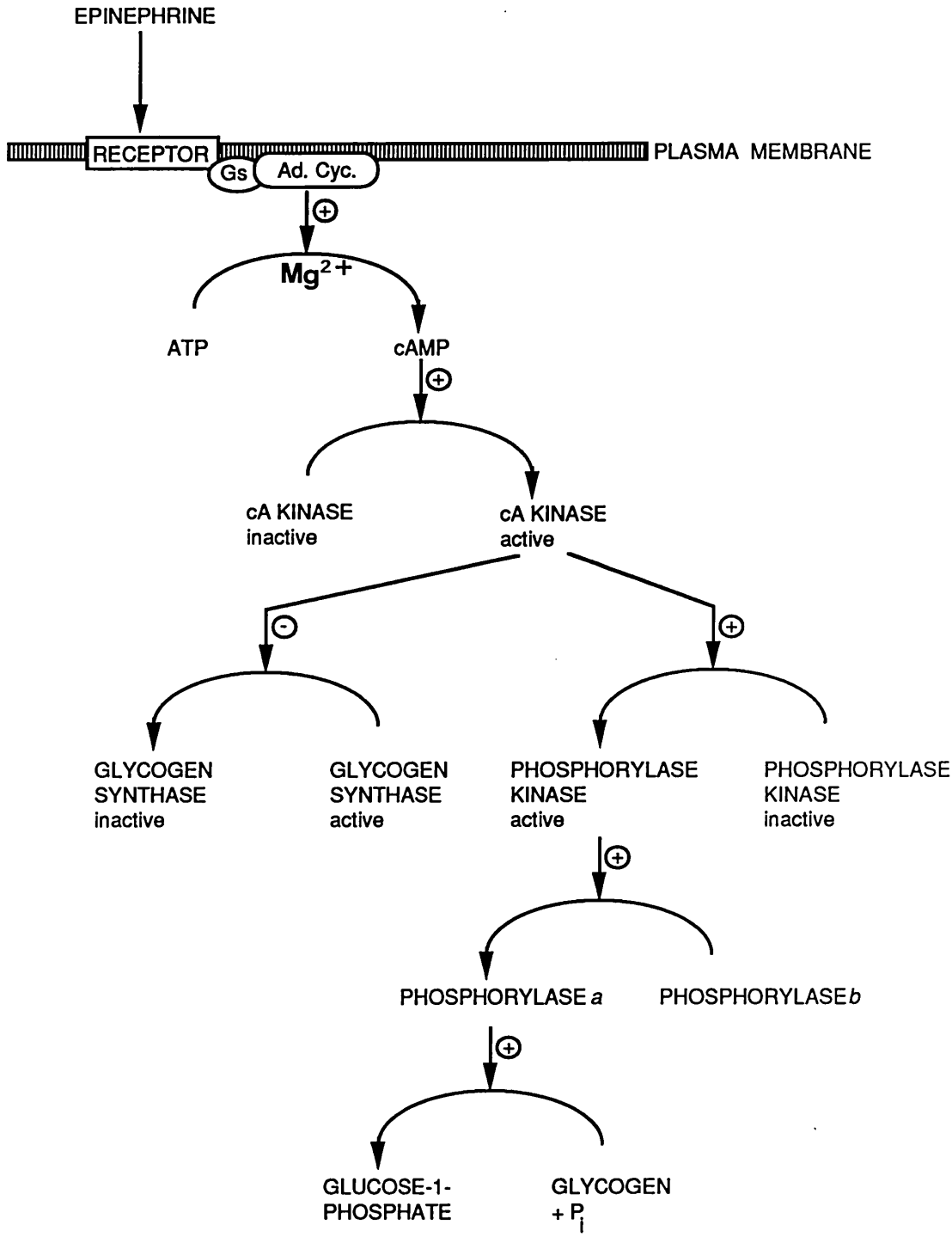
It was subsequently shown that phosphorylation of phosphorylase *b* could be enhanced by cyclic 3',5'-AMP (cAMP) (Krebs *et al*, 1959) and this lead to speculation that phosphorylase kinase was itself regulated by a kinase that was dependent upon cAMP. This was indeed shown to be the case, when the cAMP dependent kinase (cA kinase) was purified and shown to phosphorylate phosphorylase kinase in the presence of cAMP (Walsh *et al*, 1969); this explained the observations that cAMP was involved in the *in vivo* conversion of phosphorylase *b* to *a* in response to epinephrine (see Krebs *et al*, 1959). The phosphorylation of phosphorylase kinase by the cA kinase increased the activity of the former enzyme (Walsh *et al*, 1969) and the pathway by which epinephrine stimulates the breakdown of glycogen has been elucidated.

1.1.1 The control of glycogen metabolism by phosphorylation

The control of glycogen metabolism by hormones has been extensively studied and represents the prototypical model for physiological responses to extracellular messengers. The model encompasses several of the basic concepts of signal transduction. Namely: a hormone binding to a specific receptor on cell surfaces activates a signal transducing mechanism which produces second messengers; the second mesengers activate a kinase cascade which alters the physiological state of the responsive cells (Cohen, 1985).

Anticipation of skeletal muscle activity or muscular activity itself leads to the release of the hormone epinephrine from the adrenal glands (Cohen, 1985). When muscle cells are activated by epinephrine, there is increased glycogen breakdown and formation of glucose-1-phosphate which is metabolised to produce ATP for muscular activity. Epinephrine binds to β-adrenergic receptors on the surfaces of cells (Levitski, 1988), see Figure 1.2. This is the first event in this signal transducing pathway. The β-adrenergic receptor is an integral membrane protein with a Mr of about 46,000 which contains seven putative membrane spanning domains (Levitski, 1988). The hormone binding domain appears to be buried in the lipid bilayer (Levitski, 1988). When epinephrine binds to the receptor, conformational changes occur which allow the receptor to associate with a stimulatory guanine binding protein (Gs, see Figure 1.2) (Levitski, 1988). Gs is one of a class of G proteins which are composed of three subunits called α, β and γ in the ratio 1:1:1 (Levitski, 1988). The α subunits of these proteins have a guanine binding domain and also an associated GTPase activity (Levitski, 1988). In the resting state, the α subunit of Gs has a molecule of GDP bound to it, but when stimulated by an epinephrine-β-adrenergic receptor complex, GDP is exchanged for GTP. The GTP-α subunit complex is then able to activate adenylate cyclase which catalyses the conversion of ATP to cAMP (Figure 1.2) and it is cAMP which is the second messenger. Hydrolysis of the GTP to GDP inactivates Gs and the production of cAMP is halted; Gs is therefore self regulating, feedback inhibiting its own activity. cAMP diffuses into the cytoplasm and activates cA kinase (Figure 1.2). cA kinase consists of two subunits; a regulatory domain (R)

Figure 1.2 The regulation of glycogen metabolism by phosphorylation



(After Krebs, 1985)

with a Mr of about 45,000 and a catalytic domain (C) with a Mr of about 40,000 (Edelman *et al*, 1987). The inactive enzyme is a tetramer (R_2C_2) in which the R subunits associate with each other and each C subunit tightly associates with one of the R subunits in a non-covalent manner (Edelman *et al*, 1987). Each regulatory domain can bind two molecules of cAMP which causes the dissociation of the holoenzyme, resulting in a $R_2.(cAMP)_4$ complex and two active catalytic subunits. The active catalytic subunit of cA kinase phosphorylates phosphorylase kinase, amongst other proteins (Figure 1.2).

Phosphorylase kinase is a large enzyme complex, with a relative molecular mass (Mr) in the order of 1.3 million; it is made up of four types of subunit (α, β, γ, δ) with the structure $[\alpha.\beta.\gamma.\delta]_4$ (Edelman *et al*, 1987). There are two forms of the α subunit (α and α') which have different tissue distribution and give particular biochemical properties to the kinases that contain them (Edelman *et al*, 1987). The δ subunit, is identical in structure to calmodulin and the binding of Ca^{++} to this subunit allows it to associate with and activate the enzyme complex (see section 1.1.2.2). The kinase is also regulated allosterically by ADP and as mentioned by phosphorylation by cA kinase (Edelman *et al*, 1987). Activated phosphorylase kinase phosphorylates phosphorylase *b*, converting it to phosphorylase *a*, the active form (Figure 1.2). Phosphorylase *a* catalyses the conversion of glycogen to glucose-1-phosphate in preparation for muscle activity, see Figure 1.2 (Edelman *et al*, 1987). Activated cA kinase also phosphorylates glycogen synthase causing the inactivation of this enzyme, thereby having a two-fold effect on glycogen metabolism and ensuring that these two opposing enzymes

do not work in opposition to each other; see Figure 1.2 (Edelman *et al*, 1987).

1.1.2 Characteristics of protein kinases

When cA kinase was purified, it was shown to have a wide substrate specificity, suggesting that this enzyme did not only control glycogen metabolism (Walsh *et al*, 1969). At the same time that cA kinase was described, other systems were being discovered that were regulated by phosphorylation/ dephosphorylation and these joint discoveries increased interest in the phenomenon which has subsequently lead to the discovery of many kinases and phosphatases (see Krebs, 1985).

Many of the protein kinases that have been described to date have been classified with respect to the mechanism by which they are regulated. However, this nomenclature system does not cover all situations because kinases are being described whose mechanism of activation is not known; these proteins have therefore tended to be named after their primary *in vitro* substrates. This is not ideal either, since some kinases of unknown regulation phosphorylate many substrates. Further, the improvements in molecular biology techniques have resulted in the definition of protein kinases that are enigmatic both in terms of substrate and regulation which have been designated as putative kinases on the basis of primary structure alone (Hanks *et al*, 1988). A brief description of some kinases is given below.

1.1.2.1 Cyclic nucleotide dependent kinases Cyclic AMP dependent kinase (cA kinase) belongs to a family of kinases that are regulated by cyclic nucleotides. cA kinase is ubiquitous in cells and its

biochemical properties are described in section 1.1.1. Cloning studies have revealed that there are multiple forms of both the R and the C subunits which are highly conserved and show different tissue distributions. These differences probably reflect distinct biochemical functions but this remains to be demonstrated, although it is known that the R subunits show differences in affinity for cAMP and the C subunits (Edelman *et al*, 1987).

Also in this family is a group of kinases that are regulated by cyclic 3',5'-GMP (cGMP) called the cGMP dependent kinases (cG kinases) (Edelman *et al*, 1987). Unlike cA kinase, cG kinase is a dimer of identical polypeptides each of which has a catalytic (C) and a regulatory (R) domain. Each R domain can bind to two molecules of cGMP but unlike cA kinase, the complex does not dissociate and is activated by conformational changes that free the kinase domain from the inhibitory constraints of the R domain (Edelman *et al*, 1987). The activity of the catalytic domains of cA and cG kinases is probably mediated by pseudosubstrate regions in the regulatory domains (Edelman *et al*, 1987). These are short stretches of primary sequence that have high affinity for the catalytic site and bind to the latter in an inhibitory fashion. The binding of activating co-factors, results in the removal of these inhibitory regions, thereby increasing the access of substrates to the catalytic site (Edelman *et al*, 1987).

1.1.2.2 Calcium/calmodulin dependent kinases A family of kinases that are regulated by calmodulin (CaM) has been described (Wang *et al*, 1985). CaM is a protein with a Mr of 17,000 which has four Ca^{++} binding sites and therefore regulates enzymes in a Ca^{++} dependent manner. When all four Ca^{++} sites of CaM are occupied, there is a

change in its conformation which enables it to interact with particular cellular enzymes. One such group of enzymes are the Ca^{++}/CaM dependent kinases which become activated when Ca^{++}/CaM binds to them (Wang *et al*, 1985). The least specific (in terms of substrate specificity) of this group of kinases is the multifunctional Ca^{++}/CaM dependent kinase. Multifunctional Ca^{++}/CaM is composed of two types of subunits (α of which one form has been described and β of which two forms have been described; β and β'), but the ratio of these varies depending on the source of the enzyme (Edelman *et al*, 1987). Both the α and the β subunits contain catalytic and CaM binding domains and autophosphorylation activates this kinase in the absence of Ca++/CaM (Edelman *et al*, 1987). Many *in vitro* substrates have been described for this kinase and some of these are substrates *in vivo*. The phosphorylation of some enzymes by the multifunctional Ca^{++}/CaM dependent kinase has been reported to alter their activity (Edelman *et al*, 1987).

Another kinase which is regulated by CaM is a kinase which is specific for myosin light chain (MLC) called myosin light chain kinase (MLCK). MLCK is a group of kinases, of which there are many tissue and species specific types. It is composed of two types of subunits and becomes autophosphorylated upon activation which renders it constitutively active (Edelman *et al*, 1987). Unlike the multifunctional Ca^{++}/CaM kinases which have a very broad range of substrates the MLCK's phosphorylate only a single Ser residue in the P-light chain of myosin (Edelman *et al*, 1987). Phosphorylation of MLC leads to an increase in the levels of actin stimulated ATPase activity of myosin and thus contributes to the control of smooth muscle contractions.

Phosphorylase kinase can also be considered to be associated with the Ca^{++}/CaM kinases, since the δ subunit of this kinase is CaM itself (Edelman *et al*, 1987).

1.1.2.3 Haem dependent kinase and double stranded RNA dependent kinase Two kinases have been described which specifically phosphorylate eukaryotic initiation factor 2 (eIF2). eIF2 is involved in the initiation of the formation of the 70S ribosomal complex responsible for protein synthesis. At the induction of protein synthesis, the 30S ribosomal subunit binds to eIF2 and two other initiation factors called eIF1 and eIF3. GTP associates with eIF2 in the 30S complex and this induces the binding of mRNA and fmet-$tRNA_f$ to the complex (see Stryer pp754-756, 1988). eIF3 is released from the 30S complex, and the 50S ribosomal subunit is able to associate with 30S. This triggers the hydrolysis of the GTP bound to eIF2 and subsequently eIF2 and eIF1 are released. Protein synthesis then proceeds under the direction of the elongation factors. A protein kinase regulated by haem which phosphorylates the α subunit of eIF2 at low concentrations of haem and thereby inhibits formation of the initiation complex has been described (Edelman *et al*, 1987). Phosphorylation at the same site can be performed by a kinase that is stimulated by double stranded RNA (ds RNA) (Edelman *et al*, 1987). The ds RNA dependent and the haem dependent kinases appear to have similarities in their structures and are thought to be positively regulated by autophosphorylation. The physiological role of these kinases is not understood, but the ds RNA dependent kinase may have a role in antiviral activity by blocking protein synthesis upon infection of cells by ds RNA viruses (Edelman *et al*, 1987).

1.1.2.4 Rhodopsin kinase Rhodopsin kinase is different from the afore mentioned kinases in that it is not regulated by co-factors, but apears to be regulated by substrate availability (Edelman *et al*, 1987). Rhodopsin is an integral membrane protein which is found in retinal rod cells (Fung, 1985). It is a receptor of photons of light and when stimulated, it activates an enzyme cascade that results in a nervous impulse that is perceived as light (Fung, 1985). Photoactivation of rhodopsin induces conformational changes in the molecule which are detected by a G protein called transducin (Fung, 1985). Transducin is a complex of three subunits (α, β and γ) and when it is stimulated by activated rhodopsin, the α subunit dissociates from the β/γ subunits and activates a phosphodiesterase which catalyses the conversion of cGMP to 5'-GMP (Fung, 1985). The concomitant decrease in the levels of cGMP causes the closing of ion channels in the plasma membrane and blocks influx of Na^+ into the rod cells. As a result, the membrane becomes hyperpolarised and a nervous impulse is generated (Fung, 1985). A kinase which is specific for rhodopsin has been described (Edelman *et al*, 1987). This kinase has a Mr of 67,000-69,000 and is not know to phosphorylate any substrates apart from rhodopsin. Rhodopsin kinase phosphorylates photobleached rhodopsin on up to seven residues (Ser and Thr) in its carboxy terminal region (Fung, 1985). These phosphorylations change the net charge in this reqion of rhodopsin from positive to negative (Fung, 1985) and appear to promote the association between rhodopsin and a protein of Mr 48,000 (Edelman *et al*, 1987). The association of the 48,000 Mr protein inhibits transducin-rhodopsin associations and therefore the α subunit of transducin is not released (Fung, 1985). Thus rhodopsin is desensitised by phosphorylation and so the phosphodiesterase is suppressed; the cell therefore returns to the resting state (Fung,

1985). Rhodopsin kinase is activated when the substrate sites on rhodopsin are exposed by photoactivation, but the sites are masked when rhodopsin is associated with transducin (Edelman *et al*, 1987). Thus rhodopsin kinase is a constitutively active kinase that is regulated at the substrate level and controls the responses of rod cells to light (Edelman *et al*, 1987). A rhodopsin-like kinase activity has been described in cells other than rod cells. In these cells it is thought to be a β-adrenergic receptor kinase which is responsible for homologous down regulation of this receptor in a similar way to the mechanism by which rhodopsin kinase regulates the activity of rhodopsin (Edelman *et al*, 1987).

1.1.2.5 Constitutively active kinases A growing list of kinases is being described whose mechanisms of regulation have not yet been elucidated; for the most part these kinases are named after their major known substrates (Edelman *et al*, 1987). Amongst these kinases are casein kinases I (CKI) and II (CKII), which phosphorylate casein and similar acidic substrates *in vitro* (Edelman *et al*, 1987). CKI is monomeric with a Mr of about 37,000; phosphorylation of some proteins by CKI activates them (eg phosphorylase kinase) while others are inhibited by CKI phosphorylation (eg glycogen synthase) (Edelman *et al*, 1987). Mammalian CKII has a tetrameric structure (Mr: 130,000), consisting of two types of subunit called α (Mr: 37,000-44,000) and β (Mr: 24,000-28,000). The α subunit is catalytic but the function of the β subunit is unknown although it does become autophosphorylated (Edelman *et al*, 1987). CKI and CKII are widely distributed in many species; both phosphorylate a large number of proteins *in vitro* although the number of physiologically important substrates is probably smaller (Edelman *et al*, 1987). These kinases outline the nomenclature problems presented by

kinases for which regulation remains a mystery. Neither CKI nor CKII is specific for casein and this is almost certainly not a physiological substrate; the name is therefore useful in describing some of its *in vitro* substrate, but not its physiological characteristics (Edelman *et al*, 1987).

As mentioned previously, the casein kinases form part of a group of kinases that are described in terms of their *in vitro* characteristics for which the physiological significance is not yet determined. Further members of this class of kinases are given in Table 1.1. Table 1.1 is by no means a comprehensive list of the known kinases but is intended to give an idea of the diversity of kinases, their substrates and their possible mechanisms of regulation. More extensive lists of the known kinases and their functions are given in reviews by Hunter (1987) and Edelman et al (1987).

1.1.2.6 Tyrosine kinases A family of kinases has been described which is distinct from the Ser/Thr kinases in that they phosphorylate substrates on Tyr residues (Sefton and Hunter, 1984). These kinases are absolutely specific for Tyr and do not phosphorylate Ser or Thr residues (Sefton and Hunter, 1984). Tyr kinases were originally described as the transforming genes of oncogenic retroviruses, although they are not retroviral in origin, but have been sequestered from host cells (Sefton and Hunter, 1984). The sequestered genes have become mutated in the viruses, resulting in genes with altered growth controlling potentials (Sefton and Hunter, 1984).

Many growth factor receptors are Tyr kinases and have two separate domains; an external domain which forms the growth factor binding

Table 1.1 Kinases of unknown regulation

ENZYME	M r	SUBSTRATE	PUTATIVE MECHANISM OF REGULATION
Pyruvate dehydrogenase kinase	α s/u: 48,000 β s/u: 45,000	pyruvate dehydrogenase	possibly acetylCoA, ADP and NADH
Branched chain α ketoacid dehydrogenase kinase	α s/u: 48,000 β s/u: 45,000	branched chain α ketoacid dehydrogenase	ketoleucine,ketovaline ketoisoleucine.
Hydroxymethylglutaryl-CoA reductase kinase.	58,000-380,000 reported	hydroxylmethyl-glutaryl- CoA reductase	possibly by a specific kinase
Growth associated histone H1 kinase	67,000-96,000	histone H1 during cell division	?
Glycogen synthase 3	47,000-51,000	glycogen synthase; protein phosphatase inhibitor-2; type II regulatory subunit of cA kinase	?
Rbiosomal protein S6 kinase	50,000-92,000	ribosomal protein S6	protein kinase C insulin receptor? proteolysis?

site, and an internal domain which is associated with the kinase activity (Foulkes and Rosner, 1985). The mechanisms of action of growth factor receptors remain unknown, but they can activate other second messenger systems, such as the induction of inositol phospholipid turnover (see section 1.2.3.1, and Foulks and Rosner, 1985). Most Tyr kinases are associated with the plasma membrane and if they are unable to associate with the membrane, their kinase activity is unaffected, but the signal transducing abilities are impaired or lost (Foulkes and Rosner, 1985). Autophosphorylation and phosphorylation by exogenous kinases (both Ser/Thr and Tyr) are important factors in the regulation of Tyr kinase activity (Foulkes and Rosner, 1985).

Tyrosine kinases and phosphotyrosine play an important role in the regulation of cellular function and proliferation, and they are intimately involved in signal transducing pathways. Tyr kinases are multifunctional and display pleiotropic effects when activated (Foulkes and Rosner, 1985). They are important because when discovered they demonstrated that single genes could influence the proliferative states of cells (Foulkes and Rosner, 1985). They are also important because they are the routes through which many growth factors exert their mitogenic effects (Foulkes and Rosner, 1985).

1.1.2.7 Oncogenic protein kinases Cells contain a large number of genes which have the ability to increase cell proliferation if they are overexpressed or mutated; these genes are therefore referred to as proto-oncogenes (Sefton, 1985). Many proto-oncogenes have been shown to have protein kinase activity and can be divided into two groups depending on whether they phosphorylate Ser/Thr or Tyr

residues (Sefton, 1985). Most of the oncogenic kinases have been identified as the transforming genes of oncogenic retroviruses (as described for the Tyr kinases in section 1.1.2.6.), although some are known to have arisen spontaneously through gene rearrangements that alter expression levels or by structural changes that are induced through mutation events (Sefton, 1985). Most oncogenic kinases have been shown to influence cell division due to increased kinase activity. In the case of kinases that are overexpressed, this is achieved by increased levels of protein and in the case of mutations, this usually arises by loss of regulatory function (Sefton, 1985).

In most cases, the precise role that oncogenic kinases have in increasing proliferation is not known, but these enzymes form an important group of kinases because they indicate the effects that single genes can have on cell proliferation (Foulkes and Rosner, 1985). In effect, these kinases 'short circuit' the normal signal transducing responses, thereby influencing cell proliferation. They therefore demonstrate the important role of kinases in regulating responses to mitogenic signals (Sefton, 1985).

1.1.2.8 Histidine kinases A class of kinases that phosphorylate histidine residues has been described (see Hess *et al*, 1988). The activity of some enzymes has been postulated to be mediated by phosphorylation on His residues and this appears to be true for bacterial chemotaxis (Hess *et al*, 1988). Bacteria respond to chemotactic stimuli by gathering information from membrane receptors and transmitting to a flagellar 'switch' which controls the direction of rotation of the flagellum (for review, see Macnab, 1985). Cells are thus able to migrate towards or away from particular compounds in their environment. Four cytoplasmic

proteins are responsible for transmitting the signal from receptors to switch and feedback regulation is controlled by methylation or demethylation of the receptors (see Hess *et al*, 1988). One of the cytoplasmic transducing protiens (CheA) is a kinase that becomes autophosphorylated and mediates the responses of some of the other cellular proteins (Hess *et al*, 1988). The autophosphorylated residue has recently been shown to be His^{48} and mutation of this residue results in chemotactic deficient mutants (Hess *et al*, 1988). This observation shows that phosphorylation is important for bacterial chemotactic responses and indicates that histidine phosphorylation is important in signal transducing systems (Hess *et al*, 1988). The relevence and the frequency of His phosphorylation in other systems has not yet been determined.

1.1.3 Protein kinase homology

Comparison of different protein kinase sequences reveals that they all contain a region of significant homology which has been defined as the kinase domain (Krebs, 1985). This domain comprises about 250-300 amino acids and comparison of 65 kinase has revealed that it can be further divided into 11 sub-domains (Hanks *et al*, 1988). Some of the sequences appear to be definitive for kinases and others can be used to distinguish between Ser/Thr or Tyr kinases (Hanks *et al*, 1988). One of the sequences which is found in all kinases is the sequence Gly-Xaa-Gly-Xaa-Xaa-Gly (where Xaa is any amino acid), followed by a Lys 15 to 20 residues C-terminal to the final Gly; this motif is thought to be the nucleotide binding site with the Lys acting as the phosphate acceptor during catalysis (Hanks *et al*, 1988). These definitive kinase regions have been used to identify the genes for protein kinases and to isolate novel protein kinases by screening DNA libraries at low stringencies (Hanks *et al*, 1988). Comparison of

the sequences for the known kinase domains has lead to the construction of a family tree that classifies kinases on the basis of structural similarities within this domain. This tree tends to classify proteins with similar biochemical properties (such as cA and cG kinases) together and the tree is proposed to represent an evolutionary classification of the kinases. The authors suggest that the tree be used as a new method for classifying kinases (Hanks *et al*, 1988).

1.1.4 Protein kinase substrate specificity

Kinases appear to recognise the primary sequences surrounding their target residues as determined by their abilities to phosphorylate peptide substrates (see Sparks and Brautigan, 1986). Native proteins that are not normally kinase substrates can become substrates upon denaturation, in agreement with this hypothesis. Higher order structure can however influence the efficiency of a substrate by hindering or increasing the access of the substrate site for the kinase (Sparks and Brautigan, 1986). One way in which access may be increased is by the presence of structures such as β-turns at the phosphorylation site and indeed the fact that some proteins are better substrates than the corresponding peptides supports this hypothesis (Sparks and Brautigan, 1986). For many Ser/Thr kinases, there is a requirement for basic residues in the vicinity of the target residue although the specific requirements for particular kinases vary. Therefore although cA kinase and cG kinase both require basic residues in the vicinity of the target for substrate recognition, cA kinase has a broader substrate range than cG kinase (Edelman *et al*, 1987). On the other hand, although rhodopsin kinase and MLCK each have only one known substrate, rhodopsin kinase phosphorylates multiple sites on its substrate, whereas MLCK

phosphorylates only a single site (Edelman *et al*, 1987). The structural features of substrates allow for different but overlapping control of enzyme activities by distinct kinases and therefore allow precise control of the extent of phosphorylation at each site (Sparks and Brautigan, 1986).

1.1.5 Conformational changes induced by phosphorylation

An understanding of how phosphorylation of glycogen phosphorylase alters the activity state ot the catalytic site has recently been elucidated (Sprang et al, 1988). This explains (for at least this one system) how phosphorylation of a single residue alters the catalytic activity of an enzyme (Sprang *et al*, 1988). The results have been likened to allosteric controlling mechanisms because the addition of phosphate at one end of the molecule induces conformational changes throughout the polypeptide which affect the activity of a distant catalytic domain (Sprang *et al*, 1988). Functional phosphorylase is a dimer in which the subunits interact in an ordered fashion (Sprang *et al*, 1988). Transition from the *b* form to the *a* form is accompanied by only minor changes that occur almost exclusively at the interface between the two molecules of the dimer (Sprang *et al*, 1988). Phosphorylation occurs at Ser^{14} in phosphorylase *b* and results in the formation of new salt bridges which alter the status of the N-terminus of the enzyme from a disordered to an ordered structure (Sprang *et al*, 1988). The formation of new hydrogen bonds between the subunits together with the new salt bridges results in the subunits being more tightly bound together. These changes increase the affinity of phosphorylase for the activator AMP and at the same time decrease the affinity of the enzyme for the inhibitor glucose-6-phosphate (Sprang *et al*, 1988). The resulting overall conformation change in the protein displaces the C-terminus, which acts as a

competitive inhibitor of activity in phosphorylase *b*. The displacement of the C-terminus exposes the catalytic site, thereby increasing the glycolytic activity of the enzyme (Sprang *et al*, 1988). It is likely that similar types of conformational changes are induced in other enzymes by phosphorylation.

1.1.6 Integrated pathways between kinases and phosphatases

Control of cellular functions occurs through reversible phosphorylations. The enzymes responsible for protein dephosphorylations, the protein phosophatases, appear to be fewer in number and are also less specific than their kinase counterparts (Cohen , 1985). Phosphatases appear to rely on higher order structures for substrate recognition, whereas kinases rely in primary sequence to a greater extent; phosphatases also display broader substrate specificity than kinases (Sparks and Brautigan, 1986). Thus phosphorylation/ dephosphorylation at a particular site may be accomplished by a distinct kinase/ phosphatase pair whereas another site phosphorylated by the same kinase could be dephosphorylated by a different phosphatase (Sparks and Brautigan, 1986).

Four classes of protein phosphatase catalytic subunits have been identified from mammalian cells and these were initially divided into two broad groups, depending on certain biochemical characteristics. The class 1 phosphatases preferentially dephosphorylate the β subunit of phosphorylase and are inhibited by two heat stable inhibitors called inhibitor 1 and 2 (Cohen, 1985). The class 2 phosphatases preferentially dephosphorylate the α subunit of phosphorylase and are insensitive to inhibitors 1 and 2 (Cohen, 1985). Further divisions of the class 2 phosphatases have been made

on the basis of structural and regulatory properties, yeilding three subclasses: 2A, 2B and 2C (Cohen, 1985). Phosphatases and kinases are often co-regulated to avoid activation of opposing enzyme activities occuring simultaneously. For example, phosphatase 1 dephosphorylates proteins that have been phosphorylated by cA kinase. However, phosphatase 1 is itself regulated by cA kinase and phosphorylation of inhibitor 1 by cA kinase activates this inhibitor and thus inactivates the phosphatase, thus amplifying the cA kinase signal (Cohen, 1985). The inactivation of phosphatase 1 by cA kinase also has more far reaching effects, since this phosphatase dephosphorylates the substrates of other kinases, thereby "passively" increasing phosphorylations by these kinases (Cohen, 1985). Some phosphatases are regulated by mechanisms similar to kinase regulation, so a Ca^{++}/CaM dependent phosphatase has been described (tyoe 2B) and other phosphatases have been described which are regulated by complex subunit structures or Mg^{++} ions (type 2C) (Cohen, 1985).

The basic concepts that arise from the study of phosphatases and kinase are two-fold. Firstly, phosphorylation and dephosphorylation often stimulate metabolic processes in opposite directions. For example, most biodegradative pathways are activated by phosphorylation (and deactivated by dephosphorylation) whereas most biosynthetic pathways are are inactivated by phosphorylation (and activated by phosphorylation) (Cohen, 1985). Second, the number of kinases and phosphatases controlling metabolic pathways is relatively small; but both kinases and phosphatases are often multifunctional and have pleiotropic actions (Cohen, 1985). Thus protein phosphorylations and dephosphorylations form the basis of networks of interlocking pathways that enable extracellular signals

(eg hormones) to control physiological responses through the production of just a few second messengers (Cohen, 1985).

1.1.7 Protein kinase C

One of the protein kinases that has an important function in mediating cellular responses to external signals is a protein kinase discovered in 1977 which has been shown to be dependent on Ca^{++} and phospholipids for activity (see Nishizuka, 1984). This kinase is regulated *in vivo* by phospholipid metabolism which occurs in response to hormone stimulation in many cells; the kinase is called protein kinase C (PKC) (Nishizuka, 1984). Activation of PKC is essential for the responses of many secretory and neuronal cells to external stimuli (Nishizuka, 1984). Recent evidence indicates that PKC exists in numerous isotypic forms, but the function of each of these forms *in vivo* is not known (Nishizuka, 1988). This thesis describes one approach to understanding the reasons for the multiplicity of this important regulatory enzyme and the remainder of the introduction describes the properties of PKC.

1.2 PROTEIN KINASE C.

1.2.1 Discovery of protein kinase C

Protein kinase C (PKC) was first described in the laboratory of Nishizuka as a novel protein kinase purified from rat brains that was activated by high concentrations of Mg^{++}; it was referred to as protein kinase M (PKM) (Takai *et al*, 1977a; Inoue *et al*, 1977). It was shown to be distinct from the cyclic nucleotide dependent kinases by virtue of the fact that it was not activated by cyclic nucleotides, it was insensitive to the regulatory domain of cA kinase and could be separated by chromatographic means from both cA and cG kinases (Takai *et al*, 1977). The mechanisms of regulation of PKM were not immediately obvious but it was shown to exist as a precursor that could be activated by proteolytic cleavage catalysed by a calcium activated neutral protease (CANP) that had been partially purified with it, or by limited digestion with trypsin (Takai *et al*, 1977). Activation in this way was irreversible, the active enzyme using salmon sperm protamine and to varying degrees histones H1, H2A, H2B, H3 and H4 as substrates (Takai *et al*, 1977a; Yamamoto *et al*, 1978). Protamine was shown to be a substrate for the precursor form of PKM as well as the proteolytically activated form, leading to early speculation that the kinase activity may be controlled at the substrate level (Yamamoto *et al*, 1978). The activation of PKM by CANP was accompanied by a reduction in size of the precursor from a Mr of 77,000 to the active form with a Mr of 51,000; cleavage with trypsin resulted in an active fragment of Mr 57,000 and activation in crude extracts resulted in a fragment with a Mr of 66,000 (Inoue *et al*, 1977).

The function of this kinase and indeed its physiological significance were enigmatic but PKM and CANP were shown to be present in many tissues (Takai *et al*, 1977b; Kishimoto *et al*, 1977). Glycogen phosphorylase kinase and glycogen synthase were both substrates for the partially purified enzyme, suggesting that PKM had a role in controlling metabolic processes (Kishimoto *et al*, 1978). However both the activation of CANP (which required mM concentrations of Ca^{++}, Kishimoto *et al*, 1977) and the subsequent irreversible nature of PKM activation were inconsistent with activation in normal cellular environments. For these reasons, the existence of PKM and its physiological interaction with CANP were regarded as possible *in vitro* artefacts (Kishimoto *et al*, 1977).

1.2.2 Activation of protein kinase C by lipids and Calcium

The functional significance of PKM was realised when it was shown that the proenzyme of PKM could be reversibly activated by cell membrane preparations in the presence of Ca^{++} (Takai *et al*, 1979a). The precursor form of PKM was called protein kinase C (PKC) and was recognised as a new class of kinase with a unique mechanism of regulation (Takai *et al*, 1979a). The *in vitro* activation of PKC was shown to be absolutely dependent on Ca^{++} and phospholipids (Takai *et al*, 1979a) of which phosphatidylserine (PtdSer) and phosphatidylinositol (PtdIns) were shown to be the most potent activators, although phosphatidic acid (PA) and phosphatidylglycerol (PtdGly) were active to a lesser degree (Takai *et al*, 1979a; Kishimoto, 1980). PKC phosphorylated similar proteins to the PKM form of the protein although the pattern of preferences for histones was different (Takai *et al*, 1977 and 1979a). In the presence of phospholipids, PKC could be alternatively activated and inactivated by the addition of Ca^{++} and excess ethylene glycol bis(β-amino-ethyl

ether)*N,N,N',N'*-tetra acetic acid (EGTA) respectively (Takai *et al*, 1979a). Upon activation PKC was shown to associate with the phospholipids and addition of EGTA reversed this association (Takai *et al*, 1979a). Under these conditions mM concentrations of Ca^{++} were required for activity, suggesting that PKC was controlled by pulses of Ca^{++} *in vivo* (Takai *et al*, 1979a).

Further experiments lead to the observation that a small amount (5% w/w) of diacylglycerol (DG) could reduce the Ca^{++} dependence of PKC from the mM to the μM range (Kishimoto *et al*, 1980). DG increases the affinity of PKC for Ca^{++}, therefore allowing for its activation at low Ca^{++} concentrations (Takai *et al*, 1979b; Kishimoto *et al*, 1980). DG could not activate PKC in the absence of phospholipid and only increased the affinity of this enzyme for Ca^{++} in the presence of PtdSer but not PtdIns, PA or PtdGly (Kishimoto *et al*, 1980). For many years it had been known that certain hormones stimulated the turnover of inositol phospholipids and that one of the products of this pathway was DG (see Michell, 1975). The demonstration that DG increased the affinity of PKC for Ca^{++} so that the kinase was active at physiological levels of the metal ion was therefore important for showing that PKC was linked to signal transduction. A new understanding of the role of PKC emerged, in which PKC was perceived as being activated by DG production in response to hormonal stimulation of cells (see Nishizuka, 1984).

Activation by DG's is very specific and only 1,2-*sn* DG's are able to activate PKC *in vitro* (Takai *et al*, 1979b). Monoacylglcyerols, triacylglycerols, cholesterol and free unsaturated fatty acids are inactive (Ganong *et al*, 1987). The potency of DG's in activating PKC depends on the acyl side chains of DG. DG with two long chain

saturated fatty acids are inactive, whereas those with one unsaturated fatty acid chain are activators of PKC, the structure of the other chain being irrelevent. However, DG's with short chain saturated fatty acids are activators of PKC, suggesting that the lack of activation with the long chained varieties was due to physical factors and under some conditions these could support kinase activity (Ganong *et al*, 1987). PKC activation in mixed micelles is absolutely dependent on DG (or TPA, see section 1.2.4.5) (Boni and Rando, 1985 and Hannun *et al*, 1986), whereas in pure lipid micelles PKC can be activated in the absence of DG at high Ca^{++} concentrations (Takai *et al*, 1979b).

Initial attempts to activate PKC *in vivo* by the addition of biological relevent DG to cells were unsuccessful (Nishizuka, 1984). This is because these compounds are not very water soluble, form micelles and therefore do not penetrate into cell membranes very efficiently (Ganong et al, 1987). The compound *sn*-1-oleoyl-2-acetylglycerol (OAG) and various short chained synthetic DG's are more soluble in aqueous environments and so have been used successfully to activate PKC *in vivo* (see Nishizuka, 1986).

1.2.3 PKC and phosphatidyl inositol turnover

A wide variety of extracellular signals cause inositol phospholipid turnover and it is known that both breakdown products of phosphatidyl 4,5 bisphosphate (4,5-PIP_2) act as second messengers. DG activates PKC and inositol 1,4,5 trisphosphate mobilises Ca^{++} from internal cellular stores (see Hokin, 1985).

1.2.3.1 Phosphatidyl inositol turnover The effect of hormones on phospholipid metabolism were first recorded by Hokin and Hokin in

1953. They were working with the cholinergic stimulation of enzyme secretion in pigeon pancreas and observed that upon stimulation, the incorporation of ^{32}P into certain tissue extracts increased by as much as ten fold. The incorporation was found to be specific to the lipid fractions and it was shown that the inositol phospholipids were the major recipients (Hokin and Hokin, 1958). The phosphate was shown to be incorporated into the inositol moiety (and not the glycerol backbone) through a cyclic pathway that did not involve the *de novo* synthesis of lipids (Hokin and Hokin, 1958). The existence of a cyclic PtdIns metabolic pathway whose turnover was controlled by external signals had been discovered and steady state levels of DG were thought to be regulated by DG-lipase and DG-kinase (Hokin and Hokin, 1960). Further experiments revealed that the PtdIns response to agonists was a feature of other secretory tissues (Eggman and Hokin, 1960) and PtdIns metabolism was believed to be connected to the control of metabolic processes such as the entry of Na^+ ions into cells (Hokin and Hokin, 1960; and see Mi chell, 1975).

Durell *et al* (1969) demonstrated that the release of inositol phosphates from PtdIns followed a shorter time course than ^{32}P incorporation into lipids. This suggested that it was the products of PtdIns metabolism rather than the effects that lipid metabolism had on membrane permeability that was responsible for the metabolic responses of cells to agonists (Durell *et al*,1969). This view was supported by the observation that there were numerous forms of inositol phospholipids (Folch, 1949) and that while inositol phospholipids represented only a small proportion of cellular phospholipids, only they were metabolised in response to agonists (Hokin-Neaverson, 1974 and see Michell,1975). Metabolism resulted in a net reduction in the amount of inositol phospholipids in tissues

but no changes could be detected in the more abundant phospholipids (Hokin-Neaverson,1974). Enzymes specific for the degradation of PtdIns to DG and the inositol headgroups were identified and are referred to as the PtdIns specific phospholipase C's (PtdIns-PLC) (see Hokin, 1985).

1.2.3.2 Diacylglycerol as a second messenger By 1975, numerous agonists were known to have an effect on PtdIns metabolism but there were still no clues as to the physiological significance of 'PtdIns turnover' (see Michell, 1975). A common mechanism of activation was thought to exist and in all the known examples the agonists did not appear to enter the cell, but operated through cell surface receptors. Michell (1975) suggested that PtdIns turnover may have a function in controlling cellular Ca^{++} levels by regulating the activity of Ca^{++} gates. However there was no direct proof that at least one of the break down products of PtdIns turn-over were second messengers. The observation that DG could potentiate the activity of PKC by increasing its affinity for Ca^{++} was therefore of great significance, since it indicated that the breakdown products of PtdIns were second messengers. The predominant DG in inositol lipids had been shown to be 1-stearoyl-2-arachidonyl diglyceride (Holub *et al*,1970), a structure that theoretically would be an excellent PKC activator (Takai *et al*, 1979b). A model of hormone signal transduction was therefore proposed in which agonists caused PtdIns degradation, forming DG and this acted as a second messenger regulator of PKC at normal cellular levels of Ca^{++} (Takai *et al*, 1979b; Kishimoto *et al*, 1980). Support for this hypothesis came from studies with human platelets (as will be described in section 1.2.6.2) and from cell studies using synthetic DG's.

1.2.3.3 A bifurcating pathway It has subsequently been shown that the other product of 4,5-PIP_2 degradation, inositol-1,4,5-trisphosphate (Ins1,4,5P_3) also acts as a second messenger, causing Ca^{++} mobilisation from internal cellular stores (see Streb *et al*, 1983; Berridge and Irvine, 1984 and Hokin, 1985). Ca^{++} was already established as a key component in many cellular responses and Michell (1975) had proposed that inositol phospholipid turnover and Ca^{++} responses were closely linked, because of the correlation between these two events in cells. It has been shown that Ins1,4,5P_3 causes Ca^{++} mobilisation from internal stores (probably the endoplasmic reticulum), resulting in a rise in intracellular Ca^{++} levels within seconds of hormonal stimulation. The effect is specific for Ins1,4,5P_3, and no Ca^{++} release is seen with inositol bisphosphates or inositol monophosphates (see Hokin., 1985). The pathway by which the inositol moiety is recycled into inositol phospholipids remains to be elucidated. The mobilisation of Ca^{++} is required for full responses of human platelets to hormones (see section 1.2.6.2 below) and this indicates that each arm of the 4,5 PIP_2 pathway is important in cellular responses to external signals (Nishizuka, 1984).

1.2.4 The phorbol ester receptor

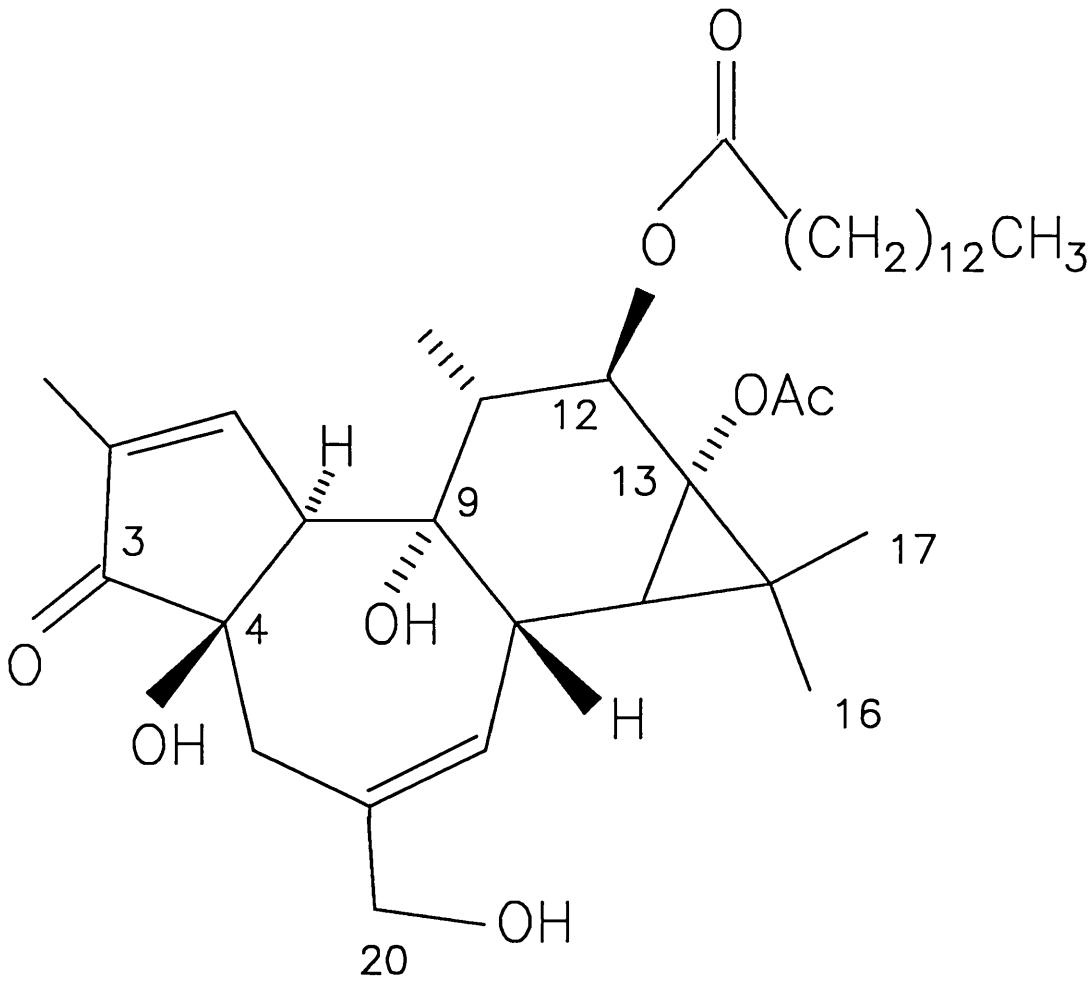
Phorbol esters have been defined as class of potent tumour promotors. PKC has been identified as the major cellular receptor for phorbol esters and PKC has therefore been implicated in cellular mitogenic responses (see Ashendel, 1985).

1.2.4.1 Tumour promotion Experimental tumours can be induced in animal models with chemicals by a method that involves multiple steps. Production of tumours by this method has been separated into

two defined stages (Diamond *et al*, 1980). In the first stage or the 'induction', tissues are exposed to a single sub-threshold level of a carcinogen. This produces a biochemical change in cells which does not appear to alter their proliferative characteristics in any detectable manner, but represents a senescent alteration that can persist for many years (Diamond *et al*,1980). If initiated cells are subsequently exposed to repeated doses of a tumour promoter, they become activated into a state of proliferation, at first producing benign tumours ('papilloma like') which will progress to malignant lesions if further applications of tumour promoters are given (Diamond *et al*,1980). Tumour promoters are therefore defined as chemical compounds which although not carcinogenic themselves, can cause the production of malignant lesions in tissues which have been previously induced into a state of readiness by treatment with a single sub-threshold dose of a carcinogenic compound.

1.2.4.2 The structure of biologically active phorbol esters In 1941 Berenblum identified oils and resins that could be extracted from the plant *Croton tiglium* as having potent tumour promoting properties when used in conjunction with the initiator benzpyrene. The active ingredients were later identified as 12,13 diesters of phorbol (Figure 1.3), a compound in the plant extracts which does not contain any tumour promoting activity by itself (Diamond *et al*, 1980). All tumour promoting phorbols are esterified at the 12 and 13 positions although other features of these compounds have effects on tumour promoting activity such as the requirement of a free allyl hydroxide at the 20 position (see Figure 1.3) (Diamond *et al*, 1980). Both symmetrical (fatty acids of equal lengths at the 12 and 13 positions) and asymmetrical (fatty acids of unequal length at the 12 and 13 positions) phorbol esters have tumour promoting activities

Figure 1.3 The structure of phorbol esters



although the lengths of the fatty acids at these positions affect their potency (Diamond *et al*, 1980). As a general rule, potent tumour promoters (both symmetrical and asymmetrical) are those with a total fatty acid side chain length of 14 to 20 carbon residues (Diamond *et al*, 1980). The most potent tumour promotor identified is 12-*O*-tetradecanoylphorbol-13-acetate (TPA; also refered to as phorbol-12-myristate-13-acetate; PMA) which has a myristate at the 12 position and an acetate at the 13 position (Diamond et al, 1980). It is interesting to note that phorbol-12-acetate-13-myristate is almost as potent as TPA (Ashendel,1985).

1.2.4.3 Biological effects of phorbol esters Phorbol esters elicit two sets of biological effects when applied to rodent skin models. The acute effects can be detected within a few hours of treatment and include oedema and erythema, followed by increased mitogenic activity which lasts for several days after which skin normality is restored (Diamond *et al*, 1980). Repeated applications of phorbol esters maintain these responses after which the chronic effects manifest themselves as benign tumours which if further stimulated by phorbol esters will progress to form malignant lesions (Diamond *et al*, 1980). The acute effects include increased protein synthesis, increased nucleic acid synthesis and by increased activity of certain enzymes (Diamond *et al*, 1980). One of the enzymes whose increased activity is extensively characterised is ornithine decarboxylase (ODC), which catalyses the formation of putrescine from ornithine and is rate limiting in polyamine synthesis (Diamond *et al*, 1980). The amount of activity of ODC in a cell may increase by as much as 200 fold following phorbol ester stimulation, the extent of induction being dependent upon the potency of the phorbol ester used and on the dose (Diamond *et al*, 1980). ODC seems to have a direct influence

on nucleic acid synthesis in response to phorbol ester treatment (Diamond *et al*, 1980).

Phorbol esters have also been shown to have numerous specific effects on cells in culture. These effects include uptake of metal ions; altered glucose transport; altered biosynthesis characteristics (eg prostaglandins); excretion of biologically active compounds and their precursors (eg arachidonic acid); and effects on enzyme activity and nucleic acid synthesis (Diamond *et al*, 1980). Further to these altered biochemical properties, TPA also alters the physiological properties of cells, leading to mitogenesis and altered differentiation patterns (Diamond *et al*, 1980).

1.2.4.4 Properties of the phorbol ester receptor Phorbol esters are extremely lipophilic and therefore it was difficult to establish whether the biological effects of these compounds were due to non-specific membrane effects or to binding to specific receptors (Ashendel, 1985). However in 1980, Drieder and Blumberg were able to demonstrate that a specific phorbol ester receptor could be detected by using [20-^{3}H]phorbol 12,13-dibutyrate (PBu_2), which was less lipophilic than TPA and therefore gave lower background binding. The receptor had a high affinity for phorbol esters, was specific for biologically active phorbol esters and could be saturated (Drieder and Blumberg, 1980). The binding of phorbols was reversible, showing that it was specific and was linear to the amount of protein in the assay (Drieder and Blumberg, 1980). Binding was also shown to be stable in a similar manner to the stability of proteins (lost by treatment at 100°C; sensitive to papain) and required the presence of divalent metal ions (Ca^{++} or Mg^{++}) (see Ashendel, 1985). Compounds that disrupted membrane integrity were

shown to alter the binding properties of phorbol esters and therefore it was concluded that the receptor was a membrane protein whose binding properties were influenced by its lipid environment (Ashendel, 1985).

The receptor was shown to have an extremely wide tissue distrubution, being detected in every nucleated cell of higher animals that was tested (Ashendel, 1985). It was found to be at its highest concentration in brain and could be detected in cells grown in culture (Ashendel, 1985). However no correlation could be made between the distribution of the receptor and the physiological or biological function of a cell type or on the proliferative state or age of a cell (Ashendel, 1985). The distribution did however appear to correlate with cellular communication, as demonstrated by its abundence in brain tissues and in the immune system, but its apparent absence in plants and in lower animals (Ashendel, 1985).

The biochemical properties of the receptor stimulated much interest because of its diverse effects on different cell lines. One of the unexplained phenomena was down-modulation (down regulation) of the receptor. When cells were exposed to phorbol esters the amount of binding seen increased initially and then decreased to a level that was below resting levels (Ashendel, 1985). This effect was only seen in cells that were biologically active and was thought to be due to either changes in the lipid or cytoplasmic environment of the cell or due to modification or compartmentalization of the receptor (Ashendel, 1985). Another interesting characteristic of the receptor was that for some cell lines, non-linear Scatchard plots were obtained for phorbol ester binding which suggested that there may be

more than a single class of receptor, which had different affinities although the effects may have been due to Ca^{++} (Ashendel, 1985).

1.2.4.5 Identification of protein kinase C as the phorbol ester receptor Evidence that PKC was the phorbol ester receptor came initially from studies with human platelets. It had been observed that phorbol esters could mimic the effects of thrombin in causing platelet activation and serotonin release (see Castagna *et al*, 1982). When stimulated in this fashion, a cytoplasmic protein with a Mr of 40,000 (40K) was shown to be rapidly and heavily phosphorylated in a manner similar to that seen when platelets were stimulated by exogenously added DG (see section 1.2.6.2 and Castagna *et al*, 1982). This suggested that DG and TPA acted on the same protein *in vivo*. *In vitro*, TPA was shown to substitute for DG in activating PKC at low concentrations of Ca^{++} by increasing the affinity of the enzyme for the metal ion (Castagna *et al*, 1982). TPA and DG did not synergise to activate PKC suggesting that TPA had a similar mechanism of activation to DG. Thus it appeared that PKC would be activated by TPA *in vivo* at physiological concentrations of Ca^{++} (Castagna *et al*, 1982). TPA was however more potent than DG, the former activating PKC at concentrations about 1000 fold less than the latter (Castagna *et al*, 1982). In platelets, the TPA effect appeared to be due to direct activation of PKC since DG was not produced (Castagna *et al*, 1982).

PKC and the phorbol ester receptor had similar tissue, species and subcellular distributions supporting the hypothesis that the two were the same (Ashendel, 1985). Further proof of this fact came when PKC and the phorbol ester receptor were purified by numerous protocols and shown to co-purify to homogeneity (Parker *et al*, 1984 and see Woodgett *et al*, 1987). Diacylglycerol was shown to compete

with phorbol esters for binding (Sharkey *et al*, 1984) and the potency of phorbol esters as tumour promotors was shown to correlate with their ability to activate PKC *in vitro* (Woodgett *et al*, 1987). TPA has been shown to mimic many of the effects of hormone stimulation on particular culture cells, resulting in similar patterns of substrate phosphorylations and in similar biological effects (see for example Castagna *et al*, 1982; and Woodgett *et al*, 1987). DG and TPA are thought to bind to PKC at the same site, activating the kinase by a similar mechanism, in which the affinity of PKC for Ca^{++} is increased. The 12, 13 position acyl groups of phorbol esters was believed to mimic the structure of the 1, 2 position acyls in DG and it is the similarity between these two structures that forms the basis of activation of PKC by phorbol esters (Nishizuka, 1984). More recently it has been suggested that the acyl groups merely allow phorbol esters to partition into lipid membranes and that it is the phorbol nucleus which is important in PKC activation (Wender *et al*, 1986; Jeffrey and Liskamp, 1986; Itai *et al*, 1988).

In vivo activation of PKC by phorbol esters is different from the activation by DG in some cell systems. For example, both TPA and OAG inhibit granulosa cell maturation, but the biochemical status of cells treated with these two compounds is different (Shinohara *et al*, 1985). Differences in effects of TPA and DG's on cell physiology is probabily a reflection of the differences in the relative stabilities of TPA and DG in membranes. DG in produced transiently in response to agonists and is rapidly metabolised by DG kinases and lipases. By contrast, TPA is quite stable in membranes and has an extremely long half life compared to DG. As a consequence, phorbol esters are present in cells for longer periods of time than DG and so may redistribute into other membranes, resulting in the activation

of PKC in unusual cellular compartments. TPA also has a higher affinity for PKC and so the responses of PKC to these compounds *in vivo* is different (see Woodgett *et al*, 1987).

1.2.5 PKC substrates

PKC is very promiscuous in terms of substrates and although many *in vitro* substrates have been described for it, the *in vivo* substrate repetoire of this kinase is almost certainly smaller (see Nishizuka, 1986; Woodgett *et al*, 1987). Numerous receptors for growth factors and hormones have been shown to be PKC substrates. These include the epidermal growth factor receptor (EGFR), the IL-2 receptor, the transferrin receptor, and the insulin and insulin like growth factor I receptors (Woodgett *et al*, 1987). The EGFR is phosphorylated on Thr^{645} which is located at the N-terminal end of the kinase domain just before the transmembrane section of this molecule (Woodgett *et al*, 1987). Phosphorylation at this residue occurs *in vitro* and *in vivo* in response to agents that activate PKC (DG and TPA). *In vivo* phosphorylation of EGFR results in the loss of a class of high affinity binding sites for the receptor ligand (EGF); in the reduction of the protein kinase activity of the catalytic domain; and in the transient internalisation of the receptors followed by their return to the plasma membrane (Woodgett *et al*, 1987). Although the physiological significance of these changes is not understood it does suggest that the activation of PKC can affect other signal transducing pathways and indicates that PKC may have a regulatory function in the mitogenic responses of cells to mitogens.

Many other potential substrates have been identified that are not growth factor receptors (Nishizuka, 1986; Woodgett *et al*, 1987). One of the substrates for PKC *in vivo* and *in vitro* is $pp60^{src}$ and the

phosphorylation of this protein *in vivo* indicates two important concepts in the interactions of PKC with its substrates. The protein $pp60^{src}$ is usually associated with the the plasma membrane, being myristylated at its N-terminus; this protein is phosphorylated *in vivo* on Ser^{12} following TPA stimulation (Woodgett *et al*, 1987). In a mutant form of $pp60^{src}$, the protein does not become myristylated and therefore no longer associates with the plasma membrane; this form of $pp60^{src}$ is not phosphorylated by PKC *in vivo* (Woodgett *et al*, 1987). Firstly, it indicates that the location of potential PKC substrates is important for phosphorylation. Secondly, it also indicates that activated PKC is associated with the plasma membrane and only phosphorylates substrates that are also found at this location (Woodgett *et al*, 1987). The identity of some PKC substrates is known, but others are identified merely by their size on polyacrylamide gels and have been identified as the major phosphorylated proteins in cells following TPA or DG stimulation (see for example the 80K substrate identified in Rozengurts laboratory, see Rozengurt, 1985). Some PKC substrates are also known to be substrates for other kinases activated by second messengers (such as cA kinase; see Nishizuka, 1986). Most of the PKC substrates almost certainly do not partake in signal transducing and tumour promoting responses and an understanding of the mechanisms of action of PKC awaits the identification of the proteins that are important in these pathways (Nishizuka, 1986).

1.2.6 Characteristics of PKC *in vivo*

PKC is a ubiquitous protiein kinase that is involved in signal transduction and tumour promotion. It is a single polypeptide with a Mr in the region of 80,000 which has distinct catalytic and regulatory domains. It is absolutely dependent upon Ca^{++} and

phospholipids for activity and the activity can be potentiated at low concentrations of Ca^{++} by either DG or TPA (Woodgett *et al*, 1987). The role of PKC in stimulus-response coupling has therefore been extensively investigated, using phorbol esters and diacylglycerols to activate PKC *in vivo*. In order to manipulate PKC with DG *in vivo*, short chain synthetic DG's are used, because these retain the ability to activate PKC and are more able to interc a late into cellular membranes than long chained and natural DG's.

PKC appears to have a pivitol role in the responses of many secretory and neuronal cells to external signals. There is an extensive list of cells which respond to phorbol esters (see Nishizuka, 1986). PKC activation in pituitary cells (endocrine system) for example stimulates the release of pituitary hormones, growth hormone, luteinising hormone, prolactin and thyrotropin (see Nishizuka, 1986). Other functions that are initiated by PKC activation include secretion of hormones and enzymes from exocrine cells; excretion of neuropeptides from nerve cells; initiation of muscular activity; activation and release of active compounds from cells of the immune system; and the alteration of biochemical functions in a variety of cell types (Nishizuka, 1986).

1.2.6.1 PKC translocation and down regulation When the Ca^{++} and phospholipid dependent activity of PKC was first demonstrated, it was observed that upon activation, PKC asociated with lipid micelles (Takai *et al*, 1979a). This appears to be consistent with activation *in vivo*. Treatment of cells with phorbol esters causes an apparent redistribution of PKC from a predominantly cytosolic (soluble) to a predominantly membrane (particulate) associated form (Anderson *et al*, 1985). Similar results are obtained when cells are

stimulated by hormones and growth factors and these observations are backed up by immunological data (see Woodgett *et al*, 1987). PKC therefore appears to move from the cytoplasm to the membrane when cells are stimulated by hormones due to the production of DG in the membrane, suggesting that the active kinase is membrane associated. The association of PKC with the membrane is probably dynamic, PKC constantly associating with and dissociating from the membrane. In the presence of DG or TPA, the association between PKC and membrane is stablised, and PKC is activated (Woodgett *et al*, 1987).

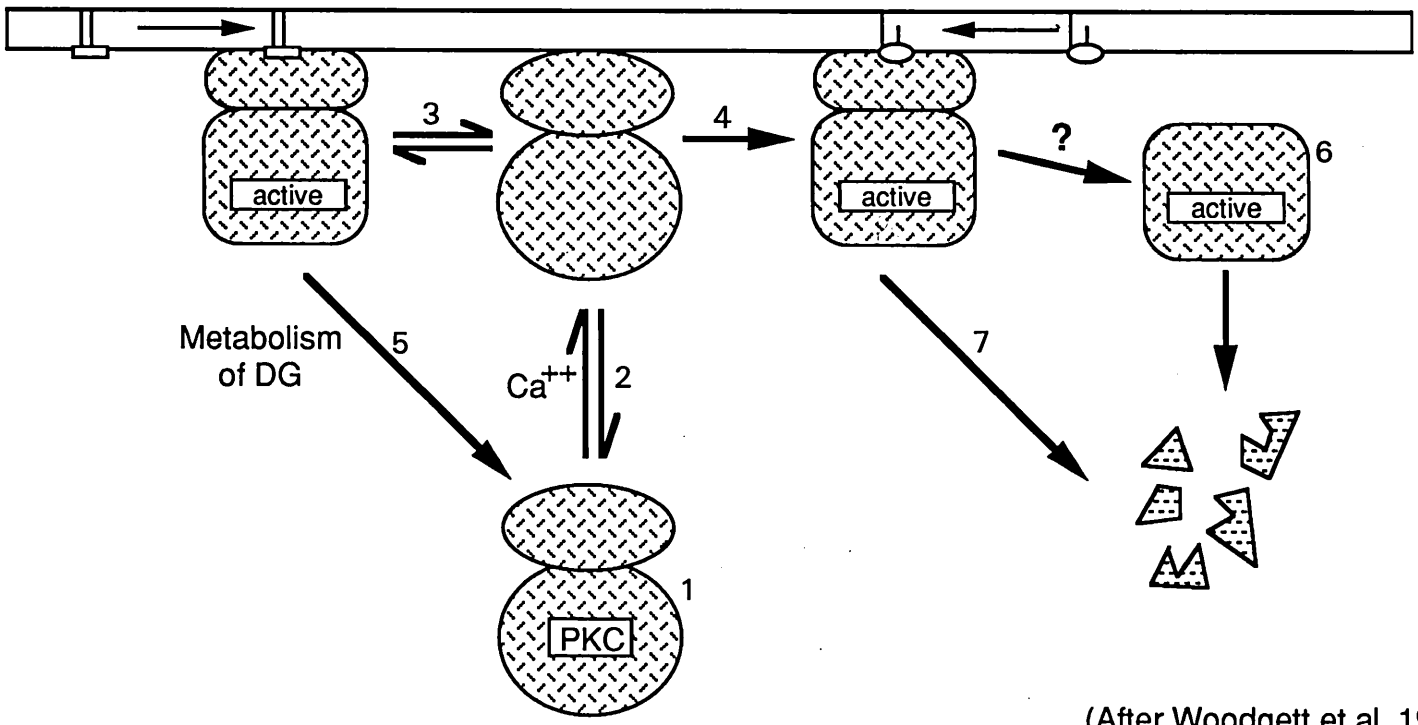
Another characteristic of PKC is down-regulation following chronic stimulation by phorbol esters. Down-regulation of the phorbol ester receptor had been documented before it had been identified as PKC (Diamond *et al*, 1980). When cells are exposed to phorbol esters, the PKC down-regulates and the cells no longer respond to stimuli that are mediated through PKC (Woodgett *et al*, 1987). The mechanism of down-regulation appears to be through a normal translocation response followed by degradation of PKC. When cells are treated with TPA, the PKC translocates to the membrane, with which it becomes chronically associated. This appears to reduce the stability of the kinase and it becomes degraded at a faster rate than unstimulated PKC (Young *et al*, 1987). The rate of PKC synthesis under these conditions is unaltered and the mRNA levels do not change, indicating that down regulation is not due to decreased synthesis but due to increased degradation. PKC down-regulation is not seen with DG because the transient nature of DG pulses in membranes do not retain PKC at the membrane for significant periods of time (Woodgett *et al*, 1987). However, it is conceivable that upon stimulation of PKC by DG a proportion of the activated

kinase is cleaved into the two domains, forming a constituitively active soluble form of PKC which becomes cytosolic (Young *et al*, 1987).

Thus a model for PKC activation by DG or TPA can be proposed (Figure 1.4). PKC is in dynamic equilibrium with the plasma membrane,associating with it in a Ca^{++} dependent manner. The production of DG from PtdIns turnover stabilises the membrane association and activates the catalytic domain (Figure 1.4). Once the DG is removed from the membrane by further metabolism, the PKC returns to the resting dynamic equilibrium (Figure 1.4). In the presence of phorbol esters however, the association of PKC with the membranes is prolonged. This exposes the catalytically sensitive regions of the molecule for longer, resulting in the degradation and therefore down-regulation of the protein (Figure 1.4). It has been suggested that down regulation of the molecule proceeds through an M form of the kinase but no definitive evidence is available to substantiate this possibility (Young *et al*, 1986).

1.2.6.2 A model for the activation of platelets In platelets the effect of PKC activation is particularly well characterised. When platelets are stimulated by a variety of secretagogues (such as thrombin), aggregation occurs and numerous biologically active compounds such as seratonin and acid hydrolases are secreted. Concomitant with activation is the rapid and extensive phosphorylation of two proteins with Mr values of 20,000 (20K) and 40,000 (40K) (Woodgett *et al*, 1987). The 20K protein has been identified as MLC and is phosphorylated by MLCK (see section 1.1.2.2). MLCK is activated in a Ca^{++}/CaM dependent manner and is therefore stimulated in response to thrombin due to Ca^{++}

Figure 1.4 Inactive cytosolic PKC (1) associates with the plasma membrane in a Ca^{++} dependent manner (2), but remains inactive until stimulated by DG (3) or TPA (4). In the case of DG, the PKC returns to the cytosol once the DG signal is removed (5). In the case of PKC activated by TPA, this activation leads to eventual protein degredation (7), possibly through the production of an active 'M form' (6).



(After Woodgett et al, 1987)

mobilisation which occurs as a result of 1,4,5$InsP_3$ production (Woodgett *et al*, 1987). The 40K substrate was shown to be a PKC substrate, the evidence coming from several lines of research. First, when platelets are stimulated by thrombin, 4,5-PIP_2 is rapidly degraded, giving a transient pulse of DG and this was considered to have the potential to activate PKC (Kawahara *et al*, 1980). Second, the phosphorylation of 40K could be stimulated by the addition of bacterial phospholipase C to platelets, which produced DG in the membranes (Kawahara *et al*, 1980). Third, purified PKC was able to phosphorylate 40K *in vitro* and gave similar phosphopeptide maps to 40K phosphorylated *in vivo* (see Nishizuka, 1984) and finally, the addition of synthetic DG to platelets caused 40K phosphorylation *in vivo* (see Nishizuka, 1984). The appearence of DG in platelet membranes was always accompanied by 40K phosphorylation, even in the absence of further metabolic processes (such as arachadonic acid production). A link had therefore been established between 40K phosphorylation and PKC activition; in this system, 40K phosphorylation is indicative of PKC activation (see Nishizuka, 1986).

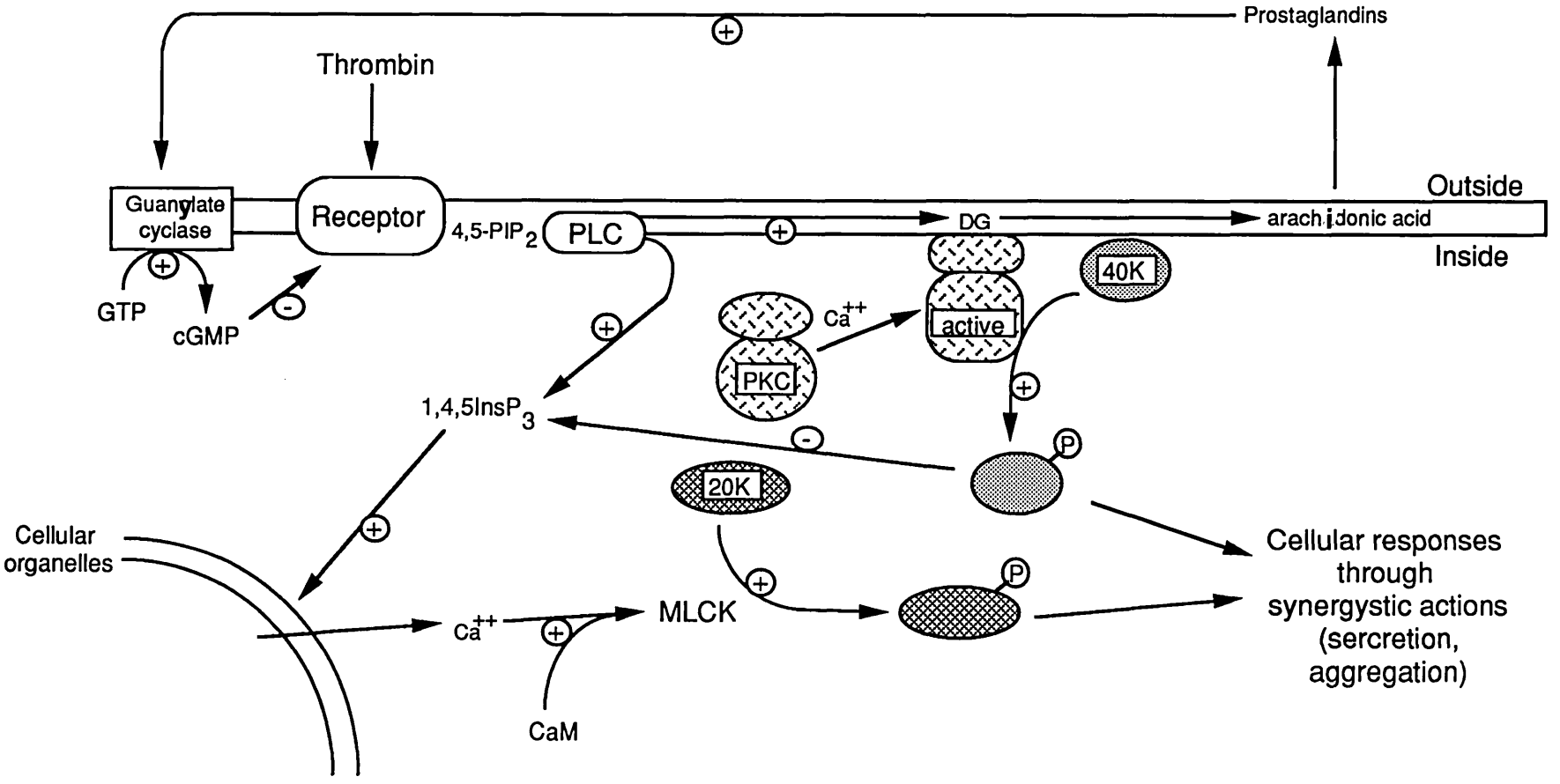
Activation of PKC does not result in 20K phosphorylation and MLCK does not appear to phosphorylate 40K, suggesting that the two arms of the pathway are stimulated independently (Nishizuka, 1984). When platelets are stimulated with synthetic DG, PKC is activated and 40K phosphorylated (Nishizuka, 1984). By careful manipulation of the Ca^{++} ionophore A23187, it is possible to increase the free Ca^{++} concentration in platelets to stimulate MLC (20K) phosphorylation in the absence of PKC activation (Nishizuka, 1984). However when either 20K or 40K is phosphorylated in the presence of A23187 or DG alone, a full thrombin-like response is not seen (Nishizuka, 1984). A

secretory response is only seen when both A23187 and DG are present and both arms of the bifurcating pathway are activated.

The 40K protein has been reported to contain Ins1,4,5P_3-5' phosphatase activity and therefore the phosphorylation of this protein may represent a feedback pathway in the activation process (see Woodgett *et al*, 1987). A model for platelet activation has been proposed in which thrombin, acting through a specific surface receptor activates a PtdIns-PLC which catalyses the breakdown of 4,5-PIP_2 to Ins1,4,5P_3 and DG (Figure 1.5) (Woodgett *et al*, 1987). The DG activates PKC which is responsible for mediating part of the secretion responses and the Ins1,4,5P_3 mobilises Ca^{++} from internal stores, which mediates the other half of the response. MLC is phosphorylated as a result of Ca^{++} mobilisation and the 40K substrate due to PKC activation. One of the functions of PKC in this model may be the activation of an Ins1,4,5P_3 phosphatase, which by dephosphorylating Ins1,4,5P_3 acts as a feedback loop to inhibit the Ca^{++} signal (Figure 1.5) (see Woodgett *et al*, 1987). This hypothesis remains to be confirmed.

Stimulation of inositol phospholipid turnover in platelets leads to arachidonic acid production (Nishi zuka, 1986). Most arachidonic acid is generated by the degradation of phosphatidylcholine and phosphatidylethanolamine, although it can also be generated from DG by the action of DG-lipases (Nishi zuka, 1986). Arachidonic acid is a precursor in prostaglandin/leukotriene synthesis and these compounds function through receptors to activate second messenger pathways, including guanylate cyclase. In platelets the cGMP produced by the cyclase activates cG kinase and this kinase like cA kinase appears to have antagonistic actions towards inositol

Figure 1.5 Activation of platelets by thrombin



(After Nishizuka, 1986)

phospholipid turnover (Nishizuka, 1986). It therefore appears that signal transduction involving inositol phospholipid turnover is feedback inhibited by other second messenger systems (Figure 1.5). The principles discussed for platelets probably represent a general model for PKC activation by inositol phospholipid turnover, although the precise details will vary from cell type to cell type (Nishizuka, 1986). PKC may therefore represent a cross-over point for many cellular pathways or it may represent a channel through which all signals pass (Nishizuka, 1986). Nevertheless, activation of this kinase results in pleiotropic responses in many cell types and in order to understand further the function of this kinase, many groups have cloned the PKC gene.

1.2.7 Cloning PKC

Following the purification and characterisation of PKC, the gene encoding this protein was cloned and sequenced (Parker *et al*, 1986 and Parker *et al*, 1989). The studies of Parker *et al* (1986) revealed that PKC (later defined as the -α isotype) is a single polypeptide with a predicted Mr in the region of 76,000 which could be divided into two functional domains (as was discussed by others in subsequent papers, see Carpenter *et al*, 1987). The N-terminus constituted a putative regulatory domain which contained a tandomly repeated Cys rich domain with the structure Cys-$(Xaa)_2$-Cys-$(Xaa)_{13}$-Cys-$(Xaa)_2$-Cys. This structure is comparable to structures in DNA binding proteins defined as zinc fingers, which are the DNA binding domain of these proteins (see Berg, 1986). This lead to speculation that these regions represented a DNA binding domain in PKC and that activation of this enzyme resulted in the binding of the regulatory domain to specific regions of DNA. To date there is no firm evidence that PKC has any DNA binding activity (see Nishizuka, 1988),

although such claims have been made (Testori *et al*, 1988). These Cys rich repeats may represent the phospholipid binding domains (Carpenter *et al*, 1987). Within the sequence, a definitive Ca^{++} binding site could not be identified but a potential site was seen in a partial 'EF Hand' structure in the N-terminal half of the molecule (Parker *et al*, 1986). The kinase domain of PKC is located in the C-terminal half of the molecule and was defined by its homology with other Ser/Thr and Tyr kinases (Parker *et al*, 1986). Within this region there is a putative nucleotide binding sites with the consensus sequences Gly-Xaa-Gly-$(Xaa)_2$-Gly-$(Xaa)_{17}$-Lys (Parker *et al*, 1986). The two domain structure of the polypeptide suggested that this kinase may have arisen from the fusion of a regulatory gene and of a kinase domain to form the present structure (Parker *et al*, 1986).

1.2.8 A family of kinases

During the cloning of PKC it became apparent that PKC was not a single entity, when three PKC distinct cDNA species were detected and sequenced (Parker *et al*, 1986 and Coussens *et al*, 1986). Similar results were obtained almost simultaneously from several groups working with PKC from different animal species and this resulted in confusion as each group adopted its own (and often contradictory) nomenclature (Carpenter *et al*, 1987). The nomenclature adopted in this thesis is that of Coussens *et al* (1986 and 1987). The isotypes are therefore referred to as PKC-α, -β_1, -β_2 and -γ. Initially only the -α, -β_1 and -γ clones were detected and although the proteins were shown to be highly homologous, the genes were found to be located on different chromosomes (Coussens *et al*, 1986). Thus it was clear that PKC was a complex enzyme, with multiple different isoforms. Further complexity was shown to exist when a fourth mRNA was

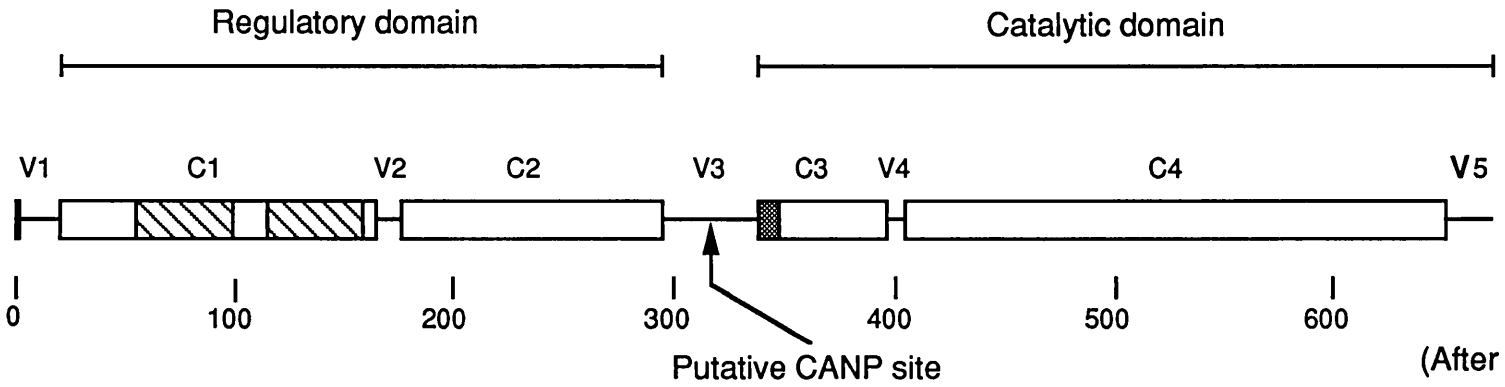
identified. This mRNA species arose from a 3' splice variation of the -β_1 gene and resulted in a polypeptides which differed from -β_1 in only its last 50 amino acids; this was called -β_2 (Coussens *et al*, 1987).

Comparison of the sequences for the four putative PKC proteins shows that they are highly homologous, with an overall identity in excess of 65%, although the -α and -β genes are more closely related to each other than either is to the -γ gene (Coussens *et al*, 1986). All the genes encode single polypeptides with predicted lengths in the region of 670 to 700 residues and predicted Mr values in the region of 77,000 to 78,000. Alignment of the protein sequences reveals that the conservation between them occurs in distinct regions which are separated by more highly variable regions (Coussens *et al*, 1986). Overall, there are four conserved and five variable regions which are designated C1-4 and V1-5 respectively (see Figure 1.6). The V regions are on the whole very short (less than 20 residues) although the V3 region is quite extended (about 50 residues).

The C1 and C2 regions form the regulatory domain and probably contain the lipid, Ca^{++} and DG/TPA binding sites (Figure 1.6) (Coussens *et al*, 1986). The Cys rich repeats described for PKC-α are conserved in -β and -γ and are located in the C1 region (Figure 1.6, hatched boxes); the overall identity in this region is about 81% (Coussens *et al*, 1986). The C2 region is less conserved than the C1 region and it is this region in which the half 'EF hand' structure described for PKC-α (section 1.2.9) is located. This structure is however not conserved in the -β and -γ isotypes leading to speculation that the isotypes may show differential responses to Ca^{++} (Coussens *et al*, 1986). The C3, V4, C4 and V5 regions together

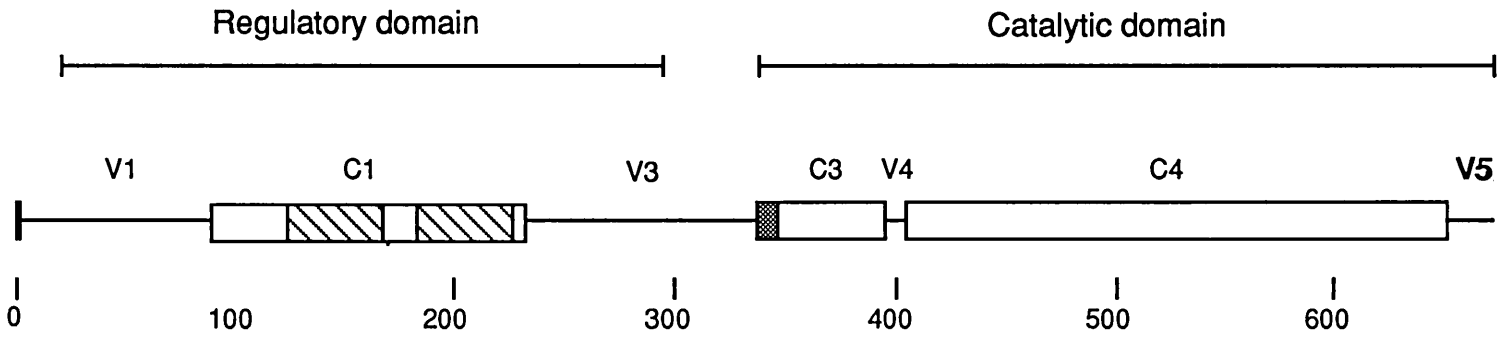
Figure 1.6 The primary structure of PKC

(a) PKC –α, –β 1, –β 2, –γ



(After Coussens *et al*, 1986)

(b) PKC –ε, –δ, –ζ



(After Nishizuka, 1988)

constitute the kinase domain (see Figure 1.6) (Coussens *et al*, 1986). The conservation of sequences in this entire region is approximately 72% and this domain shows extensive homology with the kinase domains of other Ser/Thr and Tyr kinases; the putative nucleotide binding site (Gly-Xa-Gly-Xaa-Xaa-Gly-$(Xaa)_{17}$-Lys) is located at the extreme N-terminal end of the C3 region (Figure 1.6, shaded box) (Coussens *et al*, 1986).

As discussed in section 1.2.6, PKC has two distinct functional domains (one of which is regulatory and the other is the kinase domain), which can be separated proteolytically (see Woodgett *et al*, 1987). The V3 region is believed to form a hinge between the catalytic and regulatory and is probably exposed on the surface of the molecule as predicted from its highly charged nature. It is in this region that proteolytic cleavage of PKC into the two functional domains is believed to occur (Coussens *et al*, 1986). This prediction is backed up by immunological evidence using the monoclonal antibody MC5, which was raised to whole PKC (Young *et al*, 1987). The epitope for MC5 has been located to the V3 region (residue 305) of the PKC-α polypeptide and upon proteolytic cleavage of the protein this epitope cannot be detected in either fragment (Young *et al*, 1987).

Recently three new PKC clones have been described which have significant sequence homology with PKC-α, -β and -γ; these have been called PKC-δ, -ε and -ζ (see Nishizuka, 1988). When aligned with the -α, -β and -γ clones the new members contain the C1 region (with conserved Cys repeats), the C3 region and C4 regions but lack the C2 region (Nishizuka, 1988). The -ε kinase also shows phospholipid and TPA dependent activity but this isotype shows

significant differences in its substrate specificity compared to -α, -β and -γ (Schaap *et al*, 1989). Initial studies indicate that the -ε isotype may be independent of Ca^{++} for acitvation and this would be consistant with the Ca^{++} binding domain being located in the C2 region (Ohno *et al*, 1988; Schaap *et al*, 1989). These isotypes show a much narrower tissue distribution than the -α and β isotypes but are similar in distribution to the -γ isotype (Schaap *et al*, 1989 and Ohno *et al*, 1988; Ono *et al*, 1988).

1.3 Aims of this thesis

The existence of a large family of PKC-like molecules posed many possibilities and questions about signal transduction regulated by this enzyme family. The multiplicity suggested that cellular responses may be affected by activation of more than one PKC pathway. Thus it could be argued that each PKC isotype was responsible for mediating the responses of cells to particular agonists. When the sequences of the PKC isotypes from different animals are compared, there is greater conservation between the equivalent isotypes from different species than between different isotypes from the same (see Parker *et al*, 1989). Of note, the conservation in the V regions between species was almost complete. The high degree of conservation of the V regions suggests that these regions represent functional domains that give the isotypes individual characteristics. In the regulatory domain, the V regions may represent the sites of interaction with particular co-factors, phospholipids, receptor proteins or membrane proteins. In the kinase domain the V regions may give the isotypes individual (but perhaps overlapping) substrate specificities. *In vivo*, the isotypes were shown to have distinct tissue distribution patterns (Coussens *et al*, 1986, 1987 and see Nishizuka, 1988). The existence of cells which

contained multiple PKC isotypes suggested that these cells may be responsive to agonists that required different PKC isotypes to mediate their signal transducing responses. This would explain the diversity of responses seen when 'PKC' was activated. It would also enable the responses of cells to particular agonists to be carefully tailored by the selective expression of the individual isotypes.

The cloning studies had revealed that the isotypes were structurally different and had distinct patterns of tissue expression, however they had not revealed anything about the biochemical properties of the individual isotypes. The possibility therefore remained that the nature of the differences was merely to do with the differential tissue expression mentioned, the important differences between the isotypes being located in the promotor regions; however the exquisite conservation of the isotypes between different animal species argues against this possibility.

In order to understand signal transduction, the role that each isotype has in these processes has to be established. As one approach to addressing this question, this thesis describes the purification, separation and subsequent biochemical analysis of the -α, -β and -γ isotypes of PKC from bovine brain. The results show that while these isotypes fall into the broad definition of Ca^{++}, phospholipid and DG/TPA dependent kinases, their *in vitro* activation characteristics and their substrate specificity ranges show subtle differences. This suggests that the *in vivo* properties of the isotypes will show differences in responses to activators, and preliminary studies in this direction appear to confirm this.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

General Laboratory reagents and solvents were purchased from BDH, England and were 'AnalaR' grade. Glutaraldehyde solution, glycine, bovine serum albumin, thyroglobulin, Triton X-100, Tween-20, β-mercaptoethanol, *o*-phenylenediamine, phorbol 12-myristate 13-acetate, arachidonic acid, DL-dithiothreitol, phenylmethylsulfonyl fluoride, betaine, aprotinin, leupeptin, benzamidine, tosyl lysine chloromethyl ketone (TLCK), tosyl phenylalanine chloromethyl ketone (TPCK), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDAC), diaminoethanetetra-acetic acid (EDTA), ethylene glycol bis(β-amino-ethyl ether)*N,N,N',N'*-tetra acetic acid (EGTA), Hepes, Bes, Mes, histone III-S, protamine sulphate, myelin basic protein (MBP), *sn*-1,2-dioctanoylglycerol (diC_8), *sn*-1,2-dihexanoylglycerol (diC_6), diolein and threonine were purchased from the Sigma Chemical Company, England. Trypsin and keyhole limpit haemocyanin (KLH) were purchased from Worthington Diagnostic Systems Ltd. Octyl-Sepharose, AH-Sepharose 4B, phenyl-Sepharose, protein A-Sepharose and S-200 gel matrices were from Pharmacia, UK Ltd. Acrylamide, sodium dodecyl sulphate (SDS), *NN'*-methylenebisacrylamide (Bis), ammonium persulphate (APS), N,N,N',N'-tetramethylethylenediamine (temed), hydroxylapatite gel matrices, mixed bed resin AG501-X8(D) and G-50 gel filtration matrix were from BioRad, England. Phosphatidylserine was from Lipid Products, England unless otherwise stated. Radio-labelled products, 'hyperfilm MP' and 'Rainbow markers' were from Amersham, England. Phosphate buffered saline (PBS) was purchased from GIBCO, England. Goat-anti-rabbit horse radish peroxidase conjugated antibodies were purchases from

Miles Laboratories, England. Calf thymus histone H1 was purchased from Boerhinger-Mannheim. Adjuvants were from Difco Laboratories, USA. The detergent 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and the coupling agent *m*-maleimidobenzoylsulfosuccinimide ester (sulpho-MBS) were from Pierce, UK Ltd.

2.2 Coupling of peptides to carrier proteins

2.2.1 Coupling with glutaraldehyde Peptides lacking Cys residues were coupled to KLH through their free α-amino groups. For each peptide, the coupling conditions were as follows: 15mg KLH (dialysed into 1ml PBS) was added to 25mg peptide (dissolved in PBS, 0.48ml) and 15µl glutaraldehyde (25% solution) was added. The mixture was incubated for 20 min (15-20°C) with occasional stirring and a second 7.5µl aliquot of glutaraldehyde was added; this was incubated as before. The reaction was stopped by the addition of 300µl 1M glycine (pH 6.0). The conjugate solution was divided into aliquots and stored at -20°C until required.

2.2.2 Coupling with MBS Peptides with Cys residues were coupled to carrier proteins with the coupling agent sulfo-MBS. The conditions were according to Rothbard *et al* (1984). Briefly: 15-20mg thyroglobulin was dissolved in 0.4ml PBS and added to 5mg sulfo-MBS dissolved in 0.4 ml water. The mixture was incubated for 60 min (15-20°C) and the MBS-thyroglobulin complex was desalted on a G-50 column equilibrated in sodium phosphate buffer (100mM, pH 6.0). Meanwhile, the peptide was reduced; 15-20mg peptide was disolved in 0.5ml of 0.1M borate buffer (pH 8.0) and 100µl of freshly made sodium borohydride solution (5mg/ml) was added. This was

incubated for 5 min (0°C) and two drops of HCl (1M) were added followed by a 5 min incubation (0°C). Two drops of NaOH (1M) were added to the peptide and the mixture was added to the desalted MBS-thyroglobulin complex. This was incubated for 18 hr (15-20°C) and desalted on a G-50 column equilibrated in ammonium hydrogen carbonate buffer (100mM). The conjugate was stored at 4°C until required.

2.3 Enzyme linked immuno-sorbant assay

Antibody titres were determined by ELISA. 96 well ELISA plates were seeded with 100µl of relevant antigen (50ng peptide or 5ng protein) in 200mM sodium carbonate buffer (pH 9.2). The plates were incubated for 18 hr (4°C). All subsequent steps were performed at room temperature. The plates were washed three times in 0.05% v/v Tween-20 in PBS (Tween/PBS) and then 200µl 0.5% w/v BSA in Tween/PBS was added to each well. The plates were incubated for 60 min and washed as before. 50µl of antiserum at relevant dilutions (diluted in PBS) was added to each well and incubated for 120 min. The wells were washed as before and incubated with 50µl goat-anti-rabbit horse radish peroxidase conjugated antibodies diluted 1 in 500 in Tween/PBS. The plates were incubated for 120 min and washed as before. 100µl of substrate* was added to each well and the reaction was allowed to proceed for 15 min in the dark. The OD_{450} of the solution in each well was recorded.

* The substrate was prepared as follows: *o*-phenylene diamine was made up to a solution of 2mg/ml in 156mM di-sodium hydrogen orthophosphate, 27mM Citric acid (pH 6.0) containing 0.1% v/v H_2O_2.

2.4 Polyacrlyamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out acording to Laemmli (1970). The concentrations of acrylamide in the resolving gels are indicated. The stack contained 5% w/v acrylamide. All reagents were electrophoresis grade. Samples for SDS-PAGE were prepared by adding three volumns of protein solution to one volume of four times concentrated Laemmli sample buffer (modified from Laemmli, 1970). These were immediately immersed in a boiling water bath for 5 min. The standard molecular weight proteins were from BioRad and the sizes are as published in the catalogue. Gels were stained either with Coomassie blue, or by silver staining according to the method of Ansorge (1985). Protein concentration determinations were performed according to the method of Bradford (1976), using an immunoglobulin control(sigma).

component	%acrylamide(w/v)					
	7%	10%	15%	12.5%	25%	5% (stack)
% acrylamide (w/v)	7	10	15	12.5	25	5
% Bis (w/v)	0.019	0.027	0.04	0.05	0.05	0.13
Tris.HCl pH 8.8 (M)	0.375	0.375	0.375	0.375	0.375	-
Tris.HCL pH 6.8 (M)	-	-	-	-	-	0.125
% SDS (w/v)	0.1	0.1	0.1	0.1	0.1	0.1
% APS (w/v)	0.1	0.1	0.1	0.033	0.033	0.1
% Temed (v/v $x10^{-4}$)	3.3	3.3	3.3	3.0	3.0	3.3

Four times concentrated Laemmli sample buffer:

8% w/v SDS

0.25M Tris.HCl, pH 6.8

10% v/v glycerol

1mM DL-dithiothrietol

0.1mg/ml bromophenol blue

2.5 Immuno-protein blotting

Immuno-protein blotting was as described by Towbin *et al*, (1979). Briefly: proteins were separated by SDS-PAGE and transferred to a BioRad electrophoresis tank, containing 3l 25 mM Tris, 192 mM glycine, 20% v/v methanol. Optimal transfer of PKC was achieved by electro-transferrence to nitrocellulose sheets at constant current (400mA) for 75 min (4°C). Non-specific sites were blocked in a solution of 5% w/v milk powder (Marvel), 0.05% Tween-20 (Sigma) in PBS. The sheets were washed three times with a solution of 0.1% v/v Triton X-100 in PBS (Triton/PBS) for 10 min each. The sheets were incubated with antibody diluted in Triton/PBS for 120 min at room temperature and washed as before. They were then incubated with [125-I]-protein A for 60 min. The sheets were then washed once more as before, dried and exposed to autoradiographic film.

2.6 Preparation of the PtdSer-acrylamide column

Six 1ml aliquots of PtdSer (10mg/ml, Sigma) were evaporated to dryness from 95:5% chloroform:methanol under a stream of nitrogen in separate polypropylene tubes (17ml, Sarstedt). For each aliquot, the PtdSer was re-dissolved in 500µl dimethylformamide (60°C, 2min) and 5ml acrylamide: Bis (15% w/v:5% w/v) was added with

constant and vigorous mixing. 100µl APS (140%w/v) and 2.5µl Temed were added with constant mixing and the acrylamide was allowed to polymerise. Once polymerised, the portions were combined and cut into small pieces (2-3mm^2). These were homogenised in a loose fitting Dounce homogeniser and the resulting acrylamide beads were washed three times with water to remove the fine particles. The beads were equilibrated in the required buffer and used immediately.

2.7 Preparation of the threonine-Sepharose column

The thr-Sepharose column was prepared as published (Kikkawa *et al*, 1986). Briefly: AH-Sepharose 4B (Pharmacia, 15g) was swollen in 200ml 0.5M NaCl and washed in a scintered glass funnel with 3l 0.5M NaCl solution, followed by 1l water at pH 4.5. The gel was resuspended in 120ml water (pH 4.5) and 150mg Thr dissolved in 5ml water was added. 2.5g EDAC was added to the gel/Thr mixture with constant stirring and the pH was adjusted to between 4.0 and 4.5. The mixture was incubated with tumbling (15-20°C) and the pH was checked every 20 min and readjusted to between 4 and 4.5 if necessary. The pH of the mixture was adjusted to between 4 and 4.5 after 120 min incubation and then incubated overnight (4°C). The gel was washed on a scintered glass funnel with alternative 500ml aliquots of sodium acetate buffer (0.1M, pH 4.0) containing 0.5M NaCl and Tris.HCl buffer (0.1M, pH 8.0) containing 0.5M NaCl (total of 4l each). The gel was stored in 20mM Tris.HCl, 0.5M NaCl at 4°C until required.

2.8 Protein kinase C assay

PKC was assayed in the following assay buffer: 50mM Hepes pH 7.5, 0.25mM EDTA, 12.5mM magnesium acetate, 0.75mM calcium chloride, 1.25mg/ml histone H1, 0.25% Triton X-100, 1.25mg/ml phosphatidyl-

L-serine (PtdSer), 100ng/ml 12-0-tetradecanoyl phorbol 13-acetate (TPA), 0.125mM ATP (containing [γ^{32}P] ATP at a specific activity of 150-200 cpm/pmol), PKC enzyme; total volume = 40µl. The assays were initiated by the addition of ATP, incubated at 30°C for 3min and terminated by spotting 25µl of the reaction mix onto 1cm x 1cm squares of P81 paper followed by immersion into 30% acetic acid. The squares were washed twice for 10min in 10% acetic acid and then counted for Cherenkov radiation.

The PtdSer was presented either in mixed micelles or in pure lipid micelles. Mixed micelles was prepared by evaporating to dryness 1mg PtdSer from a chloroform/methanol stock together with 80ng TPA from an ethanol stock. The lipids were redissolved 200µl of 20mM Hepes pH 7.5, 1% Triton X-100 (30°C, 2 min). Pure lipid micelles were prepared by dissolving 1mg PtdSer dried down from choloroform/methanol stock in 30µl 95:5% chloroform:methanol. The dissolved lipids were then dispersed into 470µl of 20mM Hepes pH 7.5 by sonication for 5min (4°C) under a stream of nitrogen. The dependence of the isoenzymes on diacylglycerols (DG) was investigated using mixed micelles in the absence of TPA. Calcium buffering solutions were calculated using the 'Metlig' Programme, kindly provided by Prof. R. Denton, Bristol University.

2.9 Phosphopeptide mapping

Samples containing phospho-proteins were separated by SDS-PAGE. The acrylamide gels were Coomassieblue stained, dried and exposed to autoradiographic film (-80°C). The phosphorylated substrates were identified and the required bands were excised from the gels. The gel pieces containing phosphopeptides were re-swollen in 1ml 50mM ammonium bicarbonate (5min) and extracted four times with

1ml aliquots of acetone (gently agitated for 15 min each). The gel pieces were re-swollen in 1ml 50mM ammonium bicarbonate (15 min) and finally resuspended in 0.5 ml 50mM ammonium bicarbonate. Trypsin (5% molar ratio with respect to test protein) was added to each gel piece and they were incubated for 18 hr (37°C). The supernatants were removed and saved. A further aliquot of trypsin (5% molar ratio) was added followed by incubation for 2 hr (37°C). This process was repeated until the recovery of radioactive peptides in the supernatant was in excess of 95% (usually after three aliquots of trypsin). The supernatants were taken to dryness in a 'Speed-Vac' and the phosphorylated peptides were disolved in 10-15µl acetic acid (100mM). The recovery of the phosphopeptides was >98%. The peptides were loaded onto a thin layer chromatography plate (20cm x 20cm, Kodak) in 2µl aliquots allowing each aliquot to air-dry completely before subsequent loadings. Once dry, the peptides were electrophoresed on a flat bed apparatus (Pharmacia) in one of two buffer systems. The buffer systems used were: (1) pyridine: acetic acid: water (1:10:189), pH 3.5; or (2) formic acid: acetic acid: water (1:4:45), pH 1.9. For both buffer systems, electrophoresis proceeded at constant current (50mA) for 25-45 min. The plates were allowed to air-dry and then subjected to the second dimension of ascending chromatography in acetic acid: butan-1-ol: water: pyridine (3:10:12:15). This was allowed to proceed until the solvent front was 2 cm from the top of the plate (about 2.5 hr). The plates were air-dried and exposed to autoradiographic film (-80°C).

2.10 Identification of phosphorylated residues in peptides

Phosphorylated serine residues were identified by the method of Holmes (1987). With this method, the phosphoserine residues are converted to *S*-ethylcysteinyl residues, which are stable to Edman

degradative chemistry (unlike phosphoserine residues) and can therefore be positively identified on sequencing chromatograms. Briefly: peptides were phosphorylated by PKC in the normal reaction mixture. The reaction was terminated by the addition of trichloro acetic acid to a final concentration of 20%. The reaction tubes were incubated at 0°C (10 min) and the precipitate removed. The peptides in the supernatant were separated from contaminating proteins and lipids by gel filtration on a G-50 column equilibrated in ammonium bicarbonate (50mM). The phospho-peptides were located by radioactive emission, evaporated to dryness and dissolved in 50µl of a reaction mixture consisting of: ethanethiol (60µl), water (200µl), dimethylsulphoxide (200µl), ethanol (100µl) and 5M NaOH (65µl). This was incubated for 60 min (50°C) under nitrogen. The reaction was terminated by cooling the mixture (4°C) and adding 10µl glacial acetic acid followed by 1ml water. The solvents were removed in a 'Speed-Vac' and the derivatised peptides were loaded directly onto polybrene treated discs and installed into the protein sequencer. The discs were given a manual wash with 10ml *n*-butyl chloride and the peptides were sequenced on an Applied Biosystems Inc. 477 automated protein sequencer according to the manufacturers protocol (this was carried out with the help of Mr. N. Totty, Ludwig Institute). The *S*-ethylcystein residues were identified as a peak that eluted immediately in front of the diphenylthiourea (DPTU) peak on the sequencing chromatographs.

CHAPTER THREE

PRODUCTION OF PKC ISOTYPE SPECIFIC ANTIBODIES

Monospecific antibodies were generated to PKC-α, -β_1, -β_2 and -γ and were used primarily to follow the chromatographic behaviour of these isotypes during purification and separation. The antisera have also proven useful for cellular expression studies and for mapping the domain structure of PKC. As a direct approach to producing monospecific antisera, peptides representing the variable regions of each isotype were used as antigens. These regions are unique to each isotype and would therefore be predicted to give selective antisera. This approach of using synthetic peptides to generate antibodies against closely related but different polypeptides has been used successfully elsewhere, eg Rothbard *et al* (1984).

In 1980 Sutcliffe *et al* (1980) reported the use of a pentadecapeptide to raise antibodies to an unidentified protein from the Molony leukaemia virus and Walter *et al* (1980) used a similar technique to produce antibodies to SV40 large T antigen. These two groups were therefore able to show that short peptides could be used as antigens to raise antibodies that would recognise the parent proteins from which the peptide sequences were derived. It was subsequently shown that it was possible to raise antibodies to internal peptides as well as peptides from the C- and N- termini of proteins (Lerner *et al*, 1981; Sutcliffe *et al*, 1983). Guidelines (based on experience) for the efficient generation of antibodies from peptides were produced (see Palfreyman *et al*, 1984; Tanaka *et al*, 1985; Gullick, 1987). On the whole these guidelines indicate that the best antigenic peptides are those which are charged, highly mobile, water soluble and at least eight residues long (see Palfreyman *et al*

1984; Kris *et al*, 1985; Tanaka *et al* 1985; Gullick, 1987). Factors, such as the carrier protein and the linkage method also affect the antigenicity of peptides possibly by affecting their availability (Palfreyman, 1984). The N- and C- termini of proteins seem to offer the best chance of raising antibodies that recognise the parent protein, probably because these regions of the molecules are often on the surface of proteins and therefore accessible (Palfreyman, 1984). A perusal of the literature suggests that while following the guidelines increases the chances of generating useful antisera, in practice success is often realised empirically.

In choosing peptides unique to PKC-α, -β_1, -β_2 and -γ, the search was restricted to the variable (or V) regions of the isotypes as defined by Coussens *et al*, (1986). These regions represent the only stretches of extended sequence divergence which may represent selective epitopes for each of the isotypes. The final peptide sequences were chosen on the basis of their hydropathic properties (as determined by the method of Kyte and Doolittle, 1982) within the V regions. Before synthesis, each peptide was screened against the polypeptide sequences of the other PKC isotypes in order to ensure that there were no significant stretches (>4 residues) of contiguous amino acids common to the other polypeptides. Short peptide sequences which are common to unrelated proteins have been reported to give unexpected cross-reactions (Stabel *et al*, 1987).

3.1 GENERATION OF ANTIBODIES

The peptides were synthesised on an 'Applied Biosystems Inc' (ABI) fully automated peptide synthesiser (Model 741) by Nick Totty, LICR, London. Rob Philp checked the integrity of the peptides by amino acid

analysis on an ABI automated amino acid analyser (Model 420A) and by reverse phase HPLC. All the peptides were judged to be >95% w/w pure and to contain <2% incompletely synthesised peptides. Peptides containing Cys residues at their N- or C- termini (Table 3.1) were conjugated to thyroglobulin using the coupling agent sulpho-MBS while the peptides lacking Cys residues were coupled to KLH with glutaraldehyde (section 2.1).

Rabbits were immunised with 200μg of protein-peptide conjugate. This was prepared by emulsifying peptide-conjugate in 1 part Freund's complete adjuvant and 1 part PBS (2ml total). The antigen was injected subcutaneously at multiple sites. The rabbits were boosted after 28 days with 200μg thyroglobulin-peptide prepared as above, but using Freund's incomplete adjuvant. Bleeds were taken 7-10 days after the boost and further immunizations were administered over a six month period. The antibody titres were determined against the peptides and against purified PKC isotypes by ELISA (section 2.2). Representitive results are shown in Tables 3.1 and 3.2 for peptide and protein titres respectively. The sequence of the peptides are shown in Table 3.1.

Only the $V_3\gamma$ peptide failed to give detectable antibodies. The basis for this lack of antigenicity is unlikely to be due to peptide size. Kris *et al* (1985) found that short peptides from a series tested sometimes failed to act as efficient antigens. However, with $V_3\gamma$ this is unlikely to be the case since three shorter peptides ($V_2\beta$, $V_5\beta_1$and $V_5\beta_2$) were found to be antigenic. It has also been suggested by Palfreyan (1984) that using KLH as carrier, MBS should be used as the coupling agent since this has been found empirically to be more successful. The $V_3\gamma$ peptide was linked to KLH using gluteraldehyde

Table 3.1 Reactivity of antisera to immunising peptides

PKC isotype	Antibody code	From variable region	Peptide sequence	Specific titre	Non-specific titre	Non-sp. titre vs. peptide
α-PKC	$V_{2}\alpha$	V2	LKAEVTDEKLHV(C)	400	25	$V_{2}\beta$
	$V_{3}\alpha$	V3	VISPSEDRRQPS	570	4.0	$V_{3}\beta$
	$V_{5}\alpha$	V5	(C)PQFVHPILQSAV	4000	2.0	$V_{5}\beta_{1}$
$\beta_{1/2}$-PKC	$V_{2}\beta$	V2	IQAHIEREVLI(C)	460	8.9	$V_{2}\alpha$
	$V_{3}\beta$	V3	GPKTPRRKTTNT	380	7.6	$V_{2}\alpha$
β_{1}-PKC	$V_{5}\beta_{1}$	V5	(C)SEFLKPEVKS	5000	1.7	$V_{5}\alpha$
β_{2}-PKC	$V_{5}\beta_{2}$	V5	(C)NPEFVINV	2000	3.3	$V_{5}\alpha$
γ-PKC	$V_{3}\gamma$	V3	PSPTDSKRGFFG	N.R.		
	$V_{5}\gamma$	V5	(C)PDARSPISPTPVPVM	3300	2.2	$V_{5}\alpha$

The sequence of the peptides used to raise antibodies specific for the PKC isotypes is shown. The titres are the reciprocal dilutions of antisera at which the colourimetric intensity of the ELISA reaction was halved (threshold set at 2.0). Specific titres relate to the titres against the peptide used to generate the antisera above a BSA background. Non-specific titres show the titres against peptides to a corresponding region in a different PKC isotype (indicated). Cysteine residues added for coupling purposes are indicated by parentheses. N.R. indicates no reaction seen.

Table 3.2 Reactivities of antisera against native PKC polypeptides

Antiserum	Specific titre vs PKC protein	Titre against PKC isoform
$V_2\alpha$	3.1	α
$V_3\alpha$	7.7	α
$V_5\alpha$	240	α
0442	50	α
$V_2\beta$	<2	β_1
$V_3\beta$	<2	β_1
$V_5\beta_1$	106	β_1
0442	25	β_1
$V_5\beta_2$	N.T.	
$V_5\gamma$	118	γ
0442	20	γ

The titres are the reciprocal of the dilutions at which the colourimetric intensity of the ELISA reaction was halved (above a background, calculated for another PKC isotype). 0442 is an anti-peptide antiserum which is non-specific for the PKC isotypes and was raised to the sequence PKC-α(280-292) (Stabel *et al*, 1987). N.T.= not tested due to lack of suitable PKC sample.

and not MBS, but so were $V_3\alpha$ and $V_3\beta$ both of which were antigenic. Therefore it seems unlikely to be the coupling agent which caused the lack of antigenicity of this peptide and it is probably an intrinsic property of the peptide.

The antisera raised to the $V_2\alpha$ peptide recognised the $V_2\beta$ peptide about 10 fold higher than it recognised other non-specific peptides (Table 3.1). While this non-specific reaction was only one tenth of the specific reaction of the $V_2\alpha$ antiserum for the $V_2\alpha$ peptide, it was unusually high (Table 3.1). The $V_2\beta$ antiserum did not have a reciprocal avidity for the $V_2\alpha$ peptide. There is no significant homology between these peptides and the lack of reciprocal cross-reactivity (between $V_2\beta$ antiserum and the $V_2\alpha$ peptide) suggests that the $V_2\alpha$ antiserum recognition of the $V_2\beta$ peptide is non-specific.

The antibodies raised against peptides with sequences taken from the V5 region (carboxyl termini) of the PKC molecule were found to give higher titres (approximately 10 fold) against their peptides than the V2/3 antisera (Table 3.1). The reason for the increased antigenicity of the V5 peptides may have been due to the method of linkage. The V5 peptides were conjugated with sulfo-MBS to thyroglobulin through N-terminal Cys residues. All other peptides were coupled to thyroglobulin through C-terminal Cys residues with sulfo-MBS or to KLH with glutaraldehyde. This may be the basis of the variation seen in the peptides. The V5 region antisera also recognised the relevant native proteins more efficiently than the V2 and V3 peptides, so although there was only a 10 fold difference in antipeptide titres between the V5 antisera and the V2/3 antisera (Table 3.1), there was at least a 100 fold difference in the titres for

the proteins; in fact, the $V_2\beta$ and $V_3\beta$ failed to recognise the native protein in ELISA (see Table 3.2). The more efficient protein recognition seen with the V5 antisera is also seen in immunoblotting systems; the V5 antisera are able to recognise purified PKC at dilutions up to 1 in 1000, wheras the antisera to V2 and V3 were only able to recognise purified PKC at dilutions of 1 in 100-250 (Figure 3.1). The more efficient recognition of protein seen with the V5 antisera against the protein is probably because these peptides are positioned at the C-termini of their PKC isotype. They are therefore less likely to be conformationally restrained and so are more available for binding. This suggestion is supported by the observation that the V2 and V3 antisera recognise the denatured proteins (*ie* in the immunoblotting system) but not the native protein (*ie* in the ELISA).

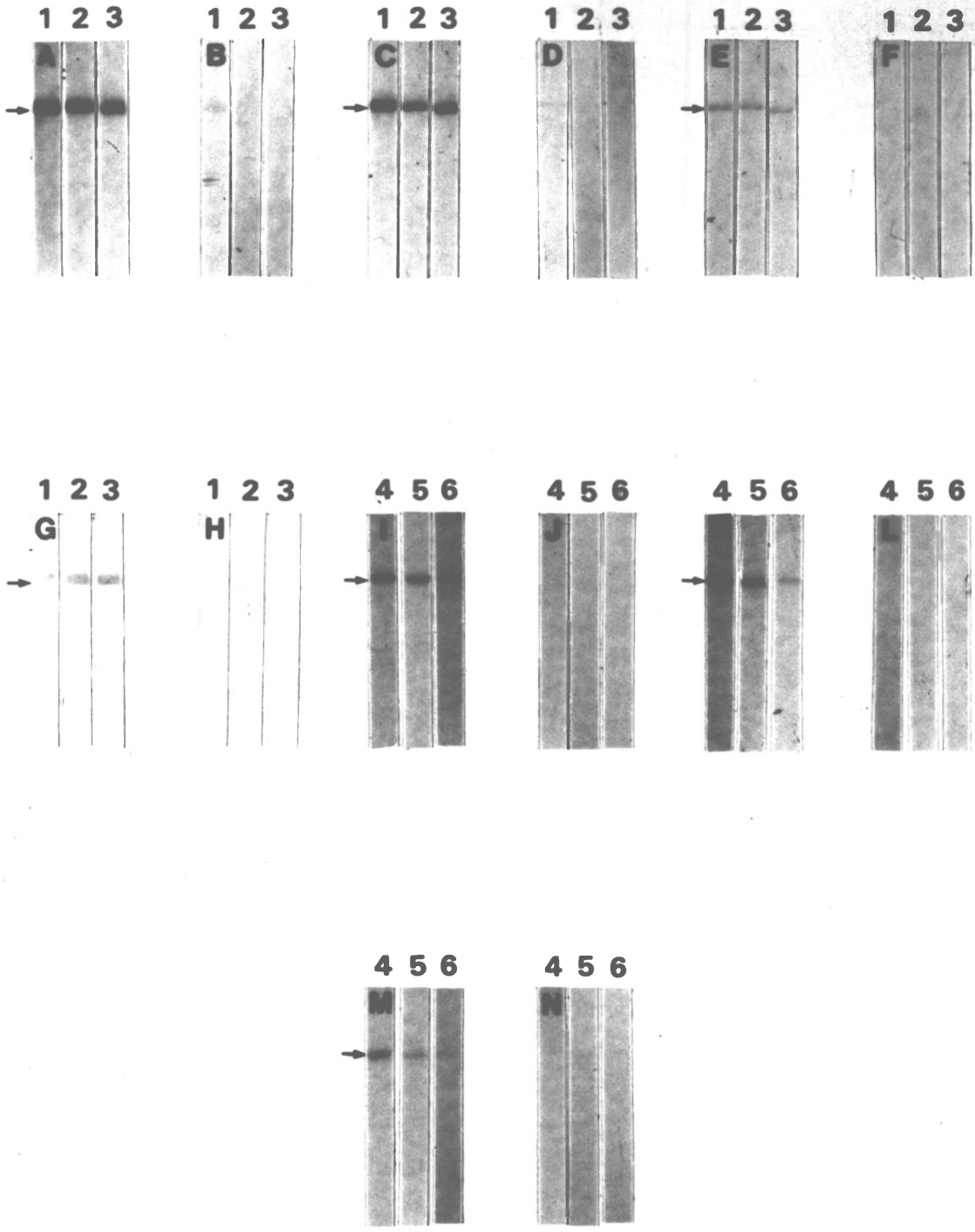
The antisera all recognise purified PKC in immunoblot analysis in a peptide competitive manner as shown in Figure 3.1. The V5 antisera were able to recognise a single protein band with a Mr of approximately 80,000 in bovine brain and rat brain extracts as shown in Figure 3.2. $V_5\alpha$ and $V_5\beta_1$ were able to detect a protein band of about Mr 80,000 in some human cell lines in a peptide competitive manner as shown in Figure 3.3. This is not unexpected, considering the exceptional conservation of the V regions of the isotypes from different animal species (Parker *et al*, 1989). The V2 and V3 antisera were unable to recognise PKC in crude extracts from any source. The $V_5\beta_2$ and $V_5\gamma$ antisera have not given positive identification of PKC in any human cell lines. However these isotypes do not have a wide tissue distribution (see Nishizuka, 1988) and therefore the failure is likely to be due to the lack of suitable cell samples. The ability of these antisera to recognise the relevent

Figure 3.1 Titration of PKC antibodies against purified PKC

Purified PKC (one unit) containing a mixture of containing -α, -β_1, -β_2 and -γ isotypes was subjected to electrophoresis (7% w/v acrylamide gels) and immuno-blot analysis as described in section 2.5. The individual strips were probed with different concentrations of antibodies, but were washed and incubated with [^{125}I]-protein A together. The nitrocellulose sheets were exposed to autoradiographic film for 48 hr. The position of PKC is indicated by the arrows. Lanes marked 1, 2 and 3 were incubated with antibody diluted to 1 in 250, 1 in 500 and 1 in 1,000 respectively. Lanes marked 4, 5, and 6 were incubated with antibody diluted to 1 in 100, 1 in 250 and 1 in 500 respectively.

Antibodies used:

panel A= $V_5\alpha$;	panel B= $V_5\alpha$ with competing peptide.
panel C= $V_5\beta_1$;	panel D= $V_5\beta_1$ with competing peptide.
panel E= $V_5\beta_2$;	panel F= $V_5\beta_2$ with competing peptide.
panel G= $V_5\gamma$;	panel H= $V_5\gamma$ with competing peptide.
panel I= $V_2\alpha$;	panel J= $V_2\alpha$ with competing peptide.
panel K= $V_3\alpha$;	panel L= $V_3\alpha$ with competing peptide.
panel M= $V_3\beta$;	panel N= $V_3\beta$ with competing peptide.


1 2 3
A
1 2 3
B
1 2 3
C
1 2 3
D
1 2 3
E
1 2 3
F
1 2 3
G
1 2 3
H
4 5 6
I
4 5 6
J
4 5 6
4 5 6
L
4 5 6
M
4 5 6
N

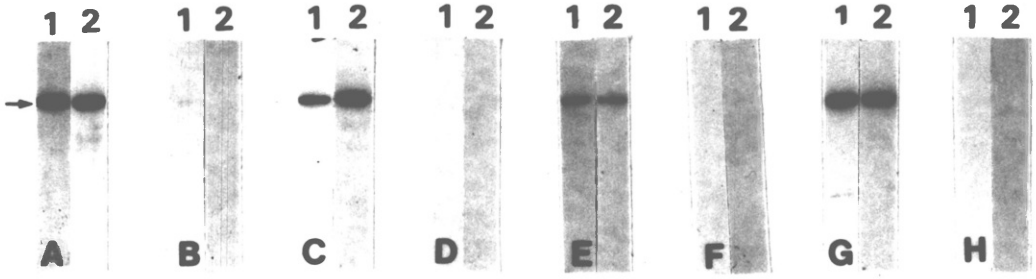


Figure 3.2 Immuno-protein blot analysis of crude bovine and rat brain extracts

Rat and bovine brains were homogenised as described in section 4.1.2 and a sample was mixed with one quarter its volume of four times Laemmli sample buffer (section 2.4). 1μg protein was subjected to electrophoresis and immuno-blot analysis as described in sections 2.4 and 2.5. Lane 1= rat brain extract; lane 2= bovine brain extract. The sheets were probed with the antisera in the absence or presence of 10μM competing peptide. The antisera were used at a dilution of 1 in 1,000. The position of PKC is indicated by the arrow.

Antibodies used:

panel A= $V_5\alpha$; panel B= $V_5\alpha$ with competing peptide.
panel C= $V_5\beta_1$; panel D= $V_5\beta_1$ with competing peptide.
panel E= $V_5\beta_2$; panel F= $V_5\beta_2$ with competing peptide.
panel G= $V_5\gamma$; panel H= $V_5\gamma$ with competing peptide.

The proteins were electrophoresed on 10% w/v acrylamide gels and the nitrocellulose sheets were exposed to the autoradiographic films for 72 hr.

Figure 3.3 Immuno-protein blot analysis of cell lines

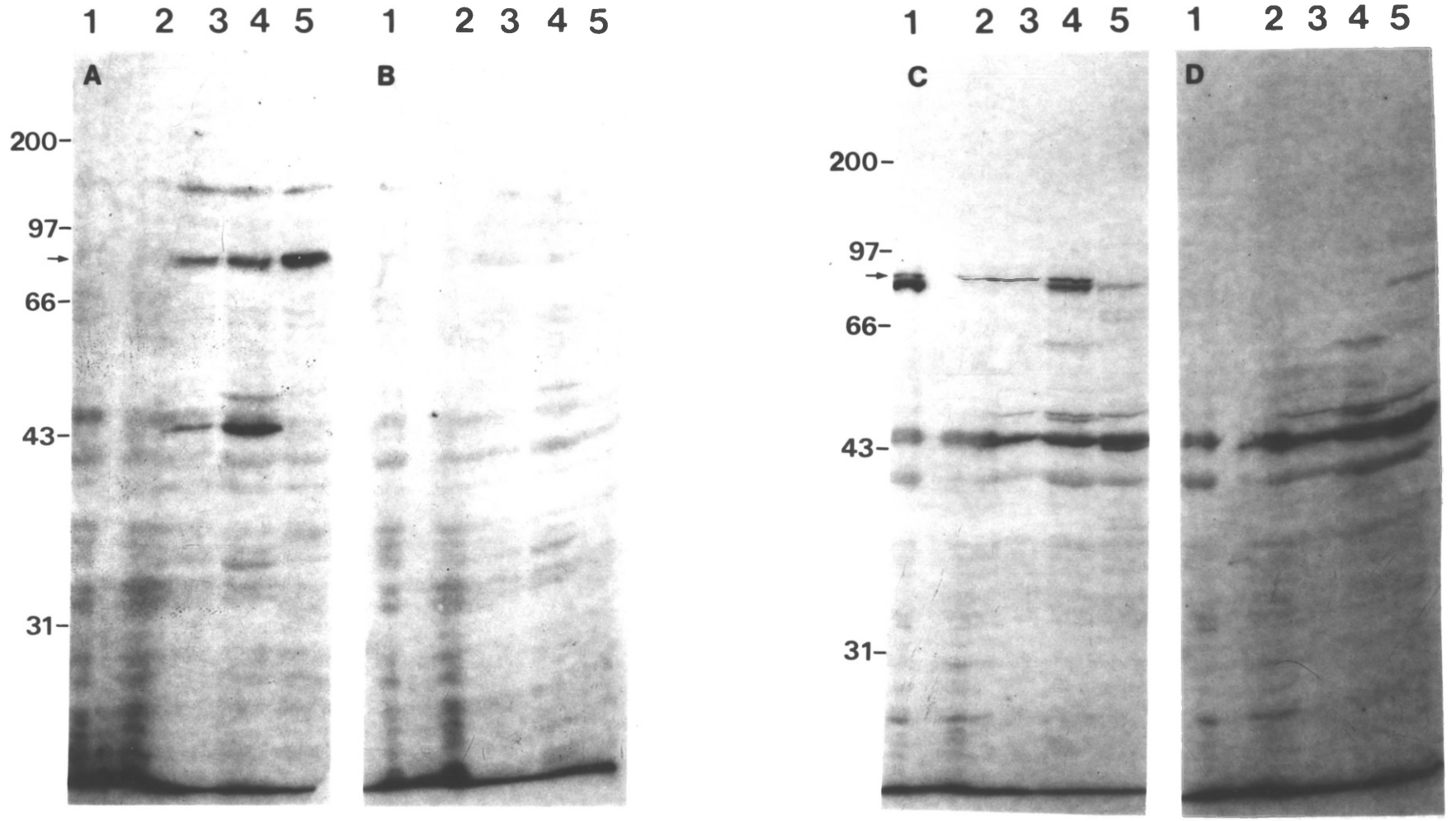
The cell lines were grown in the media as described below. The cells were passaged and while growth was still in the exponential phase, they were harvested. For each cell line, 10^7 cells were washed twice with 10ml PBS and the then lysed directly into 500µl of four times concentrated Laemmli sample buffer (Section 2.4). The lysates were immersed in a boiling water bath (5 min) and the DNA was sheared by direct probe sonication (30 sec). For each lane, 10^6 cell equivalent were loaded and these were electrophoresed until the dye front was 1cm from the bottom of the gel (10% w/v acrylamide gels). The proteins were transferred to nitrocellulose and probed with the V5 region antisera in the absence or presence of competing peptide. Lane 1= IM9 cells, lane 2= HL60 cells, lane 3= Ramos cells, lane 4= AW Ramos cells and lane 5= 328 cells. The position of PKC is indicated by the arrows and the position of migration of 'Rainbow' molecular mass markers is also indicated (x 10^{-3}). The blots were exposed to autoradiographic film for 72 hr. No positive signals could be seen for similar blots probed with $V_5\beta_2$ and V5γ antisera even after exposure of the blots to film for 56 days. The antisera were used at a dilution of 1 in 1,000.

Antibodies used:

panel A= $V_5\alpha$; panel B= $V_5\alpha$ with competing peptide.

panel C= $V_5\beta_1$; panel D= $V_5\beta_1$ with competing peptide.

The cell lines tested are: IM9 cells which are human lymphoblastoid line; HL60 cells which are a human promyelocytic line; Ramos cells, which are an Epstein Barr virus (EBV) negative lymphoblastoid line; AW Ramos cells which are Ramos cells which have been infected with EBV; and 328 cells which are a rodent glioma cell line. IM9 cells, HL60 cells, Ramos and AW Ramos cells were provided by the ICRF cell culture unit and were grown in 2% RPMI medium, supplemented with 10% foetal calf serum (FCS). 328 cells were provided by Dr M. Noble and were grown in Dulbecco's modified Eagle medium, supplemented with 10% FCS. All cell culture media were purchased from GIBCO, England.


1 2 3 4 5
A
200-
97-
66-
43-
31-
1 2 3 4 5
B
1 2 3 4 5
C
200-
97-
66-
43-
31-
1 2 3 4 5
D

PKC from brain extracts suggests that they would recognise PKC in cell lines if present. This ability of the V5 antisera to detect PKC from different mammalian species has made these antisera potentially very useful for studies with cell lines. It was noticed from these studies that PKC-β_1 in cell lines often migrates as a doublet, although the significance of this is unknown (see Figure 3.3).

The V5 antisera were able to immunoprecipitate a protein of Mr 80,000 from purified PKC preparations and from crude bovine brain extracts (Figure 3.4). The precipitation of PKC was inhibited by competing peptides. The recognition of the precipitated proteins by the 0442 antiserum (see Figure 3.4 legend) confirms their identification as PKC. In order to show that the V5 region antisera were specific for their relevent isotype, insect cell expressed PKC-α, -β_1, -β_2 and -γ were individually probed in immuno-blot with the V5 antisera. The results show that each antiserum was exquisitely specific for the relevent PKC isotype and that no cross-reaction could be detected in any of the antisera (Figure 3.5). In insect cells the PKC-γ isotype is detected as a doublet, which is in agreement with the observations of Patel and Stabel (1988). The different forms probably arise due to differential phosphorylation (Patel and Stabel, 1988). The protein samples for this analysis were kindly provided by S. Stabel.

Makowske *et al* (1988) have generated PKC monospecific antisera to the V3 regions of PKC-α, -β and -γ, and Ase *et al* (1988) have produced monospecific antisera with peptides to the V1 region of PKC-α and to the V5 regions of -β_1 and -β_2. It is difficult to compare directly the results of these workers with the results from this thesis, because of the different uses to which the antisera have

Figure 3.4 Immuno-precipitation of PKC

PKC was immuno-procipitated from crude bovine brain extracts and from purified samples.

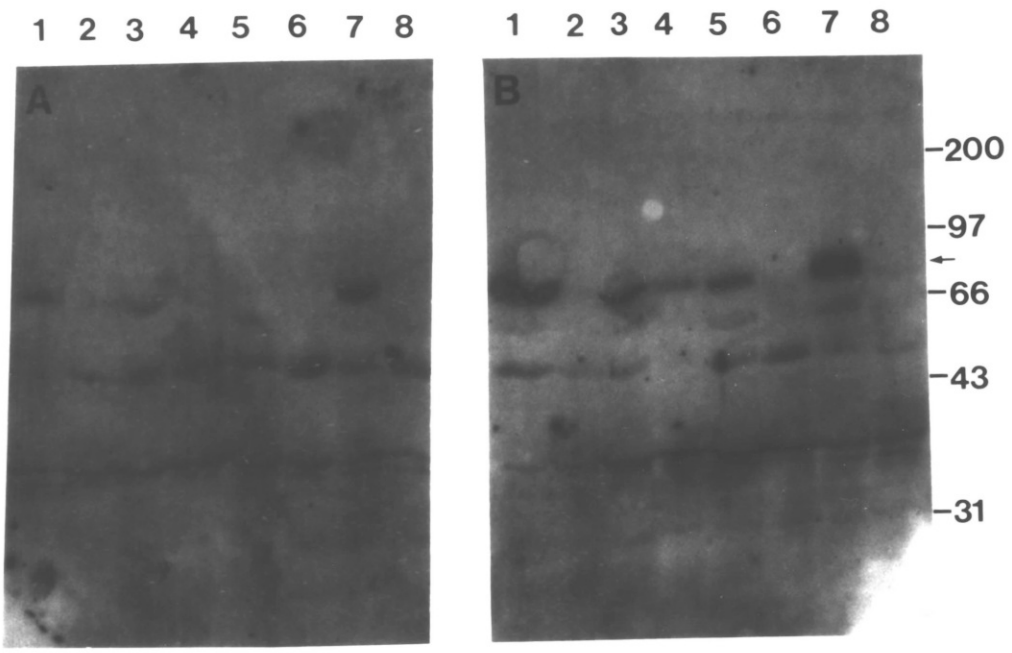
Panel A Crude brain extract

Brain extract was prepared by adding three volumes of bovine brain 27,000 x g supernatant (Section 4.1.2) to one volume of four times concentrated Laemmli sample buffer (Section 2.4). The sample was immersed in a boiling water bath (5min) and frozen until reguired. For each antibody, 30µl of extract was diluted into 90µl RIPA buffer (20mM sodium phosphate pH 7.2, 5mM EDTA, 1% v/v Triton X-100, 0.4% dexoycholate, 4µg/ml leupeptin, 10µg/ml aprotinin, 50µg/ml PMSF). 20µl antiserum was added to each vial, which had either been incubated for 30 min at 4°C with 20µl PBS containing 10mM competing peptide or 20µl PBS. The mixture was incubated at room temperature with tumbling for 120 min. 40µl of a 1:1 slurry of protein A-Sepharose was added to each vial and the mixture was incubated at room temperature with tumbling for a further 60 min. The protein A-Sepharose was collected by centrifugation and washed three times with RIPA buffer. 20µl of four times concentrated Laemmli sample buffer was added to each sample, followed by immersion into a boiling water bath (5 min). The proteins in the supernatant were analysed by SDS-PAGE (10% w/v acrylamide gels), transferred to nitrocellulose and probed with the 0442 antiserum, which recognises all four PKC isotypes (Stabel *et al*, 1987).

Panel B Purified PKC

10µg of PKC purified by threonine-Sepharose (Section 4.1.2) was diluted into 120µl RIPA buffer and treated as for the brain extracts above.

The nitrocellulose blots were exposed to autoradiographic film for 7 days. The position of PKC is indicated by the arrow and the position of migration of 'Rainbow' molecular mass markers is also indicated (x 10^{-3}). Lane 1= PKC immuno-precipitated with the $V_5\alpha$ antiserum; lane 2= PKC immuno-precipitated with $V_5\alpha$ antiserum pre-incubated with competing peptide; lane 3= PKC immuno-precipitated with $V_5\beta_1$ antiserum; lane4= PKC immuno-precipitated with $V_5\beta_1$ antiserum pre-incubated with competing peptide; lane 5= PKC immuno-precipitated with $V_5\beta_2$ antiserum; lane 6= PKC immuno-precipitated with $V_5\beta_2$ antiserum pre-incubated with competing peptide; lane 7= PKC immuno-precipitated with $V_5\gamma$ antiserum; lane 8= PKC immuno-precipitated with $V_5\gamma$ antiserum pre-incubated with competing antiserum.


1 2 3 4 5 6 7 8
A
1 2 3 4 5 6 7 8
B
200
97
66
43
31

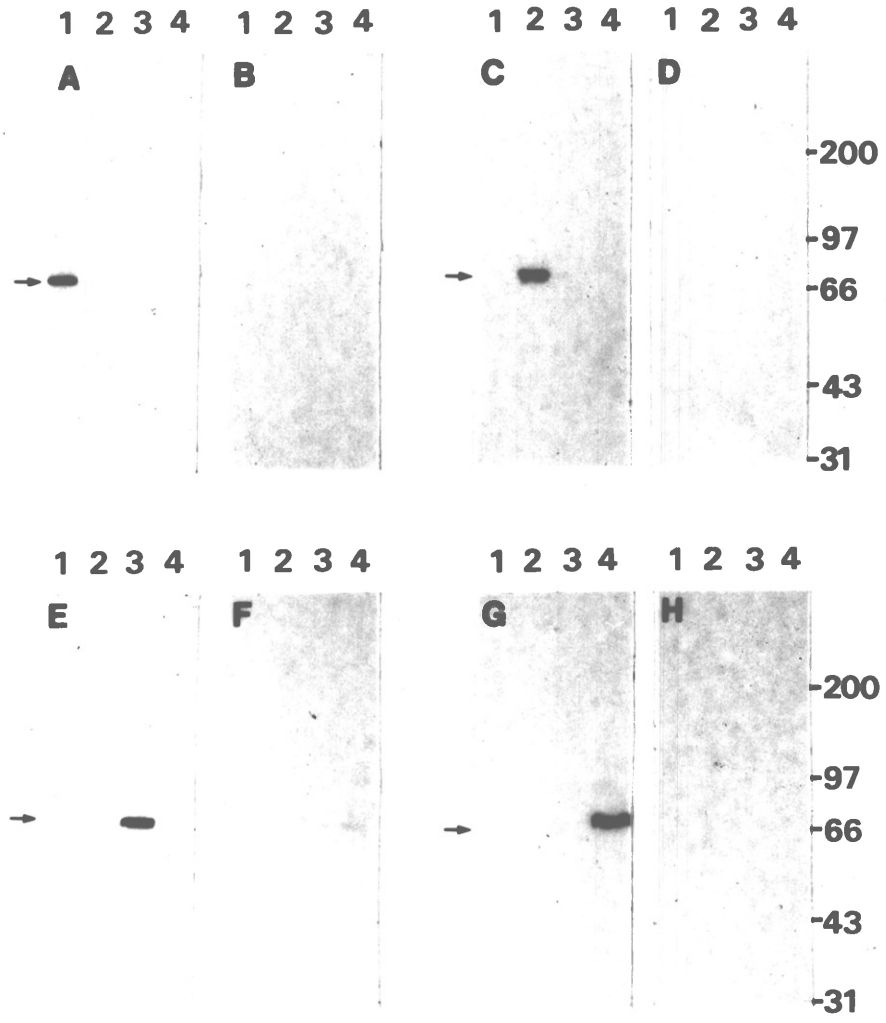


Figure 3.5 Immuno-protein blot analysis of PKC expressed in insect cells

PKC-α, -β_1, -β_2 and -γ were expressed in insect cells and purified to homogeneity according to Patel and Stabel (1988). 1.0μg of protein was subjected to electrophoresis and immuno-blot analysis as described in sections 2.5. The proteins were loaded in the following order: lane 1= PKC-α, lane 2= PKC-β_1, lane 3= PKC-β_2 and lane 4= PKC-γ. The sheets were probed with the antisera in the absence or presence of 10μM competing peptide. The antisera were used at a dilution of 1 in 1,000. The position of PKC is indicated by the arrow and the positions of migration of 'Rainbow' molecular mass markers are indicated (x10^{-3}). The proteins were electrophoresed on 7% w/v acrylamide gels and the blots were exposed to the autoradiographic films for 48 hr.

Antibodies used:

panel A= $V_5\alpha$;	panel B= $V_5\alpha$ with competing peptide
panel C= $V_5\beta_1$;	panel D= $V_5\beta_1$ with competing peptide
panel E= $V_5\beta_2$;	panel F= $V_5\beta_2$ with competing peptide
panel G= $V_5\gamma$;	panel H= $V_5\gamma$ with competing peptide.

been put and due to the lack of published characterisation of the antisera generated by Makowske *et al* and Ase *et al*. It is interesting to note that the V3 antisera from Makowske *et al* were used at a low dilution (1 in 100) on partially purified PKC for immuno-blotting, suggesting that the titres were not high. These results are as would be predicted from the titres of the V3 antisera seen in this thesis. Ase *et al* do not discuss the properties of their antisera nor do they show immuno-blot or immuno-precipitation data to indicate what the titres of their antisera were. Makwoske *et al* state that their antisera do not cross-react with peptides for the other isotypes but Ase *et al* do not make any such claim.

A different approach to the production of monospecific antibodies for the PKC isotypes has been described by Huang *et al* (1987b). In these studies, antisera were raised to a mixture of PKC isotypes in rabbits and goats. Once the PKC isotypes had been separated (into three pools, minimally containing PKC-α; PKC-β_{1+2} and PKC-γ), using hydroxylapatite chromatography (see section 4.2.3), the PKC in each pool was used to immuno-purify antibodies specific for themselves from the total anti-PKC antisera (Huang *et al*, 1987b). The antibody samples thus generated were found to be specific for the isotypes in as much as, at the concentrations used, they only reacted (in immuno-blot analysis) with the PKC from the pool against which they were purified. However, the authors did not know whether the three pools represented separated PKC isotypes and therefore had to resort to molecular biological techniques to provide the evidence for this (Huang *et al*, 1987b). Their results suggest that the major epitopes on PKC are in the variable regions. However this method of producing monospecific antisera is not as systematic as is the use of peptides. The method relies on the ability to separate the

isotypes. These authors have therefore not been able to produce antibodies that can distinguish between the β_1 and β_2 isotypes, because the separation of the native forms of these isotypes has not yet been achieved. They have also been unable to determine the precise isotype content of the -β pool from their hydroxylapatite column due to the lack of -β_1 and -β_2 specific antisera. They have therefore always assumed that the -β pool contains both the -β_1 and -β_2 isotypes, but as the results presented in section 4.2.3 of this thesis will show, this is not necessarily the case. The specific antibodies produced by Huang *et al* (1987b) rely on the efficient separation of the isotypes, and if this is incomplete (which cannot be determined by use of antibodies unless they are known to be absolutely specific), the antibody pools produced will be contaminated by antibodies directed to other isotypes. This has important consequences in cell expression studies, because if signals are seen with antibody pools that are reactive to more than one isotype, the results may be misleading. The conclusions will therefore depend on the concentration of antibody used. Similar confusion will arise from antibody pools that are cross-contaminated with low concentrations of antibodies against epitopes that are common to all the isotypes.

3.2 THE DOMAIN STRUCTURE OF PKC

The antisera described here have been used to examine the domain structure of PKC and to map the cleavage site in PKC digested with calpain. This investigation was conducted in colaboration with Dr. C. Crawford (Oxford University) to whom I am indebted for supplying the porcine kidney calpain. For each assay, 5.0μg purified PKC(-α,

-β_1, -β_2 and γ mixture) was added to reaction mixture containing 50mM Tris.HCl, pH7.5; 10mM Ca^{++}; 0.07% v/v β-mercaptoethanol 0.4μg calpain (total volumn = 25μl). The reaction was incubated for 30 min (30°C) and terminated by the addition of 5μl four times concentrated Laemmli sample buffer (section 2.3). The resultant fragments were immuno-blotted with antisera to the V5 region for all the isotypes and to the V2 and V3 region for PKC-α. They were also probed with the 0442 antiserum (Stabel *et al*, 1987) and the monoclonal antibody C5 (Young *et al*, 1988). The results show that all the isotypes are substrates for calpain and cleavage of the isotypes by this proteolytic enzyme results in the production of C-terminal fragments in each case, with Mr values in the range of 45,000 to 50,000. These constitute the kinase domain of each enzyme. The -β_1 and -γ isotypes appear to have two calpain cleavage sites and produce fragments which differ in Mr by about 2,000 to 3,000 (Figure 3.6). The -α and -β_2 isotypes by contrast appear to have only one major calpain cleavage site, resulting in one major C-terminal fragment. The significance of these findings is unknown.

The domain structure of PKC-α was further investigated with the $V_2\alpha$ and the $V_3\alpha$ antisera. The $V_3\alpha$ antiserum (panel E, Figure 3.6) recognises a fragment of Mr 47,000 (the catalytic fragment) and the $V_2\alpha$ antiserum a fragment of Mr 32,000 (the regulatory fragment). The major fragment recognised by the 0442 antiserum is the regulatory fragment and the monoclonal antibody C5 fails to recognise either fragment after cleavage. A similar result is also seen with C5 in the case of cleavage of PKC with trypsin and from those observations the C5 epitope has been localised to the region surrounding residue 305 of PKC-α (Young *et al*, 1988). From the results presented in Figure 3.6, the cleavage site of calpain in PKC-α

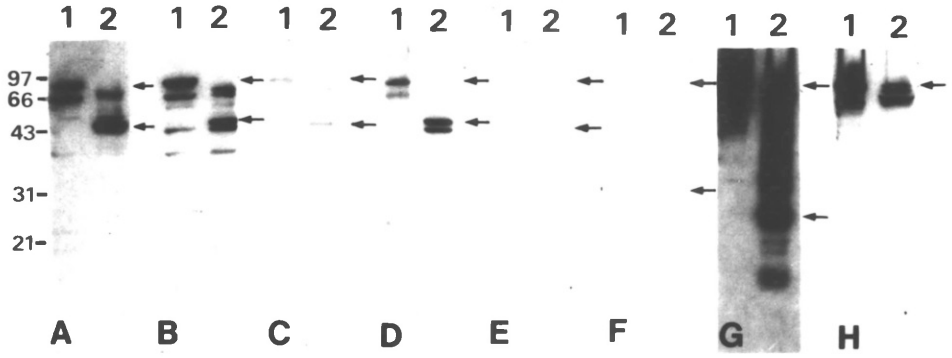


Figure 3.6 Immuno-blot analysis of PKC fragments after digestion with calpain

PKC was digested as described in the text. The fragments were electrophoresed and immunoblotted as described (section 2.4 and 2.5). The position of the major fragment recognised by the particular antibody in question is indicated by the lower arrow in each blot (in the case of a doublet, only one fragment is shown) and the position of undigested PKC by the upper arrows. 10% w/v polyacrylamide gels were used. Lane 1= undigested PKC, lane 2= PKC digested with calpain. The position of migration of 'Rainbow' molecular mass markers is indicated. The nitrocellulose sheets were exposed to autoradiographic film for 48 hr.

Antibody used:

panel A= $V_5\alpha$;
panel B= $V_5\beta1$;
panel C= $V_5\beta2$;
panel D= $V_5\gamma$;
panel E= $V_3\alpha$;
panel F= $V_2\alpha$;
panel G = 0442 antiserum;
panel H= C5 monoclonal.

can be mapped to within a small region of the polypeptide between the 0442 and $V_3\alpha$ peptides, and also within the region of residue 305, the C5 epitope. The positions of the 0442 and $V_3\alpha$ peptides in PKC-α are shown in Figure 3.7. The 0442 peptide is present in the regulatory fragment and the $V_3\alpha$ peptide in the catalytic fragment; the C5 epitope is destroyed following calpain cleavage. The site of cleavage must therefore lie between residues 292 and 316 in PKC-α and is predicted to be in the region of residues 305. The number and precise location of the cleavage site(s) remains to be determined, as do the positions of the cleavage sites in the other three isotypes.

Finally, from the calpain results it is interesting to note that the reactivity of the $V_3\alpha$ antiserum towards PKC-α increases after cleavage. This is demonstrated in Figure 3.6 (panel E) where it can be seen that the immuno-reactivity in lane 2 (post cleavage) is greater than in lane 1 (prior to cleavage). This result agrees with the suggestion that N- and C- terminal peptides form more reactive epitopes than internal peptides, as discussed above. If the predicted position of the cleavage is right, the new N-terminus of the kinase domain will be located close to the $V_3\alpha$ peptide, thus increasing its mobility, accessibility and therefore immuno-reactivity.

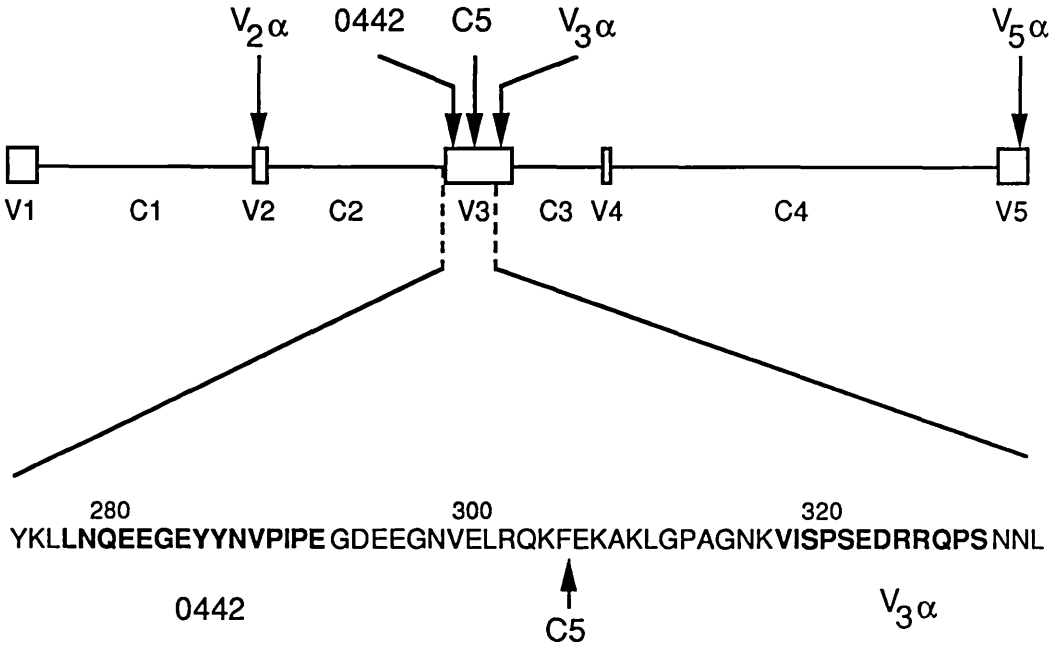


Figure 3.7 Peptides used to raise antibodies to PKC-α

The diagram is a representation of the four conserved (C1-4) and the five variable (V1-5) regions of PKC-α (see section 1.2.8). The descending arrows mark the positions of the peptides $V_2\alpha$, $V_3\alpha$ and $V_5\alpha$ which were used to raise antisera specific for this isotype. Also marked are the positions of the peptide 0442, which was used to raise antisera that are non-specific for the isotypes, and the position of the C5 monoclonal antibody epitope. The expanded region shows the sequence of part of the C2/V3 region. Marked in bold are the peptide sequences of the 0442 and the $V_3\alpha$ peptides; the ascending arrow indicates the position of the C5 epitope. Cleavage with calpain appears to occur in the region between the boundaries of the 0442 and $V_3\alpha$ peptides.

CHAPTER FOUR

PURIFICATION AND SEPARATION OF PROTEIN KINASE C ISOTYPES

4.1 PURIFICATION OF PROTEIN KINASE C

For accurate biochemical analysis, pure samples of enzymes should be used. In the case of kinases, the samples should not contain phosphatases, other kinases or endogenous kinase substrates. A reliable purification is therefore a pre-requisite to a biochemical study involving native kinases. Further, for a study involving isotypes of an enzyme, it is desirable that they co-purify so that separation does not result in samples of different purity. A PKC purification protocol was analysed for co-purification of the isotypes and found to be unsuitable. This lead to the development of a purification protocol that produced milligram amounts of PKC-α, -β_1, -β_2 and -γ mixture in a highly pure state. This sample was subsequently separated into three PKC fractions, containing the -α, -β_1 and -γ isotypes respectively. These samples were judged to be suitable for biochemical analysis, the results from which are described in Chapter 5.

A purification procedure incorporating the first three columns of the purification of Parker *et al* (1984) with a phosphatidylserine-acrylamide (PtdSer-acrylamide) column based on the method of Uchida and Filburn (1984) was used to purify PKC. The purification involved DE-52 anion exchange chromatography, octyl-Sepharose hydrophobic chromatography, S-200 gel filtration and phosphatidylserine affinity (PtdSer-affinity) chromatography. From this protocol it was possible to produce up to 10 milligrams of highly purified PKC from 25 to 30 bovine brains. However this

method was abandoned because it did not give reproducible results. The amount of PKC produced, its purity and isotype content varied from preparation to preparation and therefore a more satisfactory purification protocol was developed. The new protocol used DE-52 chromatography, phenyl-Sepharose hydrophobic chromatography, S-200 gel filtration chromatography and threonine-Sepharose chromatography. It was more efficient in terms of time and materials, producing 5 to 10 milligrams of a mixture of PKC-α, -β_1, -β_2 and -γ from a single bovine brain.

4.1.1 Purification of PKC by phosphatidylserine-affinity chromatography

The binding of PKC to PtdSer micelles in the presence of Ca^{++} can be reversed by the addition of Ca^{++} chelators (Takai *et al*, 1979a). This phenomenon has been exploited to facilitate the purification of PKC by the selective absorption of PKC to PtdSer micelles in the presence of Ca^{++}, followed by its elution with chelators (Wise *et al*,1982; Uchida and Filburn, 1984; Kelleher and Johnson, 1985). These methods often give low recovery (Wise *et al*, 1982; Keller and Johnson, 1985) because the binding of PKC to PtdSer is inefficient and the presence of Ca^{++} in the crude extract presents a potential proteolysis problem (Takai *et al*, 1977). Uchida and Filburn (1984) have developed a column system in which PtdSer micelles are supported within beads made from high concentrations of crosslinked polyacrylamide (PtdSer-acrylamide column). The Ca^{++} dependent binding of PKC to this matrix has been shown to be efficient and can be used during the purification of PKC. However this column does not give single step purification and must be used in conjunction with other columns. This technique has been used successfully in other laboratories (Gould *et al*, 1985).

A combination of the first three columns from the Parker *et al* (1984) purification with a PtdSer-acrylamide column, was used to purify milligram amounts of PKC.

Crude extract Typically, thirty bovine brains were homogenised at a local abbatoir in 10 x 3.3l of ice cold 20mM Tris.HCl pH 7.5, 10 mM EDTA, 10mM EGTA, 10 mM benzamidine, 0.3% β-mercaptoethanol. The homogenate was transported back to the laboratory on ice. All subsequent steps were carried out at 4°C. The cellular debris was removed from the crude extract by centrifugation (5,000 x g, 30 min).

DE-52 column The supernatant from the crude extract was adjusted to pH 8.2 and poured through glass wool onto 3l of settled DE-52 equilibrated in 20mM Tris.HCl pH 7.5, 2mM EDTA, 0.3% β-mercaptoethanol, 10mM benzamidine (buffer A.). The pH of the slurry was re-adjusted to pH 8.2 and stirred at 5 minute intervals for one hour after which it was washed batch-wise with 30l buffer A in two 5l Buchner funnnels under vacuum. The gel slurry was packed into a glass column (55cm x 9cm, 8ml/min), washed with a further 1l buffer A and the PKC was eluted by application of buffer A containing 120mM NaCl. Fractions (15ml) were collected and those containing Ca^{++}/PtdSer dependent kinase activity were pooled (Figure 4.1.1). DE-52 is a useful first step because of its high capacity for PKC (see Yeng *et al*, 1986) although as used here, only 40 to 60% of the PKC (measured by activity) in the crude extract bound to the gel matrix. This was partially due to an overloading problem and partially due to the inefficiency of the loading technique. The recovery from the column was low (15-25%) but the

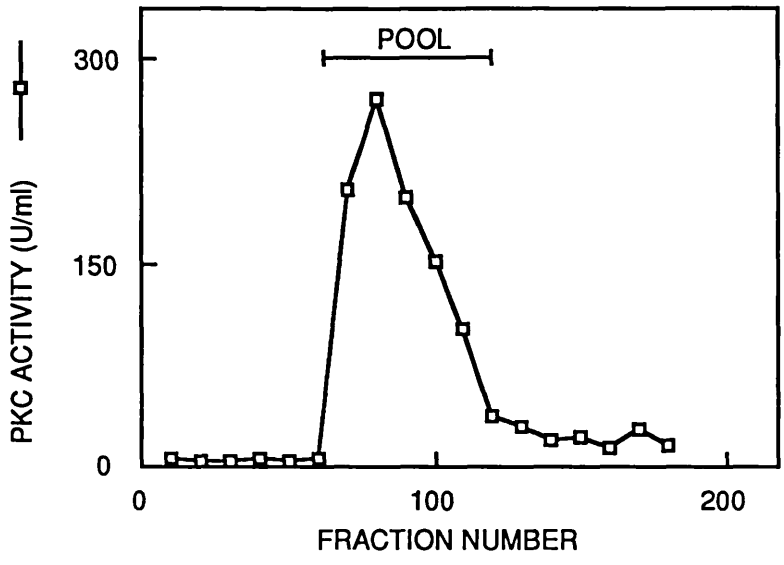


Figure 4.1.1 DEAE-cellulose chromatography
The supernatant from the brain extract was loaded batchwise onto DE-52 and washed with 30l equilibration buffer in Buchner funnels. The column gel slurry was poured into a column and the PKC eluted with 0.12 M NaCl. The Ca^{++}/PtdSer kinase activity (□—□) was determined in every tenth fraction and a pool was collected as indicated by the bar.

volume of the pool was about 20 fold smaller than the load and there was a 3-4 fold purification (see Table 4.1).

Octyl-Sepharose column Ammonium sulphate was added to the DE-52 pool (1-1.5l) to a final concentration of 18% w/v and the precipitate was removed by centrifugation (5,000 x g, 30 min). The supernatant was loaded onto an octyl-Sepharose column (9cm x 6cm, 5ml/min) equilibrated in buffer A containing 1.2M ammonium sulphate (buffer B). The column was washed with 500ml buffer B, and eluted with buffer A containing 200mM ammonium sulphate. It was difficult to quantitate accurately the PKC activity at this step due to the high concentration of ammonium sulphate which interfered with the assay. The protein that was eluted was therefore pooled without determining the PKC activity profile. This step was useful for concentrating the PKC sample prior to loading onto the Sephacryl S-200 (S-200) column and for removing calcium activated neutral proteases. The purification on this step could not be determined due to the inability to reproducibly assay the pool.

Sephacryl S-200 column The pool from the octyl-Sepharose column (200 to 400ml) was loaded onto an S-200 column (900cm x 10cm, 2ml/min) equilibrated in buffer A containing 0.02% sodium azide. Fractions (16ml) were collected and those containing PKC activity were pooled (Figure 4.1.2). The octyl-Sepharose and S-200 column together give a 2-3 fold purification and a recovery in the order of 20%. The PKC elutes from the S-200 column in a single major peak with a characteristic Mr of 70,000. There was a variable smaller peak that eluted before the major PKC peak. This peak probably arises due to protein aggregation (Figure 4.1.2 and see Parker *et al*, 1984).

PtdSer-acrylamide column The S-200 pool (500-1000ml) was adjusted to 10mM MES pH 6.5, 5mM Ca^{++}, 200mM NaCl and 4μg/ml

Table 4.1.1 Purification of bovine brain PKC by PtdSer-affinity chromatography

Fraction	Volume	Protein conc.	Specific activity	Total activity	Recovery	Purification fold
	ml	mg/ml	U/mg	units	%	
Crude supernatant	20000	18.0	12.5	4500000	100	1
DE-52 pool	1020	16.5	48.0	808000	17.9	3.8
Phenyl-Sepharose/ S-200 pool	600	3.4	86.3	174000	3.9	6.9
PtsSer-acrylamide pool (post dialysis)	27	0.25	3290	22300	0.5	263
PtdSer-acrylamide flow-through	760	2.3	19.1	33200	0.7	-

Details of bovine PKC purified from 27 brains. The high concentration of ammonium sulphate in the phenyl-Sepharose pool interferes with the assay reproducibility, therefore this pool is not analysed for activity. 40 to 60 % of the kinase in the extract failed to bind to the DE-52 column.

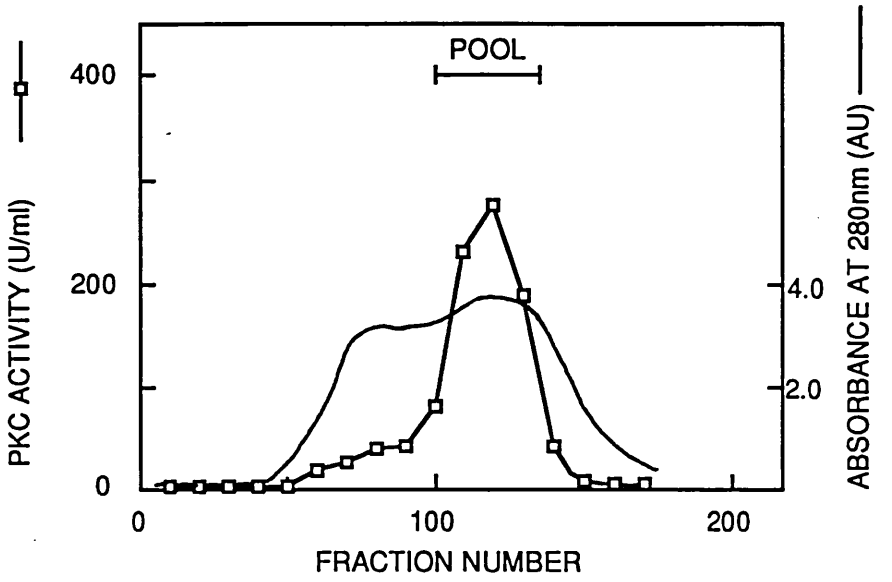


Figure 4.1.2 Gel filtration on Sephacryl S-200
The protein pool from the octyl-Sepharose column was loaded onto an S-200 column and eluted in buffer A containing 0.02% azide. The Ca^{++}/PtdSer dependent kinase activity (□—□) of every tenth fraction was determined and a pool was collected as shown by the bar. The PKC eluted as a single peak of activity but was sometimes preceeded by a minor peak of activity. The presence of this peak was dependent on the protein concentration and was due to protein aggregation.

leupeptin and loaded onto a PtdSer-acrylamide column (6cm x 2.5 cm, 2ml/min; see section 2.5) equilibrated in 10mM MES pH 6.5, 200mM NaCl, 4mg/ml leupeptin, 0.03% β-mercaptoethanol (buffer C) containing 5mM Ca^{++}. The column was washed with 100ml buffer C containing 5mM Ca^{++} and then with 200ml buffer C containing 0.1mM Ca^{++} (or until the OD_{280} was < 0.01). The PKC was eluted with buffer C containing 2mM EGTA and 20 ml fractions were collected. The fractions containing PKC activity were pooled, dialysed against 50% glycerol, 20mM Tris.HCl pH 7.5, 2mM EDTA, 1mM DTT (buffer D) and stored at -20°C. In order to reduce the losses due to proteolysis, the leupeptin was added to the S-200 pool prior to the Ca^{++}.

The PKC pool had a specific activity of 3,000-4,500 units/mg and a protein concentration of 50-400μg/ml. It had a Mr of 80,000 as judged by its mobility on SDS-PAGE and was generally found to be highly purified although often one or more contaminant(s) were found in the preparation (Figure 4.1.3). The PKC was absolutely dependent on Ca^{++}/PtdSer for activity and was stable when stored at -20°C for up to one year, with no changes in catalytic activity or activator dependence being detected. The purification of PKC by this method is shown in Table 4.1.1.

Unfortunately this protocol did not give reproducible results. The amount of PKC that was produced varied by as much as 100 fold (0.12-13 mg) from preparation to preparation. Further to this, the purity as well as the isotype content were subject to variation. The basis of these variations was primarily due to the poor reproducibility of the PtdSer-affinity column which was made freshly for each preparation and used only once. The column of Uchida and Filburn (1984) gave recoveries of the PKC in the region of

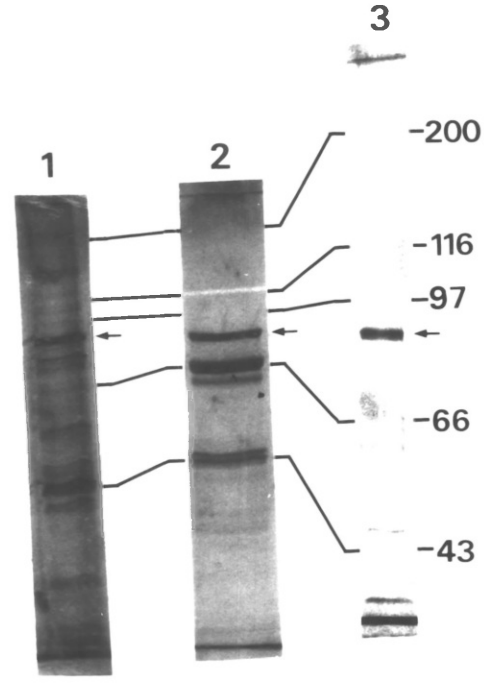


Figure 4.1.3 SDS-polyacrylamide gel electrophoresis of PKC
The PKC samples from the PtdSer-acrylamide columns from three different preparations were analysed by SDS-PAGE. Each lane represents the sample from a different preparation. Lanes 1 and 2 were analysed on 10% w/v acrylamide gels and lane 3 was analysed on a 7% w/v acrylamide gel. The PKC bands are indicated by arrows. The position of migration of standard molecular mass proteins is indicated ($x10^{-3}$). The gels were silver stained.

50%, however under the conditions outlined above, the recovery was sometimes as low as 15%, but could be as high as 90%. The yield of PKC varied from preparation to preparation, depending on the efficiency of PKC binding to the column. Further, the individual PKC isotypes had different binding properties for each new column, with the result that the PKC samples varied in their relative amounts of each isotype. For some preparations the -α isotype bound to the column in the presence of Ca^{++} while the -β isotype(s) did not bind to the column but were found in the flow-through (Figure 4.1.4). With other preparations, both isotypes bound in the presence of Ca^{++} and were eluted by EGTA; when tested, PKC-γ co-eluted with PKC-α. On the occasions when the -β isotype(s) passed through the column, it(they) could not be further purified from the contaminants by either protamine-affinity chromatography (as described by Walton *et al*, 1987 or Wooten *et al*, 1987) or by threonine-Sepharose chromatography (as described by Kikkawa *et al*, 1986). Separation of the -α and -γ isotypes from each other also proved to be unsuccessful by these chromatographic methods because of the large losses encountered.

The different binding that the isotypes had for the PtdSer-acrylamide column were considered to have a potential role in the separation of the isotypes. Attempts to increase the reproducibility of the PtdSer-acrylamide column (such as by adding TPA to the micelles to increase binding affinity) did not overcome these problems. Further to this, the purification as a whole was inconvenient and expensive in terms of time and materials. The large volumes of the pools and columns and the time taken (seven days) to process thirty bovine brains made the protocol physically tiresome.

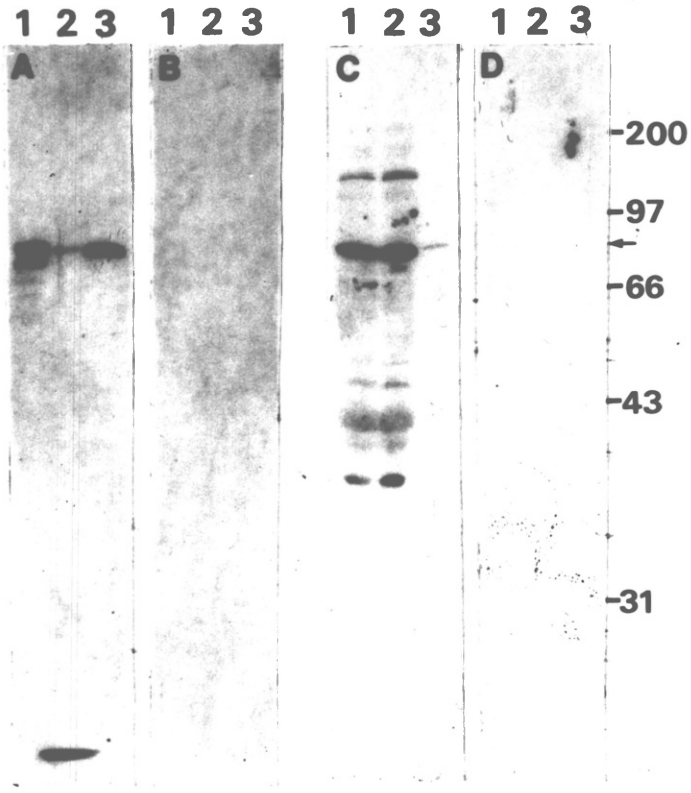


Figure 4.1.4 Binding of PKC-α and -β to the PtdSer acrylamide column

The pool from the S-200 column was loaded onto a PtdSer-acrylamide column in the presence of Ca^{++}. After washing, the PKC was eluted with EGTA. One unit of PKC from the load, the flow through and the EGTA pool was loaded per lane onto 10% w/v polyacrylamide gels. These were immunoblotted with the $V_3\alpha$ and $V_3\beta$. Lane 1= S-200 pool, lane 2= PtdSer-acrylamide flow through, lane 3= EGTA pool. The position of PKC is indicated by the arrow and the positions of migration of standard molecular mass markers are shown ($x10^{-3}$). The nitrocellulose sheets were exposed to autoradiographic film for one week.

Antibodies used:

panel A= $V_3\alpha$; panel B= $V_3\alpha$ with competing peptide

panel C= $V_3\beta$; panel D= $V_3\beta$ with competing peptide.

4.1.2 Purification of PKC by threonine-Sepharose chromatography

For an efficient preparation to be developed it was important to overcome the low efficiency of binding of PKC to the DE-52 column due to the loading method. In order to improve the efficiency of the preparation, the scale of the preparation was reduced and the PtdSer-acrylamide column was replaced by a threonine-Sepharose column (see section 2.7). The new purification used DE-52 anion exchange chromatography, phenyl-Sepharose hydrophobic chromatography, S-200 gel filtration and threonine-Sepharose chromatography. With this protocol, milligram amounts of a mixture of PKC-α, -β_1, -β_2 and -γ in a highly pure state could be produced from a single bovine brain.

Crude extract A fresh bovine brain (500g) was homogenised within 3 minutes of animal sacrifice in 1l of 20 mM Tris-HCl pH8.0, 10 mM EDTA, 10 mM EGTA, 10 mM benzamidine, 0.3% (v/v) β-mercaptoethanol, 50 mg/ml PMSF (homogenisation buffer) at 4°C in a Waring commercial blender (1l capacity; 3x5 second bursts on high speed setting). It was important that the brain was homogenised within three minutes of animal sacrifice otherwise the recovery of PKC from the extract was inefficient. This was probably due to degradation of the kinase, but could not be overcome by the protease inhibitors TPCK and TLCK. All subsequent procedures were carried out at 4°C. The homogenate (transported to the laboratory on ice) was centrifuged at 27,000 x g for 30 minutes.

DE-52 Column The supernatant (500ml) was adjusted to pH8.0, diluted 1:1 with homogenisation buffer and loaded onto a DE-52 column (6cm x 18cm; 5ml/min) equilibrated with 20 mM Tris-HCl pH 7.5; 2 mM EDTA; 10 mM benzamidine; 0.3% (v/v) β-mercaptoethanol (buffer E). After loading, the column was washed with 600 ml buffer

E and the PKC was eluted with buffer E containing 125 mM NaCl. The PKC eluted from the column in a single peak towards the trailing edge of the protein peak (Figure 4.1.5). Under these conditions, there was no detectable Ca^{++}/PtdSer dependent activity in the flow-through. The recovery from the column was twice as good as the recovery when the column was loaded batch-wise although the purification was no better (compare Tables 4.1.1 and 4.1.2).

Phenyl-Sepharose column The pool from the DE-52 column was made up to 18% w/v with ammonium sulphate and the precipitated proteins were removed by centrifugation (5,000g for 20minutes). The supernatant was loaded onto a phenyl-Sepharose column (3.5cm x 5cm; 2ml/min) equilibrated with buffer E containing 1.2 M ammonium sulphate. The column was washed with 100 ml of equilibration buffer and eluted by application of buffer E; fractions (5 ml) were collected. As described in section 4.1.1, the PKC activity was not determined due to the high content of ammonium sulphate and the eluted protein was pooled (Figure 4.1.6).

Sephacryl S-200 column The phenyl-Sepharose pool was loaded onto an S-200 column (5cm x 90cm; 2ml/min) equilibrated with buffer E containing 0.02% (w/v) sodium azide; the column was developed with the same buffer. The PKC eluted as a single peak (Figure 4.1.7), with a characteristic Mr of 70,000, which is lower than its predicted mass. This suggests that the enzyme interacts in some way with the column matrix. The joint recovery of PKC from the phenyl-Sepharose and S-200 columns was 60-80% loaded and the purification was 3-5 fold (see Table 4.1.2).

Threonine-Sepharose column. S-200 column fractions (15ml) containing PKC activity were pooled (100-120ml) and NaCl was added to a final concentration of 380 mM. This was loaded onto a threonine-Sepharose column (1.5cm x 29cm; 1ml/min) (see section

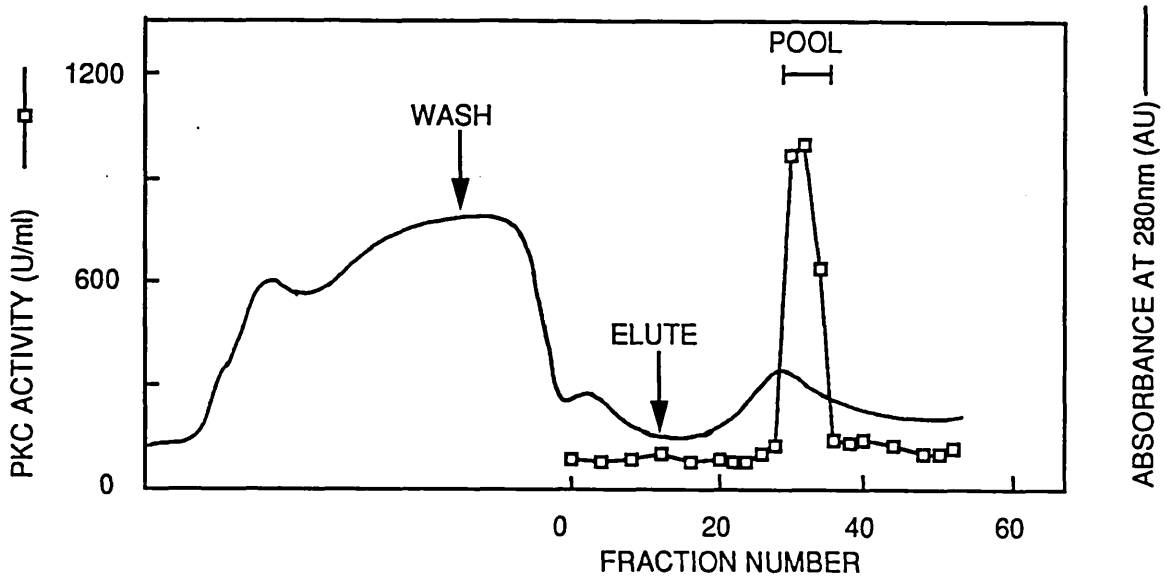


Figure 4.1.5 DEAE-cellulose chromatography
Bovine brain extract was loaded onto the DE-52 column and the flow through was collected as a single fraction. There was no detectable PKC activity in this fraction. The column was washed with buffer E and the PKC eluted by the application of 0.125 mM NaCl in buffer E. The Ca^{++}/PtdSer dependent kinase activity (□—□) in every second fraction was determined and a pool was collected as indicated by the bar.

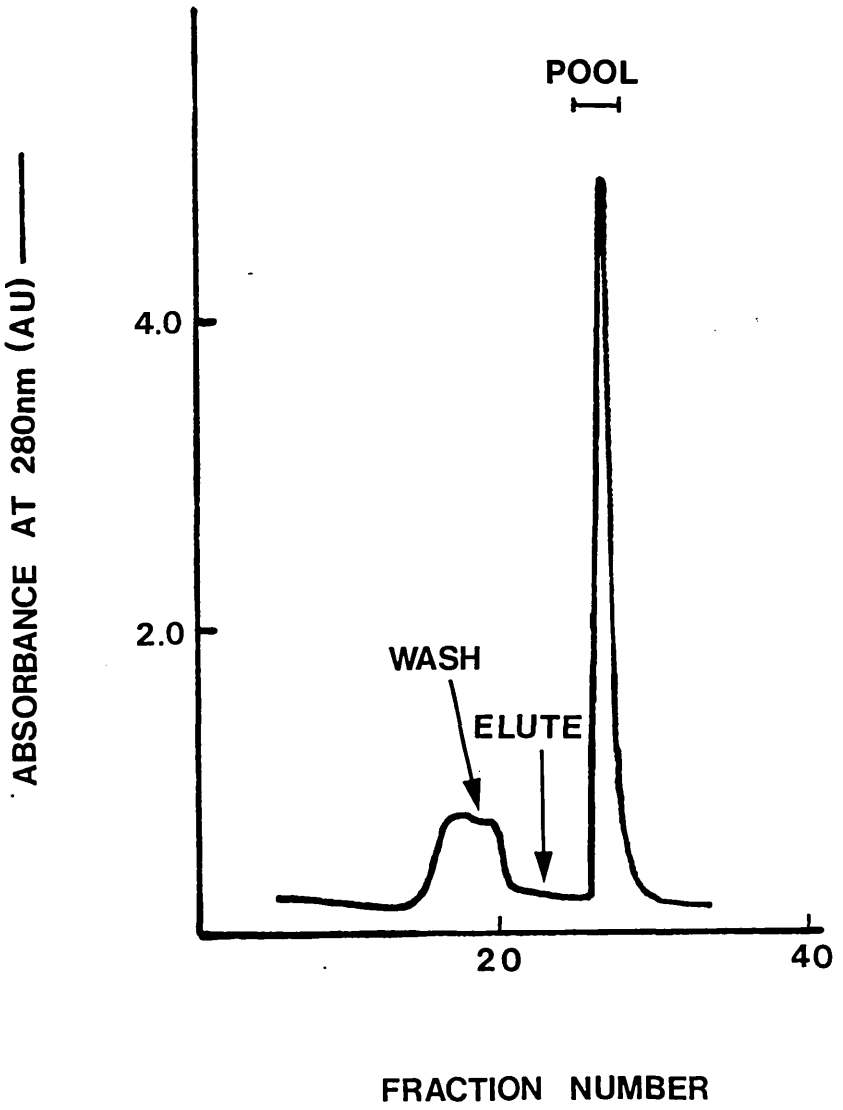


Figure 4.1.6 Phenyl-Sepharose chromatography
The pool from the DE-52 column was adjusted to 18% w/v ammonium sulphate and the supernatant was loaded onto a phenyl-Sepharose column equilibrated with buffer E containing 1.2M ammonium sulphate. The column was washed until the OD_{280} was < 0.01 and the PKC was eluted by the application of buffer E. The eluted protein was collected as indicated by the bar.

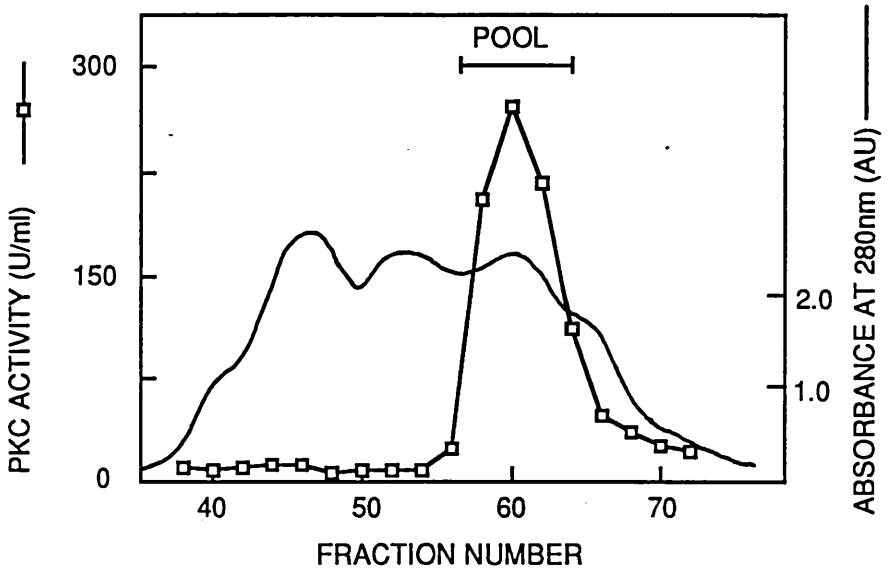


Figure 4.1.7 Sephacryl S-200 gel filtration
The pool from the phenyl-Sepharose column was loaded onto a Sephacryl S-200 column. This column was used essentially to desalt the sample but also gave some purification. The Ca^{++}/PtdSer dependent activity (□—□) of every second fraction was determined and the PKC eluted as a single peak. Unlike the larger S-200 column discussed in section 4.1.1, there was no aggregation of PKC on this column, possibly due to the lower concentration of protein loaded.

Table 4.1.2 Purification of bovine brain PKC by threonine-Sepharose chromatography

Fraction	Volume	Protein conc.	Specific activity	Total activity	Recovery	Purification fold
	ml	mg/ml	U/mg	units	%	
Crude supernatant	1000	14.0	14.3	200000	100	1.00
DE-52 pool	80.0	22.0	41.0	73000	36.5	2.80
Phenyl-Sepharose/ S-200pool	117	2.25	186	49000	24.5	13.0
Threonine-Sepharose pool (post dialysis)	16.2	0.42	1640	11200	5.6	115

Details of bovine PKC purified from a single brain (500g). The high concentration of ammonium sulphate in the phenyl-Sepharose pool interferes with the assay reproducibility, therefore this pool is not analysed for activity. The PKC is homogenous after purification to 115 fold, indicating the abundance of this protein in bovine brain.

2.6) which was equilibrated with buffer E containing 380 mM NaCl. The column was washed with 120 ml equilibration buffer and eluted with a 50 ml gradient from 380 mM to 1.2 M NaCl in buffer E. Fractions (5 ml) containing PKC activity (Figure 4.1.8) were pooled and dialysed against 50% glycerol (v/v), 20 mM Tris.HCl pH 7.5, 2 mM EDTA, 1 mM DTT, (buffer D) and stored at -20°C.

A typical purification protocol is shown in Table 4.1.2. For most preparations, the PKC concentration in the sample was between 300 and 500µg/ml and had a specific activity of 1,500-2,000 U/mg. The kinase was absolutely dependent on PtdSer and Ca^{++} for activity when tested using pure lipid vesicles and on PtdSer, Ca^{++} and TPA or DG when tested in the mixed micelle assays. About 5-10% of the PKC in the soluble fraction from the brain was recovered and interestingly, the PKC was homogenous after a purification of only 100-130 fold, indicating that this is a very abundant protein in the cytosol of bovine brain cells; this is comparable to observations for rodent brain PKC (Jeng *et al*, 1986). The PKC from this procedure migrated as a doublet on SDS-polyacrylamide gels, with Mr values of 79,000 and 81,000 (Figure 4.1.9). This doublet was due to differences in migration of the isotypes on polyacrylamide gels as will be discussed in section 4.2.3. Immuno-blotting analysis of samples from each pool collected during the purification shows that PKC-α, -β_1, -β_2 and -γ co-purify through this preparation to the threonine-Sepharose column (Figure 4.1.10). This co-purification of the isotypes was important for the subsequent separation and biochemical analysis of the isotypes because the resulting isotype pools that were generated were of similar purity (see section 4.2.3). The relative amounts of isotype in the pool are approximately 7:7:1:5 PKC-α:β_1:β_2:γ, based on the immuno-blotting data. The PKC was

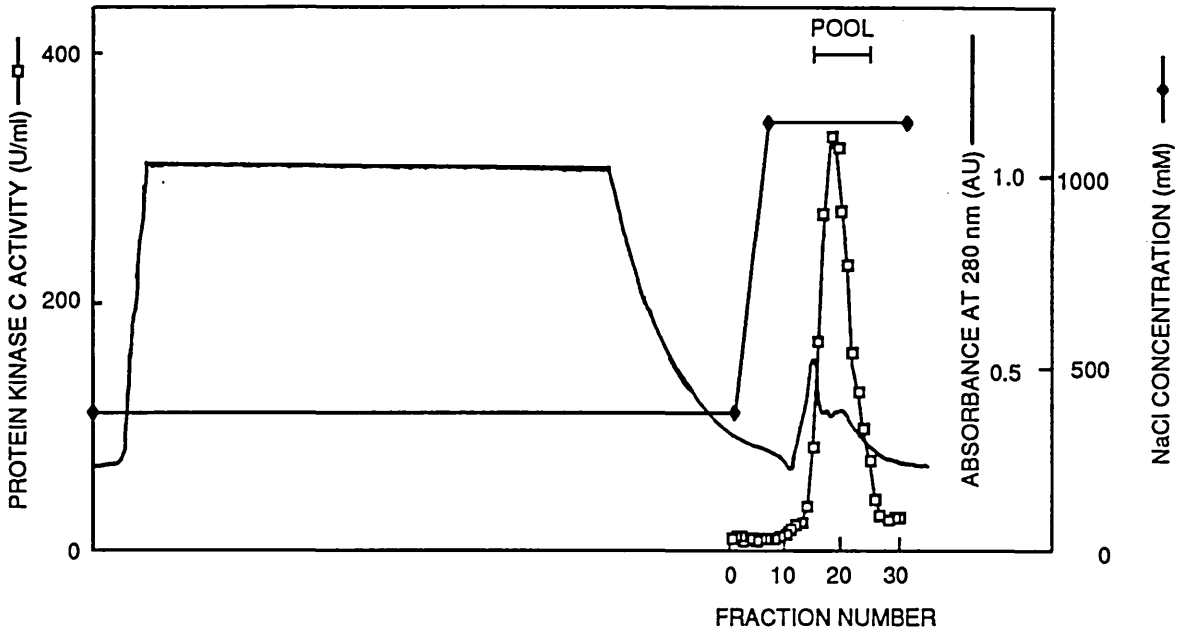


Figure 4.1.8 Threonine-Sepharose chromatography
The pool from the S-200 column was adjusted to 380mM NaCl and loaded onto a threonine-Sepharose column. The majority of the protein in the sample did not bind to the column as judged by the absorbance at 280nm (———). The flow through proteins were collected as a single fraction and did not contain any detectable PKC activity. The PKC was eluted by a NaCl gradient as shown (♦-♦), which was generated by a FPLC system. The Ca^{++}/PtdSer activity (□—□) in the fractions was determined and a pool was collected as indicated by the bar.

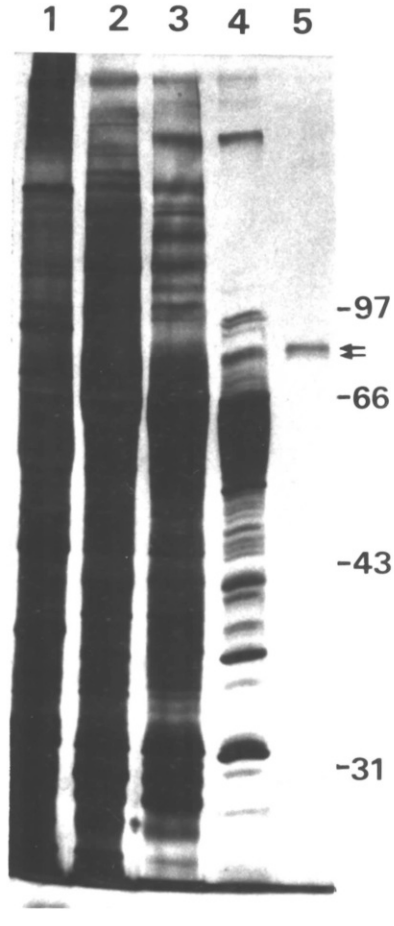


Fig 4.1.9 SDS-PAGE analysis of purified PKC
Samples from the stages of the purification were analysed by SDS-PAGE, on 12.5% acrylamide gels. One unit of PKC activity was loaded onto each lane. The proteins were detected by silver staining. Lane 1: crude extract; lane 2: DE-52 pool; lane 3: phenyl-Sepharose pool; lane 4: S-200 pool; lane 5: threonine-Sepharose pool. The position of PKC is indicated by the arrow and the migration of standard molecular mass proteins are indicated (x 10^{-3}).

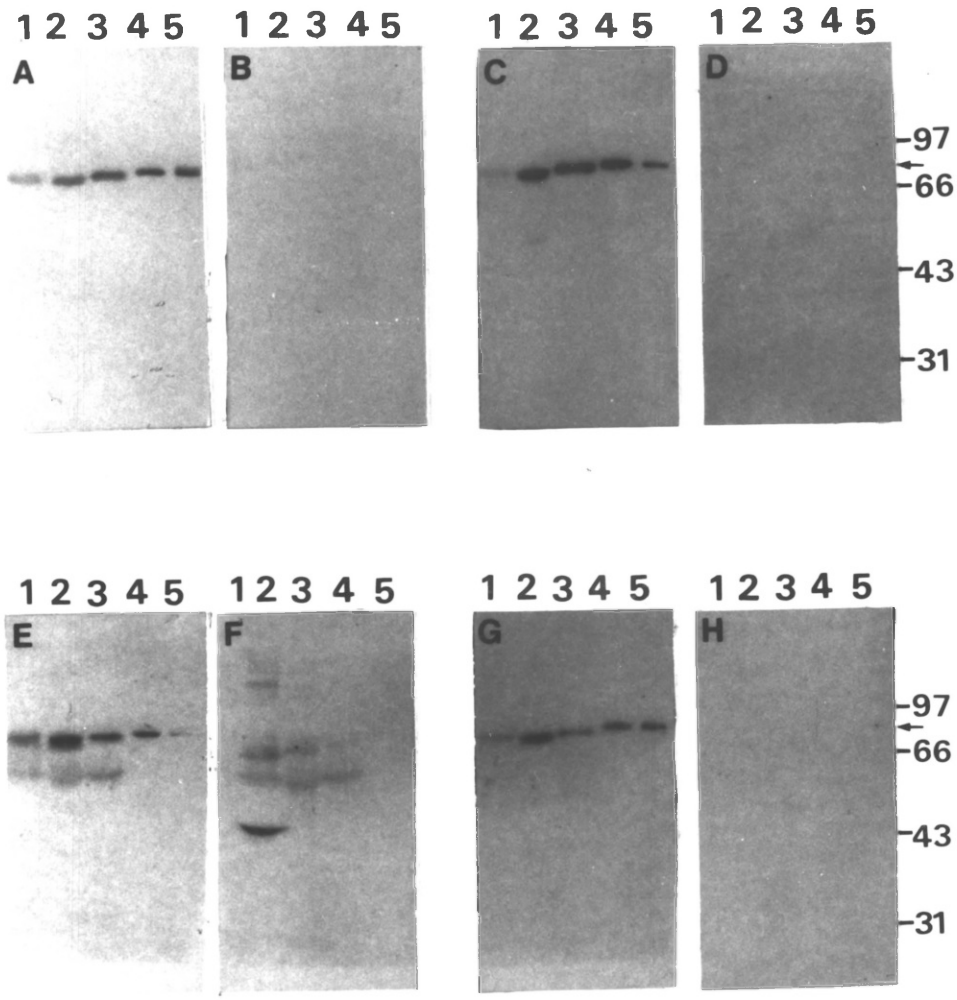


Fig 4.1.10 Co-purification of the PKC isotypes

Samples from each pool in the purification were immuno-blotted with the V5 region antisera. 0.5 units of PKC was loaded onto each lane; lane 1: crude extract; lane 2: DE-52 pool; lane 3: phenyl-Sepharose pool; lane 4: S-200 pool; lane 5: threonine-Sepharose pool. The position of PKC is indicated by the arrows and the positions of migration of 'Rainbow' molecular mass markers are shown ($x10^{-3}$). Blots with the $V_5\alpha$, $V_5\beta_1$ and $V_5\gamma$ antibodies were exposed for 24 hours; the $V_5\beta_2$ blots was exposed for 28 days. Smearing of PKC in lane A is seen due to the high concentration of protein in the sample which interferes with migration in the gels.

Antisera used:

panel A= $V_5\alpha$; panel B= $V_5\alpha$ with competing peptide,
panel C= $V_5\beta_1$; panel D= $V_5\beta_1$ with competing peptide,
panel E= $V_5\beta_2$; panel F= $V_5\beta_2$ with competing peptide,
panel G= $V_5\gamma$; panel H= $V_5\gamma$ with competing peptide.

stable when stored at -20°C in buffer D for up to one year. After this time, there was a gradual deterioration in the effector dependence but not the kinase activity. This deterioration in effector dependence was not due to proteolysis but appeared to be due to the selective denaturation of the regulatory domain.

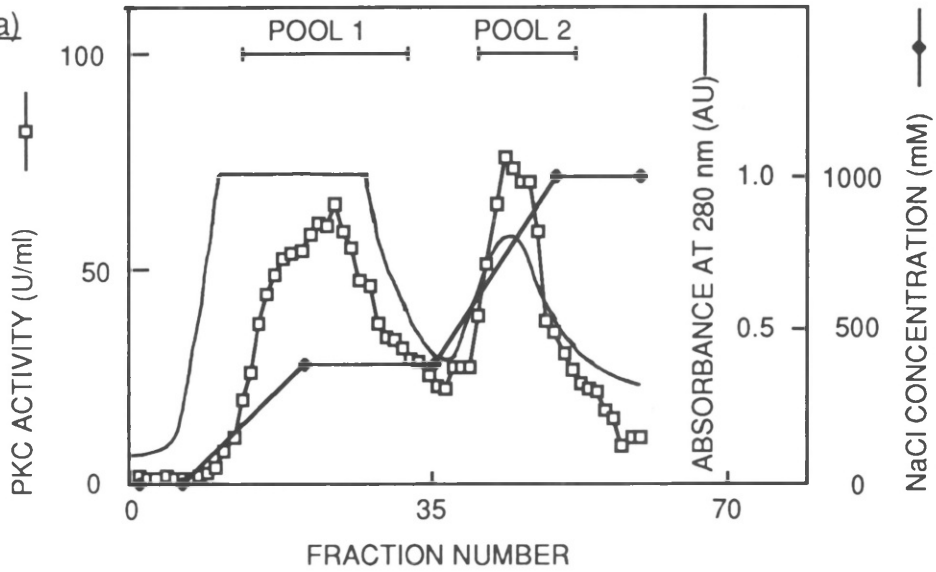
This purification is a better procedure than the protocol based on PtdSer-acrylamide for a number of reasons. Both protocols produced approximately the same amount of material but the threonine-Sepharose protocol was more efficient (about 10 fold) and the PKC produced was of higher purity as determined by SDS-PAGE analysis (compare Figures 4.1.3 and 4.1.10). The threonine-Sepharose protocol was more efficeint than the previous protocol and required only a single bovine brain as compared to the 25-30 that were needed before; this was in part due to the use of very fresh material. The processing was therefore physically less demanding and could be completed in only two days (as opposed to seven), requiring only a fraction of the buffers, chemicals and gel materials that had previously been required. Further, the new protocol was more reproducible and because PKC-α, -β_1, -β_2 and -γ co-purified through the procedure, the sample was better suited to a single separation step.

The critical step in this preparation was the observation that by loading the threonine-Sepharose column at 380mM NaCl, PKC bound to the matrix while the contaminating proteins in the S-200 sample did not (Figure 4.1.8). When the column was chromatographed under the published conditions (i.e. equilibrated and loaded in buffers lacking NaCl and then eluted with a discontinious buffer system; Kikkawa *et al*, 1986 and see Figure 4.1.11), a significant amount of

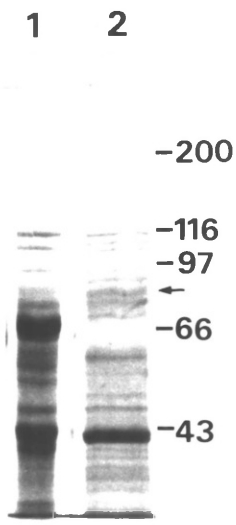
Figure 4.1.11(a) Threonine-Sepharose chromatography
The pool from an S-200 column was loaded directly onto the threonine-Sepharose column equilibrated in 20mM Tris.HCl pH 7.5, 2mM EDTA, 0.3% v/v β-mercaptoethanol. After loading, the column was washed until the OD_{280} had returned to zero and the PKC was eluted with the discontinuous gradient shown (◊-◊), which was generated by an FPLC system. The Ca^{++}/PtdSer dependent kinase activity (□—□) in each fraction was determined and the PKC eluted in two peaks. The first was associated with the major protein peak which was eluted by the first part of the gradient, and the second with the minor protein peak associated with the second ascending gradient. Two pools of activity were collected as indicated by the bars.

Figure 4.1.11(b) SDS-PAGE analysis of purified PKC
Samples from the two pools of activity collected from the column in Figure 4.1.11(a) were analysed by SDS PAGE on 10% w/v acrylamide gels. One unit of PKC was loaded onto each lane and the proteins were detected by silver staining. Lane 1= pool 1, lane 2= pool 2. The position of PKC is indicated by the arrow and the positions of migration of standard molecular mass proteins are also indicated (x 10^{-3}). The second pool was found to be more pure than the first.

(a)


POOL 1
POOL 2
100
50
0
PKC ACTIVITY (U/ml)
0
35
70
FRACTION NUMBER
ABSORBANCE AT 280 nm (AU)
1.0
0.5
0
NaCl CONCENTRATION (mM)
1000
500
0

(b)


1
2
-200
-116
-97
-66
-43

the PKC was lost in the first part of the gradient because it eluted with the contaminating proteins (Figure 4.1.11). The PKC that eluted with the second, high salt gradient was also found to be less pure than if the column was loaded in the high salt conditions (compare Figures 4.1.9 and 4.1.11). The reason for the loss of PKC in the first part of the gradient appears to have been due to ionic protein-protein interactions which were overcome by loading the column in the presence of NaCl. These conditions therefore give better recoveries since no PKC is lost in the early part of the gradient. The length of time required for the run was also reduced and the PKC could be eluted with shorter gradients, resulting in more concentrated samples.

Care must be exercised when the threonine-Sepharose column is loaded in the presence of NaCl however, because the PKC does not bind to the matrix as avidly as in its absence. As a result, the PKC tends to migrate through the column during loading and washing. The size of the load, the duration of the wash step and the column dimensions all affected the amount of migration that occured. For an S-200 pool of 120ml, the column dimensions and wash conditions shown above were found to give optimal recovery without compromising purification. Larger loads or longer washes all resulted in lower recoveries of the PKC due to its 'leaching from the column before the gradient was applied.

4.2 SEPARATION OF PKC -α, -β_1, -β_2 and -γ

This section describes the attempts to separate the PKC isotypes. Several chromatographic techniques were used to separate the isotypes, the choice of columns being based on the known chromatographic properties of PKC. Limited success was achieved with some of these methods and only hydroxylapatite chromatography based on the method of Huang *et al* (1986) gave complete separation.

The aim of this project was to analyse the biochemical properties of native PKC-α, -β and -γ. These kinds of studies should be conducted with enzyme preparations in the highest state of purity possible, to avoid interference from contaminating proteins which may sequester activating co-factors or act as phosphate acceptors. As a minimum requirement the sample must be free of contaminating kinases. The thr-Sepharose purified PKC (section 4.1.2.) was therefore well suited to the separation studies, because of its highly pure nature. Further to this, the isotypes of interest were known to co-purify and therefore since they had all been treated in the same manner during purification, they were comparable. The purification is also rapid and produces large amounts of relatively concentrated kinase that give good recoveries on subsequent columns.

4.2.1. Separation by Mono Q column chromatography

Many columns are commercially available for use with high pressure chromatographic systems. These systems are aided by high flow rates at relatively high pressures with column matrices that give high resolution; they are routinely used during the purification of many proteins. One such column is the 'MonoQ' column developed by

Pharmacia for use with the 'FPLC' system. This anion exchange column has been used under two different conditions to purify PKC from rodent brains (Jeng *et al*, 1986).

Methods All the buffers were filtered and chilled to 4°C (unless otherwise stated) prior to use. PKC samples in buffer D (section 4.1.1) were mixed with 4 volumes of column equilibration buffer and centrifuged at 10,000rpm (10 min in Eppendorf tubes) prior to loading. For most cases 1.0ml fractions were collected and the activity in these determined immediately after the run was completed. The detergent Triton X-100 was added to the buffers (0.02%) to reduce the loss of PKC due to adsorption to the plastic surfaces of the tubes. The recoveries from this column were in excess of 80% for all conditions tested. After the activity profile had been determined, pools were made of the peaks as appropriate. These were dialysed against buffer D (section 4.1.1.) and stored at -20°C prior to immuno-blotting analysis.

The basic column conditions were: PKC (0.5-1ml) was loaded onto an HR 5/5 Mono Q column equilibrated in 20mM Tris pH 7.2, 2mM EDTA, 0.3% β-mercaptoethanol, 0.02% Triton X-100 (buffer F) at a flow rate of 0.5 ml/min. The column was washed with 5 ml buffer F and the PKC was eluted with linear gradients from buffer F to buffer F containing 1M NaCl. Under these conditions, two peaks of activity were resolved. The first contained PKC-γ and the second a mixture of PKC-α and -β (Figure 4.2.1). Separation under these conditions was found to be dependent on the purity of the sample. PKC samples containing contaminating proteins gave activity profiles different from the profiles of more pure samples when chromatographed under the same conditions. In the presence of contaminants the PKC

Figure 4.2.1(a) Mono Q column chromatography

The column was equilibrated with 20mM Tris.HCl pH 7.2, 2mM EDTA, 0.3% β-mercaptoethanol, 0.02% Triiton X-100 (buffer F). The PKC sample was mixed with four volumes of buffer F and loaded onto the column. The column was washed with 5ml buffer F and the PKC eluted with the NaCl gradient shown (♦-♦). The Ca^{++}/PtdSer dependent kinase activity (□—□) in the fractions was determined and PKC eluted in two peaks. Two pools were collected as shown by the bars.

Figure 4.2.1(b) Immuno-blot analysis of PKC

Samples from pools 1 and 2 from the column represented in Figure 4.2.1(a) were immuno-blotted with the monospecific antisera. 10μl of each pool was loaded per lane; lane 1= pool 1, lane 2= pool 2. The position of PKC is indicated by the arrow.

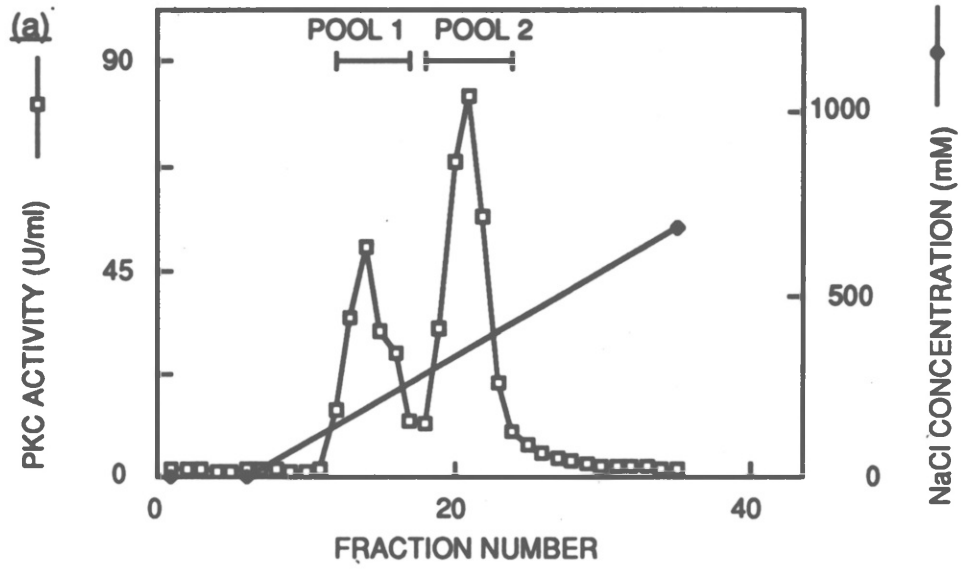
Antisera used:

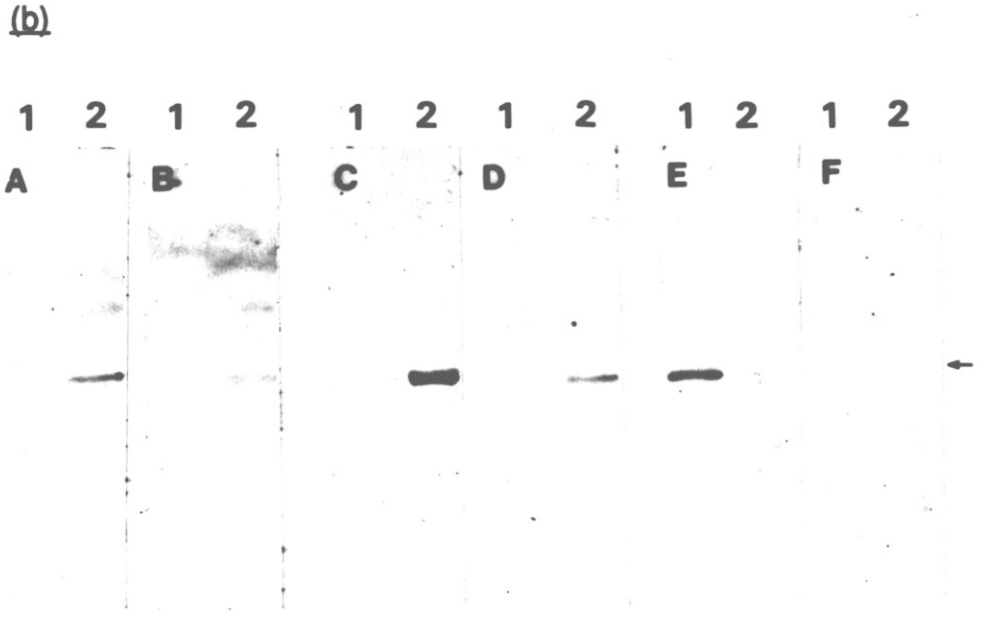
panel A= $V_5\alpha$; panel B= $V_5\alpha$ with competing peptide

panel C= $V_5\beta_1$; panel D= $V_5\beta_1$ with competing peptide

panel E= $V_5\gamma$; panel F= $V_5\gamma$ with competing peptide.

At the time that these studies were being undertaken, an antiserum specific for PKC-β_2 was not available.


(a)
POOL 1
POOL 2
90
45
0
PKC ACTIVITY (U/ml)
1000
500
0
NaCl CONCENTRATION (mM)
0
20
40
FRACTION NUMBER


(b)
1 2 1 2 1 2 1 2 1 2 1 2
A
B
C
D
E
F

isotypes co-eluted from the column in a single peak (Figure 4.2.2). Protein-protein interactions therefore appear to affect the chromatographic behaviour of the isotypes on the Mono Q column and this emphasises the need for pure PKC samples.

Under the correct conditions (*ie* those used in Figure 4.2.1) it was possible to separate the PKC into two peaks of activity. The first contained PKC-γ and the second -α and -β. Further column conditions were tested to try to separate the -α and -β isotypes in the second peak from each other. The conditions are as described above, with the additions as indicated (Table 4.2.1). All the conditions were aimed at reducing protein-protein interactions since these appeared to affect the separation of the isotypes on this column.

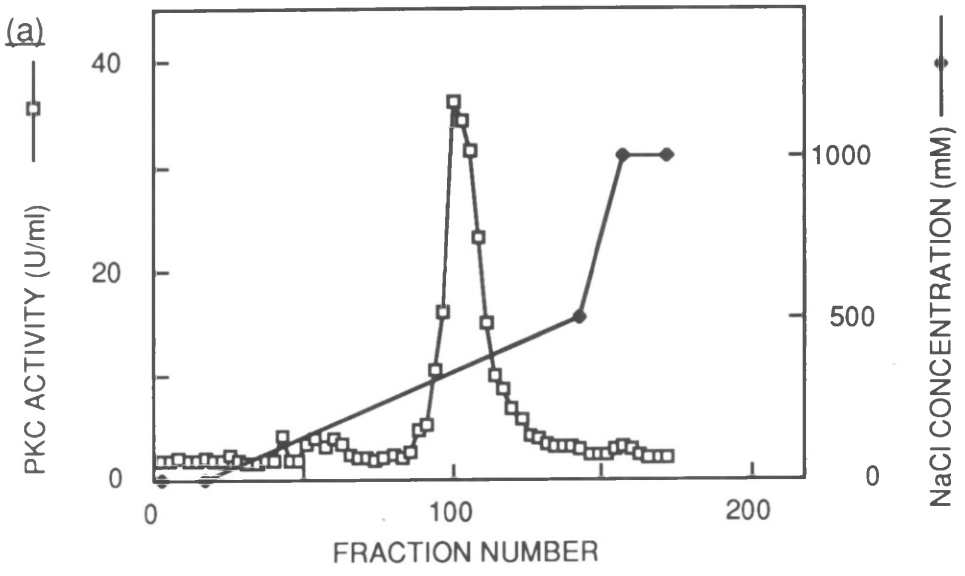
Calcium The observation that Ca^{++} differentially affected the binding of PKC to the PtdSer-acrylamide column suggested that addition of Ca^{++} may alter the behaviour of the isotypes on the Mono Q column. The column was equilibrated in 20mM Tris pH 7.2, 0.3% β-mercaptoethanol, 0.02% Triton X-100 and 0.6mM or 2.0mM Ca^{++}. The PKC samples were mixed with four volumes of equilibration buffer and incubated on ice for 15 min before loading. At both concentrations of Ca^{++}, the separation of the isotypes was unaffected and two peaks of activity were detected. The distribution of the isotypes was as before, with PKC-γ in the first and PKC-α and -β in the second (Figure 4.2.3, Table 4.2.1).

pH Altering the pH of buffers alters the net charge on proteins, causing a reduction in charge as the pH approaches the pI of the proteins. The effect of pH on the separation of the isotypes on the Mono Q column was therefore investigated. The column was

Figure 4.2.2(a) Mono Q column chromatography
The conditions were as in Figure 4.2.1(a). The PKC was mixed with four volumes of buffer F and loaded onto the column. The PKC sample used was less pure than the sample used in Figure 4.2.1 (see below). The PKC was eluted with the NaCl gradient shown (♦-♦) and the Ca^{++}/PtdSer dependent kinase activity (□–□) in the fractions was determined. The PKC eluted as a single peak.

Figure 4.2.2(b) SDS-PAGE anlaysis of PKC samples
The samples loaded onto the Mono Q column in Figures 4.2.1(a) and 4.2.2(a) were analysed by SDS-PAGE. For each lane, 1μg of protein was analysed. The proteins were detected by silver staining. Lane 1= load for Figure 4.2.2(a); lane 2= load from Figure 4.2.1(a). The position of PKC is indicated by the arrows and the migration of standard molecular mass proteins is also indicated (x 10^{-3}). The results show that the PKC sample loaded in Figure 4.2.1(a) was more highly purified than the sample from Figure 4.2.2(a).

(a)


40
20
0
PKC ACTIVITY (U/ml)
0
100
200
FRACTION NUMBER
1000
500
0
NaCl CONCENTRATION (mM)

(b)

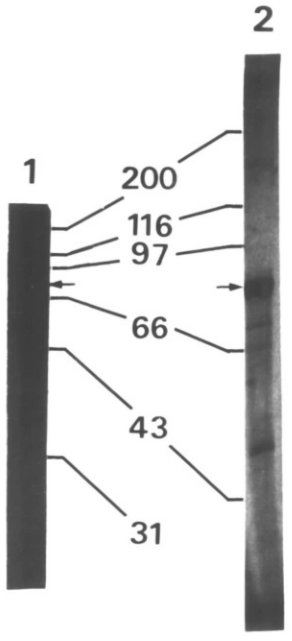

1
2
200
116
97
66
43
31

Table 4.2.1 Mono Q column chromatography

Buffer	additions	No peaks	Isotypes detected in pool numbers -α	-β	-γ	Fig
20mM Tris pH7.2	None	2	2	2	1	Fig 4.2.1
20mM Tris pH7.2	0.6mM Ca++	2	2	2	1	Fig 4.2.3(a)
20mM Tris pH7.2	2.0mM Ca++	2	2	2	1	Fig 4.2.3(b)
20mM Bes pH 6.5		2	N.D			Fig 4.2.4(a)
20mM Bes pH 7.0		2	N.D.			Fig 4.2.4(b)
20mM Tris pH7.5		1	N.D			Fig4.2.4(c)
20mM Bes pH 7.0	Discontinious gradient	3	2,(3)	2,3	1	Fig 4.2.5
20mM Bes pH 7.0	5% triton X-100	3	2,3	2,3	1,3	Fig 4.2.6(a)
20mM Bes pH 7.0	20% ethanediol	6	throughout	throughout	1,2 and 6	Fig 4.2.6(b)
20mM Bes pH 7.0	10% betaine	4 / 5	N.D.			Fig 4.2.7(a)
20mM Bes pH 7.0	run at 20°C	4 / 5	N.D.			Fig 4.2.7(b)
20mM Bes pH 7.0	0.02% CHAPS	4	2,3,(4)	(2),3,4	1	Fig 4.2.8(a)
20mM Bes pH7.5	0.02% CHAPS, flow 0.15ml/min	9	throughout	throughout	1,2,3,7	Fig 4.2.8(b)

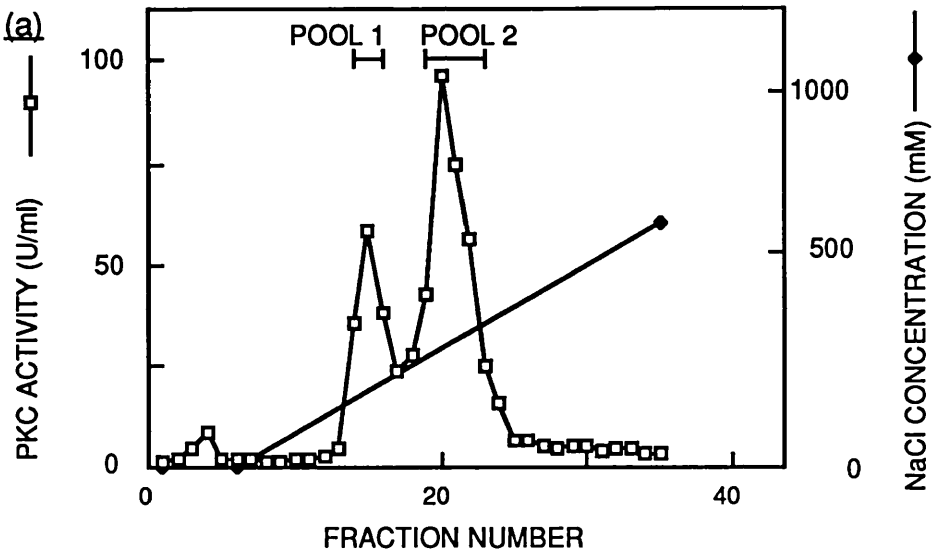
The monoQ column was chromatographed under the conditions ststed for Figure 4.2.1 but with the additions indicated. The PKC samples were highly purified. The elution positions of the -α, -β and -γ isotypes were determined by immuno-blotting analysis. N.D= not determined. When CHAPS was added to the buffers, the Triton X-100 was excluded.

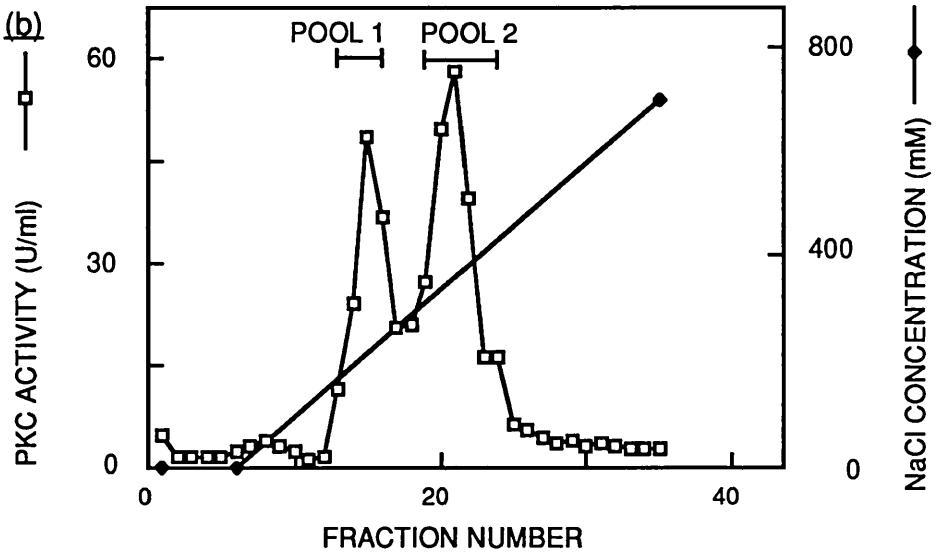
Figure 4.2.3(a) Mono Q column chromatography at 0.6mM calcium

The column was equilibrated in 20mM Tris.HCl pH 7.2, 0.6mM Ca^{++}, 0.3% β-mercaptoethanol, 0.02% Triton X-100. The PKC sample was mixed with four volumes of equilibration buffer, incubated on ice for 15 min and loaded onto the column. After loading, the column was washed with 5ml equilibration buffer and the PKC was eluted with the NaCl gradient shown (♦-♦). The Ca^{++}/PtdSer dependent kinase activity (□—□) in the fractions was determined and two peaks of activity were detected. Two pools were collected as shown by the bars. Subsequent immuno-blot analysis revealed that the first pool contained PKC-γ and the second a mixture of the -α and -β isotypes (Table 4.2.1).

Figure 4.2.3(b) Mono Q column chromatography at 2.0mM calcium

The column was equilibrated in 20mM Tris.HCl pH 7.2, 2.0mM Ca^{++}, 0.3% β-mercaptoethanol, 0.02% Triton X-100. The PKC sample was mixed with four volumes of equilibration buffer and treated as in Figure 4.2.3(a). Two peaks of activity were detected and two pools were collected as indicated by the bars. The distribution of the isotypes under these conditions was similar to those for Figure 4.2.3(a).


(a)
POOL 1
POOL 2
100
50
0
PKC ACTIVITY (U/ml)
1000
500
0
NaCl CONCENTRATION (mM)
0
20
40
FRACTION NUMBER


(b)
POOL 1
POOL 2
60
30
0
PKC ACTIVITY (U/ml)
800
400
0
NaCl CONCENTRATION (mM)
0
20
40
FRACTION NUMBER

chromatographed at pH 6.5, 7.0 and 7.5. The greatest separation of the two peaks was achieved at pH 7.0, under which conditions the two peaks of activity were baseline separated {Figure 4.2.4(a)}. At pH 6.5, there were two major peaks and a third minor peak, but the major peaks were not completely separated {Figure 4.2.4(b)}. At pH 7.5, there was a single major peak and three minor peaks, indicating that the isotypes co-eluted {Figure 4.2.4(c)}. Further separation of the isotypes could be achieved at pH 7.0 by using the discontinous gradient shown in Figure 4.2.5. Under these conditions it was possible to generate three peaks of activity. The first contained PKC-γ almost exclusively, the second a mixture of -α and -β, and the third was predominantly -β, but contained detectable amounts of -α (Table 4.2.1). This indicated that it was possible to separate the isotypes on the Mono Q column if conditions were used which would reduce the protein-protein interactions of the isotypes. All subsequent separations were conducted at pH7.0 with conditions that were designed to reduce these interactions.

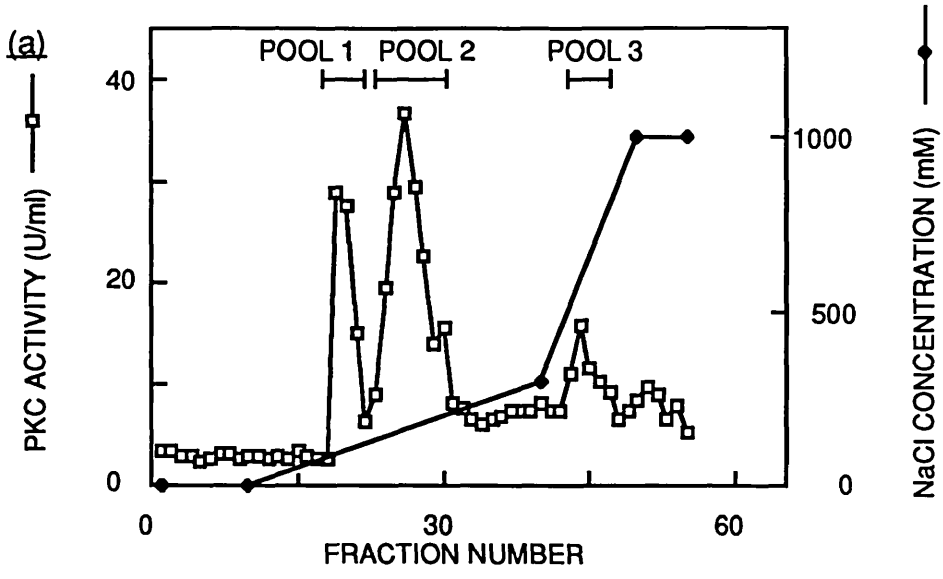
Triton X-100 and ethanediol Triton X-100 and ethanediol were added to the buffers to reduce hydrophobic protein interactions. In the presence of 5% Triton X-100 there were still three peaks of activity, as shown in Figure 4.2.6. However, the co-elution fo the isotypes appeared to increase and -γ was no longer restricted to only the first peak, but was also found in the third {Figure 4.2.6(a)}. The second peak contained a mixture of -α and -β and the first peak contained only -γ (Figure 4.2.6 and Table 4.2.1). In the presence of 20% ethanediol there were still three major peaks, but the second peak was separated into four overlapping minor peaks; these were pooled separately (Figure 4.2.6). Under these conditions the co-elution of the isotypes increased. The -γ isotype was present in the first and

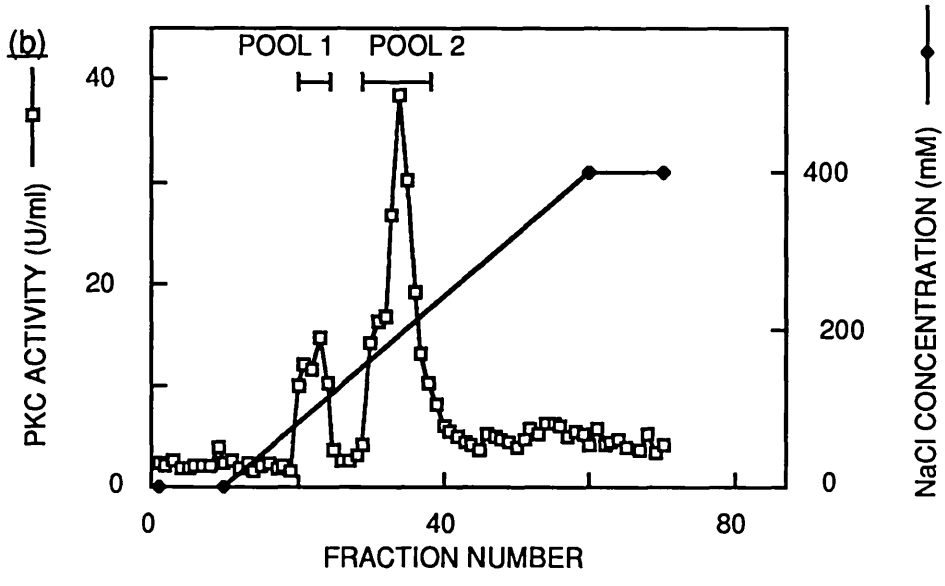
Figure 4.2.4(a) Mono Q column chromatography at pH 6.5
The column was equilibrated in 20mM Bes.NaOH pH 6.5, 2mM EDTA, 0.3% β- mercaptoethanol, 0.02% Triton X-100. The PKC sample was mixed with four volumes of equilibration buffer and incubated on ice for 15 min prior to loading. The PKC was eluted with the NaCl gradient shown (♦-♦) and the PKC activity (□—□) in the fractions was determined. Two major peaks of activity and one minor peak were detected.

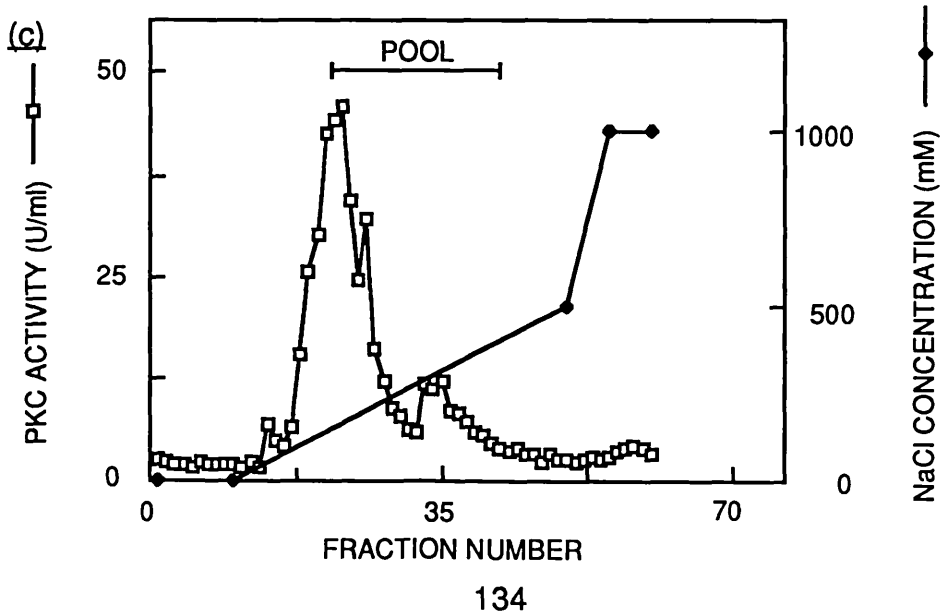
Figure 4.2.4(b) Mono Q column chromatography at pH 7.0
The column was equilibrated in 20mM Bes.NaOH pH 7.0, 2mM EDTA, 0.3% β-mercaptoethanol, 0.02% Triton x-100. The PKC sample was mixed with four volumes of equilibration buffer and treated as in Figure 4.2.4(a) above. The PKC was eluted by the NaCl gradient shown (♦-♦) and the activity in the fractions determined (□—□). Two peaks of activity were detected which were baseline separated.

Figure 4.2.4(c) Mono Q column chromatography at pH 7.5
The column was equilibrated in 20mM Tris.HCl pH7.5, 2mM EDTA, 0.3% β-mercaptoethanol, 0.02% Triton X-100. The PKC was mixed with four volumes of equilibration buffer and treated as in Figure 4.2.4(a) above. The PKC was eluted with the NaCl gradient shown (♦-♦) and the PKC activity (□—□) in each fraction determined. Under these conditions. the isotypes appeared to co-elute in a single major peak, with a minor trailing peak.

The PKC samples were the same for the three columns conditions used. Similar amounts of activity were loaded and recovered in all three cases.


(a)
POOL 1
POOL 2
POOL 3
PKC ACTIVITY (U/ml)
NaCl CONCENTRATION (mM)
40
20
0
1000
500
0
0
30
60
FRACTION NUMBER


(b)
POOL 1
POOL 2
PKC ACTIVITY (U/ml)
NaCl CONCENTRATION (mM)
40
20
0
400
200
0
0
40
80
FRACTION NUMBER


(c)
POOL
PKC ACTIVITY (U/ml)
NaCl CONCENTRATION (mM)
50
25
0
1000
500
0
0
35
70
FRACTION NUMBER

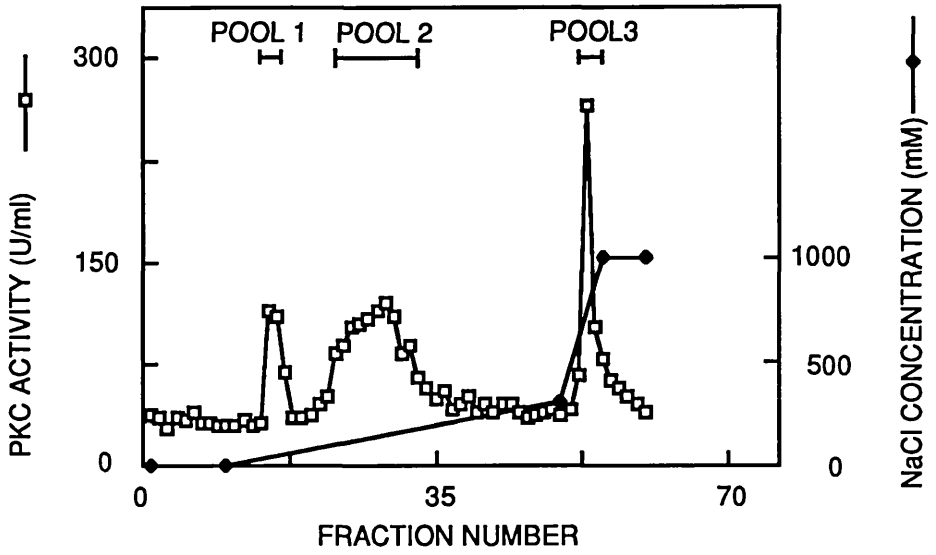


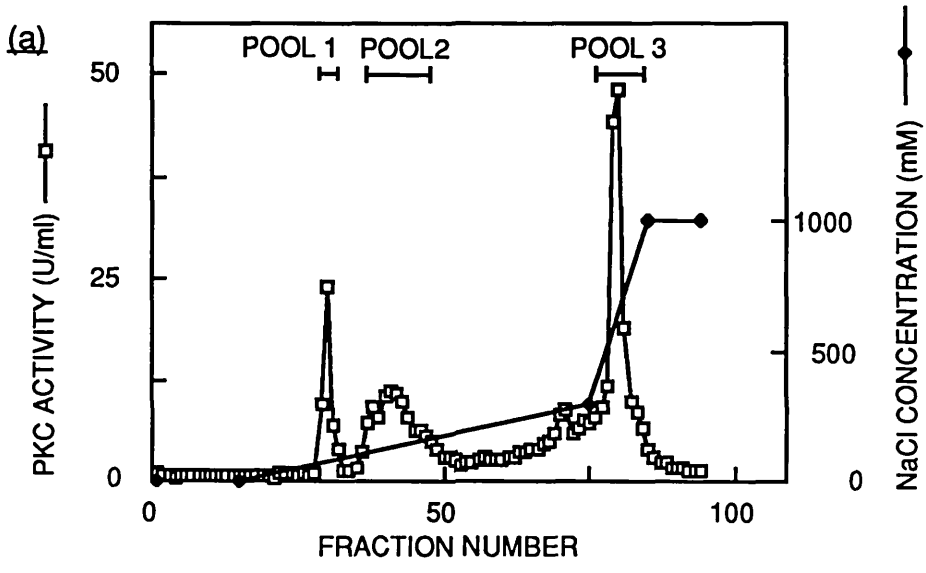
Figure 4.2.5 Mono Q column chromatography at pH 7.0
The column was equilibrated in 20mM Bes.NaOH pH 7.0, 2mM EDTA, 0.3% β-mercaptoethanol, 0.02% Triton X-100. The PKC was mixed with four volumes of equilibration buffer and loaded onto the column. After washing the PKC was eluted with the discontinious gradient shown (♦-♦). The PKC activity (□—□) in the fractions was determined and found to elute in three peaks which were baseline separated. The peaks were pooled as indicated by the bars. Immuno-blot analysis of the pools revealed that peak 1 contained PKC-γ almost exclusively, peak 2 contained both -α and -β and peak 3 was predominantly -β, although -α was also present (Table 4.2.1).

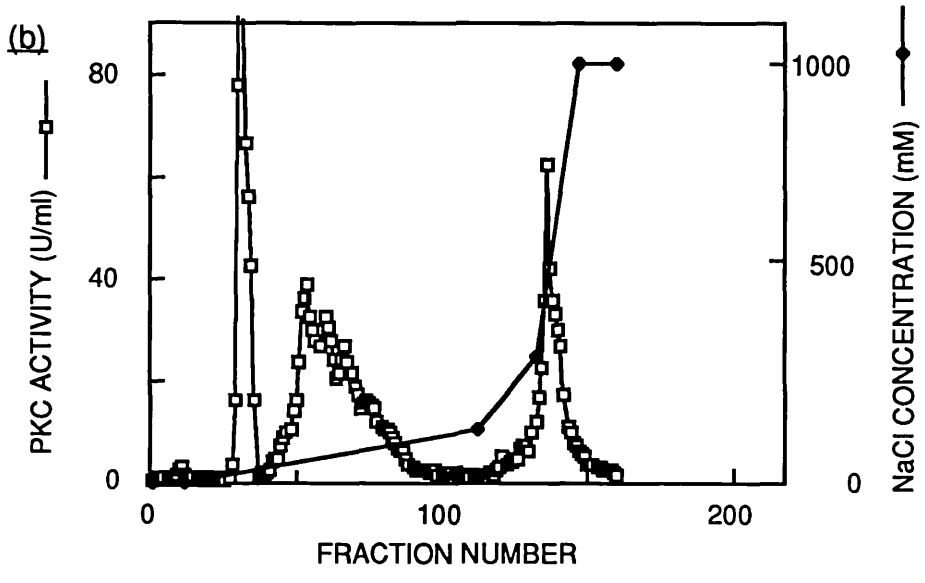
Figure 4.2.6(a) Mono Q column chromatography with 5% v/v Triton X-100

The column was equilibrated in 20mM Bes.NaOH pH 7.0, 2mM EDTA, 0.3% β-meracaptoethanol, 5% Triton X-100. The PKC sample was mixed with four volumes of equilibration buffer and incubated on ice for 15 min. The sample was loaded onto the column, washed and eluted with the NaCl gradient shown (♦-♦). 0.5ml fractions were collected and the Ca^{++}/PtdSer dependent kinase activity (□—□) in these was determined. Three pools were collected as shown by the bars. Immuno-blot analysis of the pools revealed that pool 1 contained only PKC-γ; pool 2 -α and -β and pool 3 a mixture of -α, -β and -γ. (Table 4.2.1).

Figure 4.2.6(b) Mono Q column chromatography with 20% v/v ethanediol

The column was equilibrated in 20mM Bes.NaOH pH 7.0, 2mM EDTA, 0.3% β-mercaptoethanol, 20% ethanediol. The PKC was mixed in four volumes of equilibration buffer on ice for 15 min and loaded onto the column. The PKC was eluted with the NaCl gradient shown (♦-♦) and 0.25ml fractions were collected. The PKC activity (□—□) in the fractions was determined and three peaks were detected. The second of these peaks was composed of four overlapping peaks, and these were pooled separately. Immuno-blot analysis of the 6 pools that were collected revealed that PKC-γ was in pools 1, 2 and 6; and PKC-α and -β were in all six of the pools (Table 4.2.1). The relative quantities of each isotype in the six pools was different.


(a)
POOL 1
POOL2
POOL 3
50
25
0
PKC ACTIVITY (U/ml)
1000
500
0
NaCl CONCENTRATION (mM)
0
50
100
FRACTION NUMBER


(b)
80
40
0
PKC ACTIVITY (U/ml)
1000
500
0
NaCl CONCENTRATION (mM)
0
100
200
FRACTION NUMBER

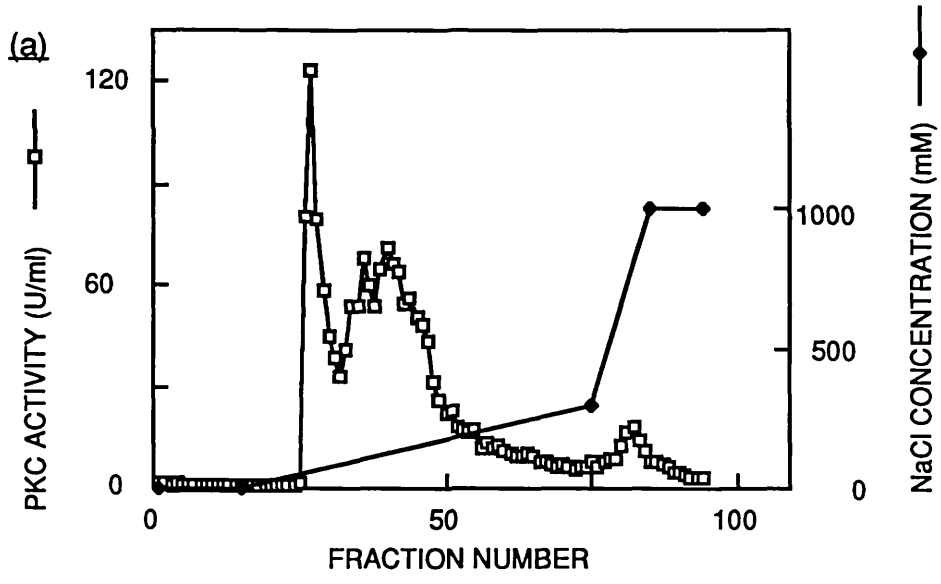
final pool as with 5% v/v Triton X-100, but was also detected in the first pool of the second peak (Figure 4.2.6). The -α and -β isotypes were detected in all the pools from this column (Table 4.2.1).

Betaine The addition of hydrophobic compounds altered the elution properties of the isotypes from the Mono Q column, but did not improve their separation and actually increased the amount of co-elution. Betaine was therefore added to the buffers. Betaine is a zwitterion and therefore interferes with ionic protein-protein interactions; it is suitable for use with the Mono Q column (Pharmacia FPLC hand book). In the presence of 10% betaine no discrete peaks of activity were detected but a complex profile was generated indicating that the isotypes co-eluted in a single peak (Figure 4.2.7). A similar trace was seen when the column was chromatographed at 20°C {Figure 4.2.7(b)}. It therefore appears that betaine and higher temperatures encourage similar protein-protein interactions between the PKC isotypes.

CHAPS Neither compounds that interfered with hydrophobic interactions, nor those that interfered with ionic interactions had improved separation of the PKC isotypes on the Mono Q column. The detergent CHAPS was therefore added to the buffers. This detergent has a zwitterionic head group and therefore can interfere with both hydrophobic and ionic interactions (Hjelmeland, 1980). In the presence of 0.02% CHAPS, four peaks of activity were detected, although peaks 2 and 3 were not completely resolved {Figure 4.2.8(a)}. The separation of the isotypes was still not complete. The -γ isotype was detected only in the first peak. The -α isotype was predominantly in the second peak, although it was present in the third peak and at very low levels in the fourth peak. The -β isotype

Figure 4.2.7(a) Mono Q column chromatography with 10% w/v betaine
The column was equilibrated in 20mM Bes.NaOH pH 7.0, 2mM EDTA, 0.3% β-mercaptoethanol, 10% w/v betaine. The PKC was mixed with four volumes of equilibration buffer and incubated on ice for 15 min prior to loading. The PKC was eluted with the NaCl gradient shown (♦-♦) and 0.5ml fractions were collected. The PKC activity in the fractions was determined (□—□) and the PKC eluted in a single peak composed of many overlapping peaks of activity.

Figure 4.2.7(b) Mono Q column chromatography at 20°C
The column was equilibrated in 20mM Bes.NaOH pH 7.0, 2mM EDTA, 0.3% β-mercaptoethanol, 0.02% Triton X-100 at room temperature (20°C). The PKC sample was mixed with four volumes of equilibration buffer and incubated at 20°C for 15 min prior to loading. After loading, the PKC was eluted with the NaCl gradient as shown (♦-♦) and 0.5 ml fractions were collected. The PKC activity profile (□—□) was similar to that seen in the presence of 10% betaine {Figure 4.2.7(a)}.


(a)
120
60
0
PKC ACTIVITY (U/ml)
1000
500
0
NaCl CONCENTRATION (mM)
0
50
100
FRACTION NUMBER

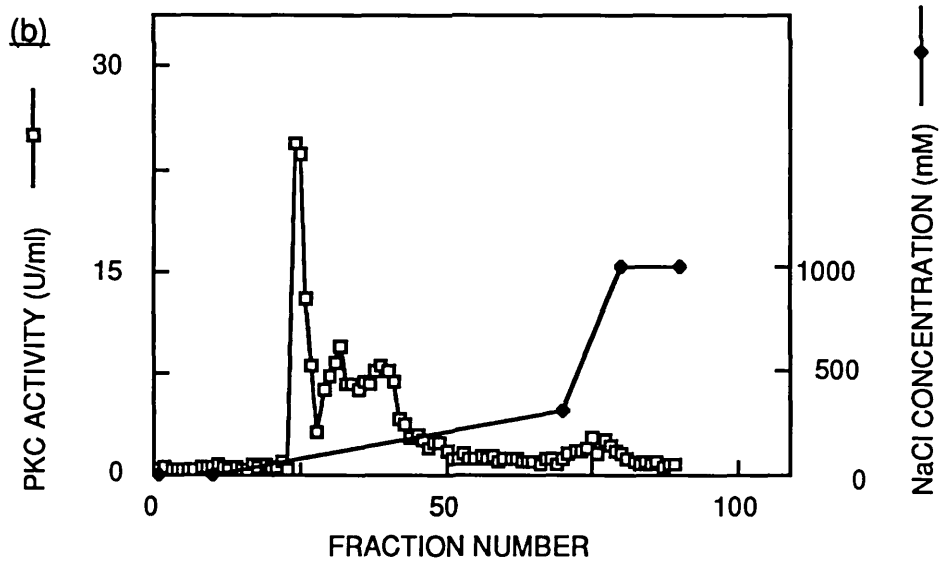
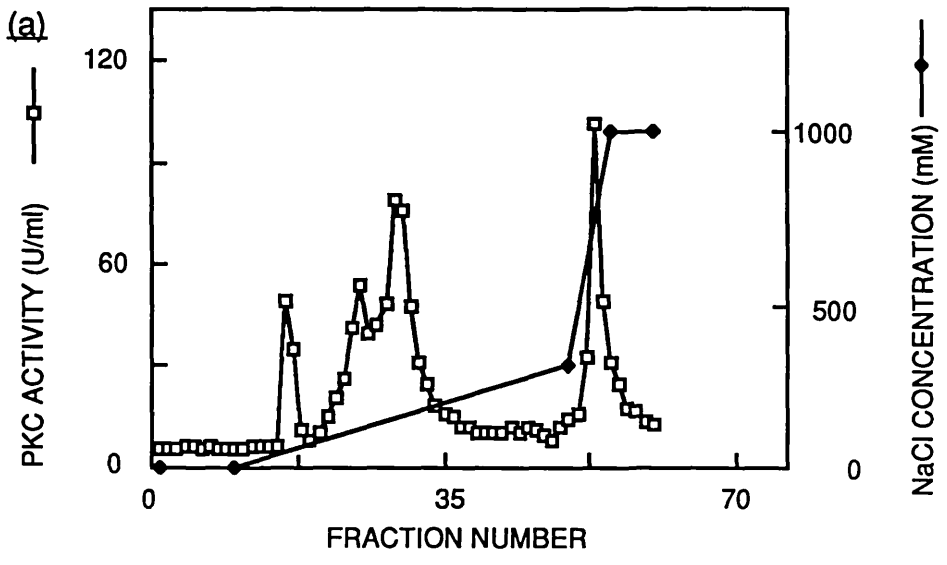
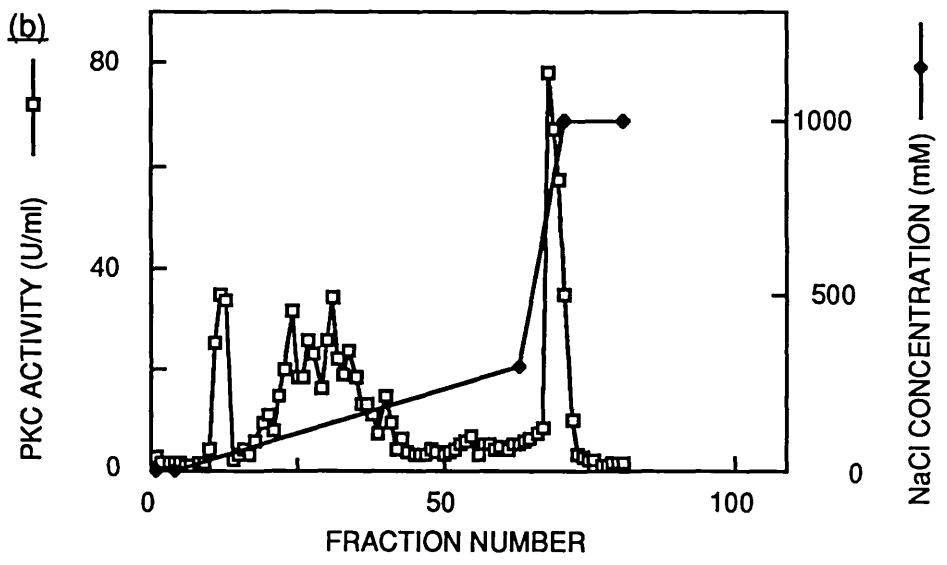

(b)
30
15
0
PKC ACTIVITY (U/ml)
1000
500
0
NaCl CONCENTRATION (mM)
0
50
100
FRACTION NUMBER

Figure 4.2.8(a) Mono Q column chromatography with 0.02% CHAPS
The column was equilibrated in 20mM Bes.NaOH pH 7.0, 2mM EDTA, 0.3% β-mercaptoethanol, 0.02% CHAPS. The PKC was mixed with four volumes of equilibration buffer and incubated on ice for 15 min. The sample was loaded onto the column and eluted with the discontinious NaCl gradient shown (♦-♦). 0.5 ml fractions were collected. The PKC activity in the fractions (□—□) was determined and four peaks of activity were detected. The second and third of these peaks were not completely resolved from each other. Four pools were collected as shown by the bars and these were analysed by immuno-blotting. Pool 1 contained only PKC-γ. Pool 2 contained predominantly PKC-α but also -β. Pool 3 contained -α and -β and pool 4 contained predominantly -β (Table 4.2.1).

Figure 4.2.8(b) Mono Q column chromatography with 0.02% CHAPS
The column was chromatographed as described in Figure 4.2.8(a) but the flow rate was reduced to 0.15ml/min and 0.3ml fractions were collected. Three major peaks of activity (□—□) were detected but the second of these was composed of five overlapping minor peaks. The first, last and five intermediate peaks of activity were pooled separately. Immuno-blot analysis of the seven pools indicated that decreasing the flow rate increased the amount of co-elution seen with the PKC isotypes under these conditions on the Mono Q column. The -γ isotype was detected in the first, second, third and final pools. The -α and -β isotypes were detected in all seven pools. The relative ratio of the isotypes in each of the pools was different but no pool contained only a single PKC isotype. This situation was similar to the results seen in the presence of 20% ethanediol {Figure 4.2.6(b)}.


(a)
120
60
0
PKC ACTIVITY (U/ml)
1000
500
0
NaCl CONCENTRATION (mM)
0
35
70
FRACTION NUMBER


(b)
80
40
0
PKC ACTIVITY (U/ml)
1000
500
0
NaCl CONCENTRATION (mM)
0
50
100
FRACTION NUMBER

was found predominantly in the final peak and it was also present in the third peak and to a lesser degree in the second. This redistribution of the isotypes under the influence of CHAPS suggested that they may be separated from each other on the Mono Q in the presence of this detergent. Reduction of the flow rate of the column (to 0.15ml/min) further fractionated the second and third peaks into numerous superimposed peaks (Figure 4.2.8). The elution of the isotypes under these conditions changed. All the isotypes were detected in more than one of the peaks and none of the peaks contained only a single PKC isotype {Figure 4.2.8(b)}.

The purified PKC isotypes were not satisfactorly separated on the Mono Q column although they could be fractionated into numerous peaks of activity. The basis for the separation was not established, but both ionic and hydrophobic interactions appeared to play important roles. Compounds that reduced one class of interactions (eg hydrophobic) appeared to promote alternative interactions (eg ionic). The Mono Q column was not investigated further because successful separation of the isotypes was achieved by hydroxylapatite chromatography (see below).

4.2.2. Miscellanious columns

The separation studies involving Mono Q columns were run concurrently with attempts to separate the PKC isotypes using columns based on matrices that had proven useful for PKC purification protocols. Initially the columns were tested for their ability to generate multiple peaks of activity from a PKC sample and if promising, the columns were tested further by immuno-blotting analysis.

Pro-RPC This is a commercially available reverse phase column for the 'FPLC' system. The column is compatable with biological buffers and can operate at high flow rates and relatively low pressures. PKC interacts with reversed phase columns and is useful in its purification as described in section 4.1. Purified PKC in buffer D (section 4.1.1.) was mixed with four volumes of 20 mM Tris.HCL pH7.5, 2mM EDTA, 0.03% v/v β-mercaptoethanol (buffer G) containing 1.5M NaCl and loaded onto an H/R 5/10 column equilibrated in buffer G containing 1.5M NaCl. The PKC was eluted with a continuous gradient from buffer G containing 1.5M NaCl to buffer G. The PKC eluted as a single, symmetrical peak (Figure 4.2.9). The recovery was about 80%. On the basis of these results, the Pro-RPC column did not appear to have separating potential.

Superose 12 column This column was also developed by Pharmacia for use with the FPLC systems and is a high resolution gel filtration column This column was chosen because the purified PKC had been shown to migrate as a doublet on SDS-polyacrylamide gels, suggesting that the isotypes may be different sizes (section 4.1.2). PKC in Buffer D (0.2-0.5ml) was loaded onto a Superose 12 column equilibrated in Buffer G (see ProRPC column). The PKC eluted as a single peak with a trailing shoulder (Figure 4.2.10). Inclusion of NaCl and Triton X-100 in the buffers and using two superose 12 columns in tandem to increase resolution did not improve the separation of the isotypes on the column.

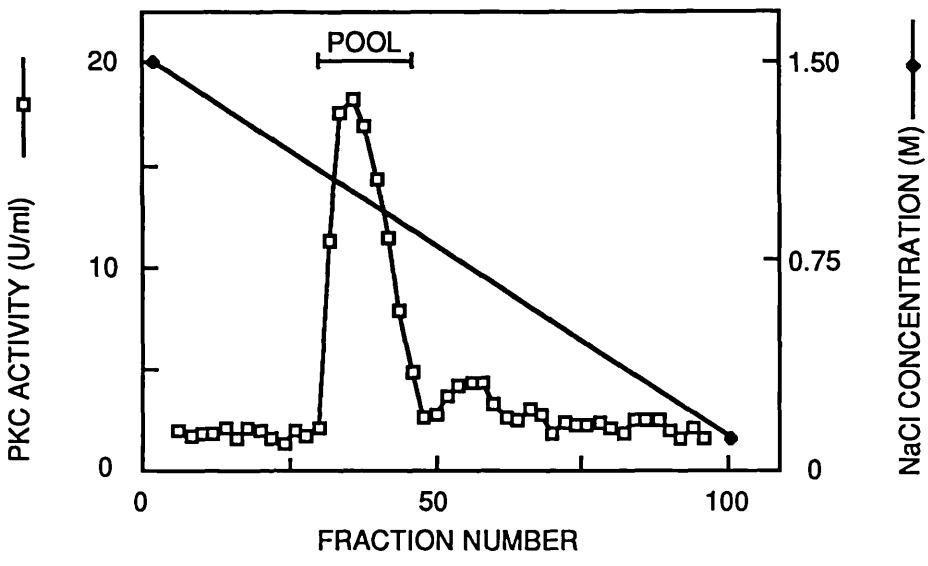


Figure 4.2.9 RPC-PRO column chromatography
The column was equilibrated in 20mM Tris.HCl pH 7.5, 2mM EDTA, 0.3% β-mercaptoethanol, 1.5NaCl. The PKC was eluted with a reverse gradient from 1.5 to 0 M NaCl (♦-♦) and 1ml fractions were collected. The PKC activity (□—□) in every second fraction was determined and PKC eluted as a single symmetrical peak. The recovery from this column was > 80%.

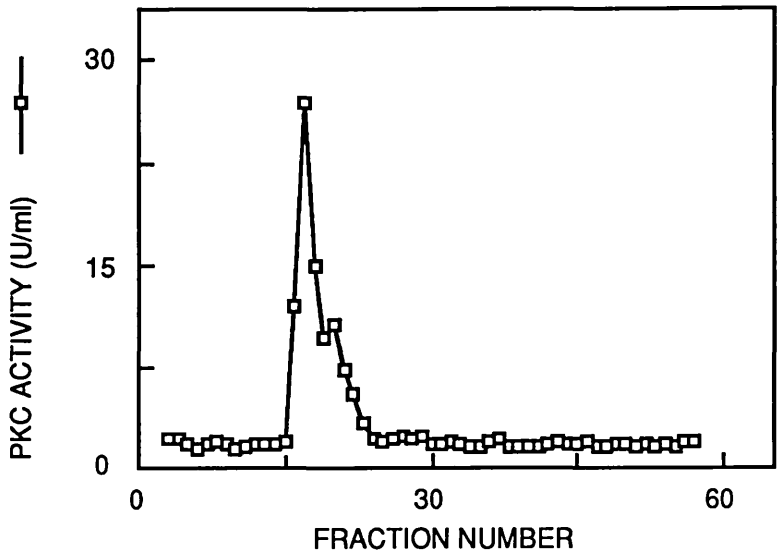


Figure 4.2.10 Gel filtration chromatography on Superose 12
The column was equilibrated in 20mM Tris.HCl pH 7.5, 2mM EDTA, 0.3% β-mercaptoethanol, 0.02% Triton X-100. PKC in buffer D was loaded directly onto the column and eluted in equilibration buffer; 0.5ml fractions were collected and the PKC activity (□—□) in these was determined. The PKC eluted as a single peak, with a trailing edge.

PS-affinity column As described in section 4.1.1., the isotypes could be separated on occasions by PtdSer-acrylamide columns. This separation was not reliable, partially due to the complex nature of the S-200 pool. The thr-Sepharose purified PKC was however a more highly purified sample which could be reproducibly produced. This was therefore chromatographed on a PtdSer-acrylamide under the conditions described in section 4.1.1. PKC in buffer D (0.2ml) was mixed with an 4 volumns of 10mM MES pH6.5, 200mM NaCl, 4μg/ml Leupeptin, 0.03% β-mercaptoethanol (buffer C), containing 5mM Ca^{++} and loaded onto a PtdSer-acrylamide column (1cm x 2cm, 0.5ml/min). The column was washed with 2ml buffer C containing 5mM Ca^{++}, followed by 2ml buffer C containing 0.1mM Ca^{++}. The PKC was eluted with 20ml buffer C containing 10mM EGTA. Two peaks of PKC activity were detected; the first in the flow-through and the second was eluted by the EGTA (Figure 4.2.11). Immuno-blotting analysis revealed that PKC-α and -β_1 were found in both fractions, while PKC-γ and -β_2 were detected only in the flow-through fraction. Increasing the column size did not improve the binding of PKC to the column, indicating that the column had not been overloaded. The results are not consistent with the results from section 4.1.1. where -α and -γ were shown to bind the column in preference to PKC-β. This illustrates that contaminating proteins can affect the chromatographic behaviour and the PtdSer binding properties of the PKC isotypes. None of the columns so far discussed were able to separate completely the isotypes from each other. Successful separation was finally achieved by hydroxylapatite chromatography as described below.

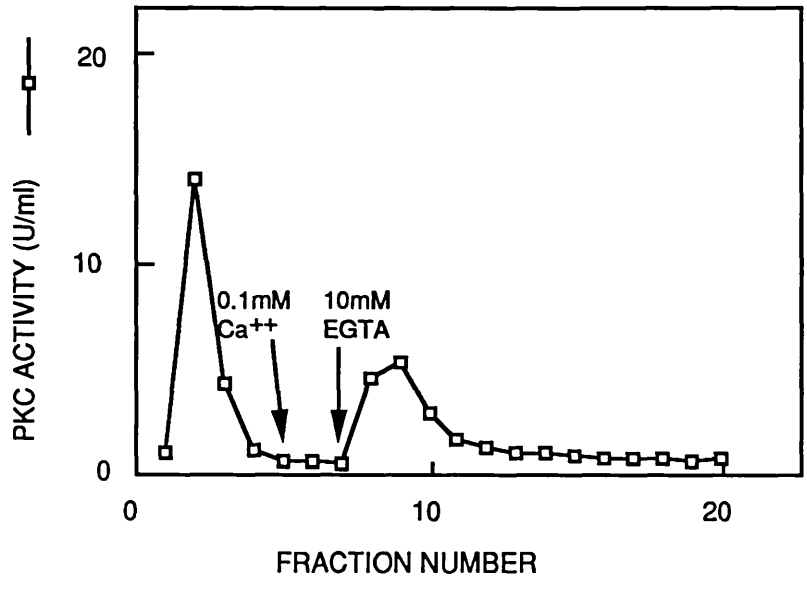


Figure 4.2.11 PtdSer-acrylamide affinity chromatography
The column (prepared as described in section 2.6; 4mm x 10mm; 0.25ml/min) was equilibrated in 10mM Mes pH 6.5, 200mM NaCl, 4µg/ml leupeptin, 0.3% β-mercaptoethanol (buffer G) containing 5mM Ca^{++}. The PKC sample was mixed with four volumes of equilibration buffer and loaded onto the column. The column was washed with 5ml equilibration buffer and then 2ml buffer G containing 0.1mM Ca^{++}. The PKC was eluted with 20ml buffer G containing 10mM EGTA; 1ml fractions were collected. The PKC activity (□—□) in the fractions was determined and two peaks of activity were detected. The first was associated with the flow through and the second was eluted by EGTA.

4.2.3. Separation of PKC-α, -β_1, -β_2 and -γ by Hydroxylapatite

While the various columns were being analysed for their abilities to separate the PKC isotypes Huang *et al* (1986) published a chromatographic method based on hydroxylapatite that was able to separate partially purified rodent brain PKC into three peaks of activity. The peaks were all dependent on Ca^{++}/Ptdser for activity and were shown to be immunologically distinct from each other (Huang *et al*, 1986, 1987a). Kikkawa *et al* (1987) subsequently demonstrated that the individual isotypes (expressed in COS cells) eluted in characteristic positions from hydroxylapatite columns and that the order of elution was PKC-γ (type I), PKC-$\beta_{(1+2)}$ (type II) and PKC-α (type III). They further demonstrated that the positions of elution corresponded to three peaks of activity seen when rat brain PKC was chromatographed under similar conditions. Huang *et al* (1987b) used immunological evidence to show that the three peaks of activity represented PKC-γ, -$\beta_{(1+2)}$ and -α in order of elution. They were however unable to determine the level of cross-contamination (if any) between the fractions, or whether there were other Ca^{++}/PtdSer dependent histone kinases in their fractions apart from PKC-α, -$\beta_{(1+2)}$ and -γ.

The observations of these two groups lead to the development of a separation protocol for the bovine brain PKC isotypes using hydroxylapatite chromatography. The monospecific antisera were used to identify the isotypes present in the three fractions thus produced. The results show that while it is possible to generate pools of separated PKC isotypes, care has to be exercised to ensure that separation is complete. The final purification protocol was a modified version of the method of Huang *et al* (1986) and was designed to give complete separation of the -α, -β_1 and -γ isotypes.

The monospecific antisera were invaluable for indicating the levels of cross-contamination in the three fractions during the development of the separation protocol.

Separation of PKC-α, -β_1 and -γ A sample (5mg) of PKC (as purified in section 4.1.2.) in buffer D (12ml, section 4.1.1) was mixed with an equal volume of 20 mM potassium phosphate pH 7.5, 0.5 mM EDTA 0.5 mM EGTA, 0.067% (v/v) β-mercaptoethanol, 10% (v/v) glycerol (buffer H). The PKC was loaded onto an hydroxylapatite column (1cm x 15cm; 0.17 ml/min) equilibated in buffer H, which was linked to a FPLC system (Pharmacia). The column was washed with 20 ml buffer H and the PKC was eluted with a discontinious gradient from buffer H to buffer H containing 280 mM potassium phosphate pH 7.5 (Figure 4.2.12). Fractions (2 ml) were collected and assayed for PKC activity. Three peaks of activity were detected under these conditions and these were individually pooled. Triton X-100 was added to the pools to a final concentration of 0.02% and the pools were dialysed against buffer D (section 4.1.1) before being stored at -20°C.

The three pools were all dependent on Ca^{++}/PtdSer and TPA (in the mixed micelle assay) for activity and no activity was detected in the absence of these activators. Immuno-blot analysis revealed that the peaks contained PKC-γ, PKC-β_1 and PKC-α in order of elution {Figure 4.2.13(a)}. The fractions were judged to be pure and to have different mobilities when analysed by silver stained SDS-PAGE {Figure 4.2.13(b)}. The apparent relative molecular masses (Mr) were: PKC-α = 80,900; PKC-β_1= 79,100 and PKC-γ ran as a smear with an apparent mass between 79,400 to 80,400. These masses are in contrast to the predicted masses of 76,800 for PKC-α; 76,900 for PKC-β_1 and

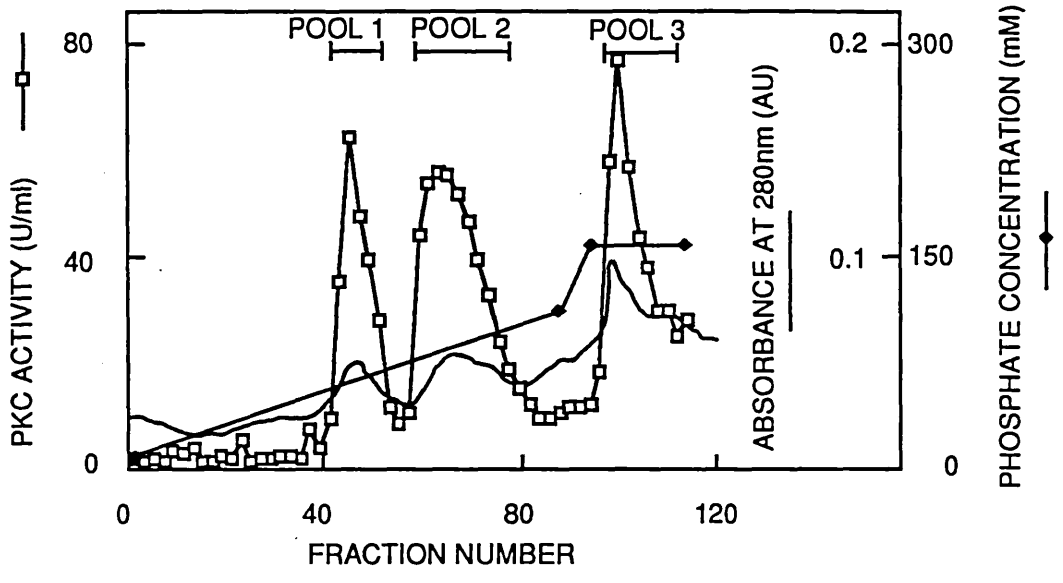


Figure 4.2.12 Hydroxylapatite column chromatography
The column was equilibrated in 20mM potassium phosphate pH 7.5, 0.5mM EDTA, 0.5mM EGTA, 0.067% v/v β-mercaptoethanol, 10% v/v glycerol (buffer H). The PKC sample was mixed with an equal volume of buffer H and loaded onto the column. After washing with 20 ml buffer H, the PKC was eluted with the discontinious potassium phosphate gradient shown (♦-♦) and 2ml fractions were collected. The Ca^{++}/PtdSer dependent kinase activity was determined (□—□) and three peaks of activity were detected which were associated with three peaks of OD_{280} absorbance (———). These were pooled as indicated by the bars.

Figure 4.2.13(a) Immuno-blot analysis of PKC

Pools 1, 2 and 3 from the hydroxylapatite column (Figure 4.2.12) were analysed by immuno-blotting with the monospecific antisera. 1μg of PKC was loaded per lane onto 10% w/v acrylamide gels. Lane 1= hydroxylapatite pool 1; lane 2= hydroxylapatite pool 2; lane 3= hydroxylapatite pool 3. The position of PKC is indicated by the arrow and the positions of migration of 'Rainbow' molecular mass markers is also indicated ($x10^{-3}$). The antibodies were used at a dilution of 1 in 1,000 and the blots for $V_5\alpha$, $V_5\beta_1$ and $V_5\gamma$ were exposed to the autoradiographic film for 48 hr. The $V_5\beta_2$ blot was exposed for 28 days.

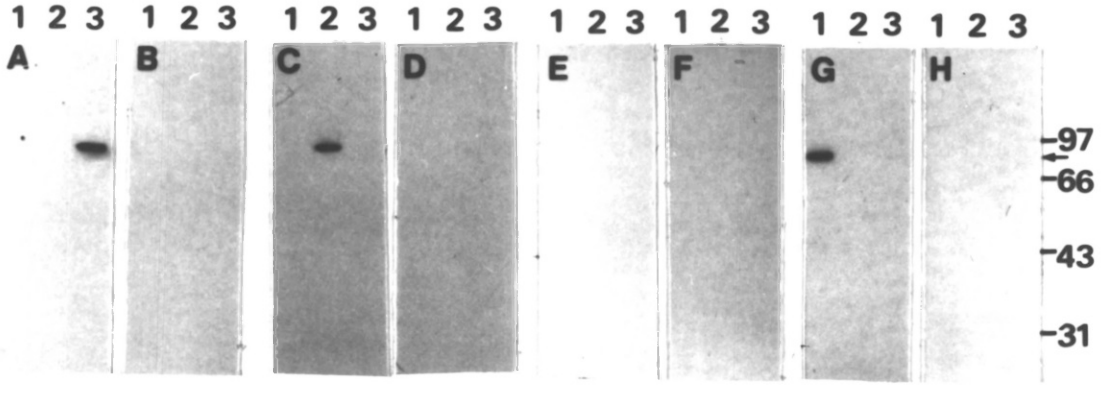
Antisera used:

panel A= $V_5\alpha$; panel B= $V_5\alpha$ with competing peptide,
panel C= $V_5\beta_1$; panel D= $V_5\beta_1$ with competing peptide,
panel E= $V_5\beta_2$; panel F= $V_5\beta_2$ with competing peptide,
panel G= $V_5\gamma$; panel H= $V_5\gamma$ with competing peptide.

Figure 4.2.13(b) SDS-PAGE analysis of PKC isotypes

Samples of the three pools from the hydroxylapatite column were analysed by SDS-PAGE on 12.5% w/v acrylamide gels. 1μg of PKC activity was loaded onto lane 1 and 0.4μg onto lanes 2, 3 and 4. The proteins were detected by silver staining. The position of PKC is indicated by the arrow and the positions of migration of standard molecular mass proteins are indicated. Lane 1= threonine-Sepharose pool; lane 2= PKC-α (hydroxylapatite pool 3); lane 3= PKB-β_1 (hydroxylapatite pool 2); lane 4= PKC-γ (hydroxylapatite pool 1).

(a)


1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
A
B
C
D
E
F
G
H
97
66
43
31

(b)


1 2 3 4
97
66
43
31

78,400 for PKC-γ (Parker *et al*, 1986; Coussens *et al*, 1986). In part the differences in the apparent and the predicted masses are due to differential phosphorylation as determined by the susceptability of the isotypes to potato acid phosphatase, which decreased the Mr of all the isotypes by 2,000-3,000. The pools were stable at -20°C for up to nine months with neither decrease in kinase activity nor co-factor dependence being detected. After that time, there was a gradual loss in co-factor dependence but not kinase activity, resulting in constitutively active kinase. This loss of activity was not due to proteolysis (their mobilities on SDS-polyacrylamide gels was unaltered) but appeared to be due to selective denaturation of the regulatory domain. The recovery of PKC from the hydroxylapatite column was in the region of 30-50% and the concentration of the pools was in the region of 100μg/ml (Table 4.2.2). This recovery is slightly better than that of Jaken and Kiley (1987).

In order to establish if any of the isotype pools contained contaminating histone kinase activity, the PKC from each was immuno-precipitated with the V5 region antisera for -α, -β_1, -β_2 and -γ. The amount of kinase remaining in the supernatant was determined. The results are shown in Table 4.2.3 and show that none of the peaks contained histone kinases other than those detected by the immuno-blotting. The kinase activity and the biochemical data can therefore be attributed to the specific PKC isotype in each pool. The -β_2 isotype was not detected in any of the pools either by immuno-blottting (Figure 4.2.13) or by immuno-depletion (Table 4.2.3). The limits of detection for immuno-blotting with the $V_5\beta_2$ antiserum in this system are in the region of 10 to 50ng/ml. It was therefore concluded that the -β_2 isotype was not present in any of the hydroxylapatite pools. This isotype is present in the threonine-

Table 4.2.2 Separation of PKC isotypes by hydroxylapatite chromatography

Fraction	Volume	Protein	Specific activity	Total activity	recovery
	ml	μg/ml	U/mg	units	%
Thr-Seph. pool	12	420	1,720	8,650	100
HPHT pool 1 (γ)	6	105	1,540	970	11
HPHT pool 2 (β_1)	11	87	890	850	10
HPHT pool 3 (α)	6	120	1,270	910	10

Details of the separation of purified PKC by hydroxylapatite column chromatography. The recovery is expressed in terms of the activity recovered, as opposed to the protein. HPHT= hydroxylapatite. The results are the characteristics of the pools after dialysis into 20mM Tris.HCl pH7.5, 2mM EDTA, 1mM DTT, 50% glycerol.

Table 4.2.3 Immuno-depletion of PKC from the hydroxylapatite pools

Antibody	% Enzyme activity remaining in supernatant		
	pool 1	pool 2	pool 3
$V_5\alpha$	103	110	5
$V_5\beta_1$	90	1	96
$V_5\beta_2$	107	100	103
$V_5\gamma$	7	108	95

Samples from pools 1, 2 and 3 of the hydroxylapatite column were immuno-depleted for PKC with the V5 region monospecific antisera. 20 units of PKC in 20mM Tris.HCl pH 7.5, 2mM EDTA, 0.3% β-mercaptoethanol, 4μg/ml leupeptin, 10μg/ml aprotanin (50μl) was mixed with 10μl antiserum (± 10μl peptide or PBS, see below). The mixture was incubated with tumbling for 6 hr (4°C). 30μl of a 1:1 slurry of protein A-Sepharose: PBS was added to each tube and the mixture was incubated for a further 2 hr (4°C) with tumbling. The protein A-Sepharose beads were removed by centrifugation and the amount of kinase activity remaining in the supernatant was determined. The results are expressed as the amount of activity remaining compared to controls in which the antisera were preincubated with 5mM competing peptide (PBS in the case of non-controls).

Sepharose pool in the lowest concentration and its recovery from the hydroxylapatite column appears to be poor. This observation is consistent with the results from Shearman *et al* (1987).

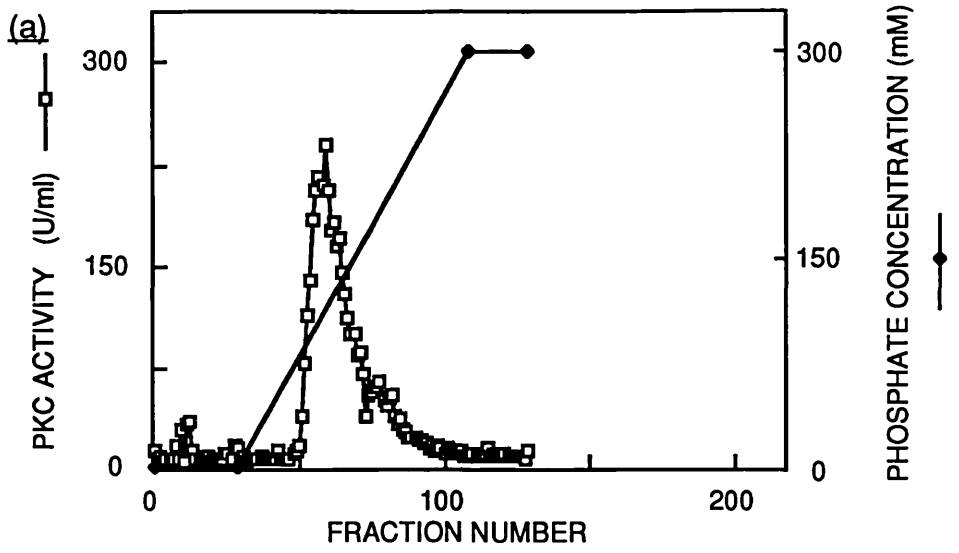
The PKC isotype fractions produced by this protocol were ideally suited for the biochemical analysis for which they were intended. They were homogenous as determined by SDS-PAGE and there was no cross-contamination between the pools. The fractions were also free from contaminating kinases and substrates. Finally, the kinase concentration was high and the pools could be produced in plentiful amounts which were stable and could therefore be used for many months.

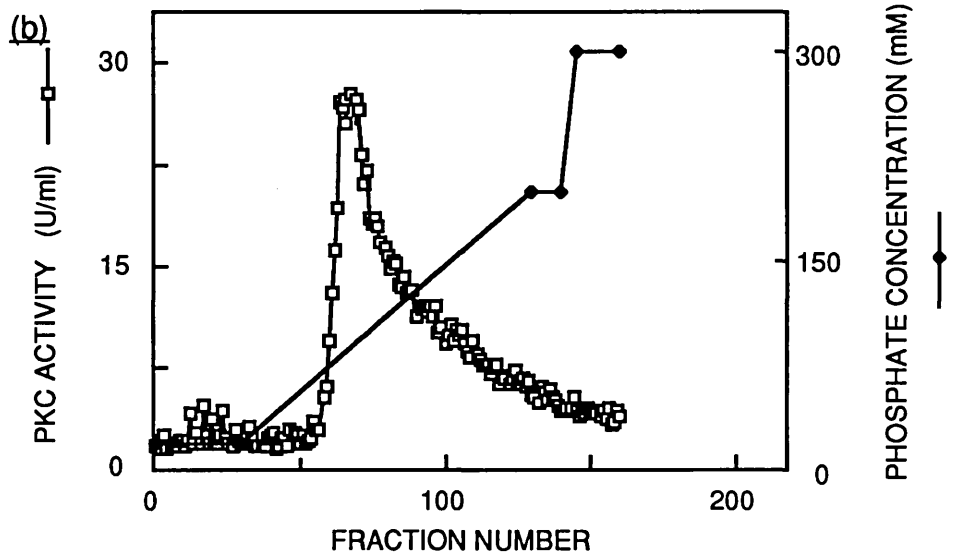
Separation of the isotypes on the hydroxylapatite column was dependent on the grade of column matrix used and could only be achieved with DNA grade hydroxylapatite (Bio-Rad). When lesser grades were used, the PKC eluted as a single peak of activity {Figure 4.2.14(a)} which contained PKC-α, -β and -γ. Shallower gradients lead to broader peaks of activity but did not improve separation {Figure 4.2.14(b)}. The use of discontinious gradients such as the one shown in Figure 4.2.14(c) which were designed to elute selectively each isotype in turn did not overcome the problem of isotype co-elution. With DNA grade hydroxylapatite by contrast, complete separation could be achieved with continious gradients. However, gradients designed to give baseline separation of -γ and -β caused -α to elute as a broad peak at low protein concentrations {Figure 4.2.15(a)}. Shorter gradients resulted in two peaks of activity, with -β and -γ co-eluting in the first peak and the -α eluting in the second {Figure 4.2.15(b)}. Complete separation was achieved with discontinious gradients as shown in Figure 4.2.12. This gradient was

Figure 4.2.14(a) Hydroxylapatite column chromatography
The column (1cm x 15cm) was equilibrated in 20mM potassium phosphate pH 7.5, 0.5mM EDTA, 0.5mM EGTA, 0.067%w/v β-mercaptoethanol, 10% v/v glycerol (buffer H). The flow rate was 0.17ml/min and the column matrix was ordinary grade hydroxylapatite (Bio-Gel HTP; BioRad). The PKC sample was mixed with an equal volume of buffer H and loaded onto the column. The PKC was eluted with a gradient from 20mM to 300mM potassium phosphate (♦-♦). 2ml fractions were collected. The PKC activity (□—□) in every fraction was determined and the PKC eluted in a single major peak, followed by a second minor peak.

Figure 4.2.14(b) Hydroxylapatite column chromatography
The column conditions were as for Figure 4.2.14(a) but a less steep gradient was used to elute the PKC. Under these conditions, a single peak of activity was seen as in Figure 4.2.14(a). The peak of activity was more broad and no separation of the isotypes was seen.

Figure 4.2.14(c) Hydroxylapatite column chromatography
The column conditions were as for Figure 4.2.14(a) but the PKC was eluted with the discontinious gradient shown (♦-♦) and the PKC activity (□—□) in every second fraction was determined. The gradient was designed to elute selectively the -γ, -β and -α isotypes in turn. Three peaks of activity were generated but subsequent immuno-blot analysis indicated that these did not contain the separated isotypes. Each isotype was detected in each of the three peaks, although their relative concentrations were different.


(a)
PKC ACTIVITY (U/ml)
300
150
0
0
100
200
FRACTION NUMBER
300
150
0
PHOSPHATE CONCENTRATION (mM)


(b)
PKC ACTIVITY (U/ml)
30
15
0
0
100
200
FRACTION NUMBER
300
150
0
PHOSPHATE CONCENTRATION (mM)

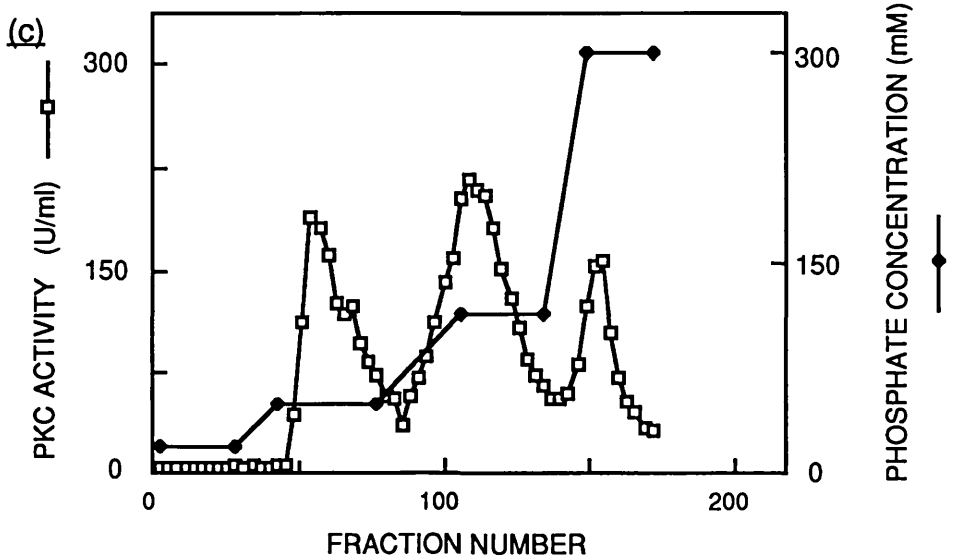
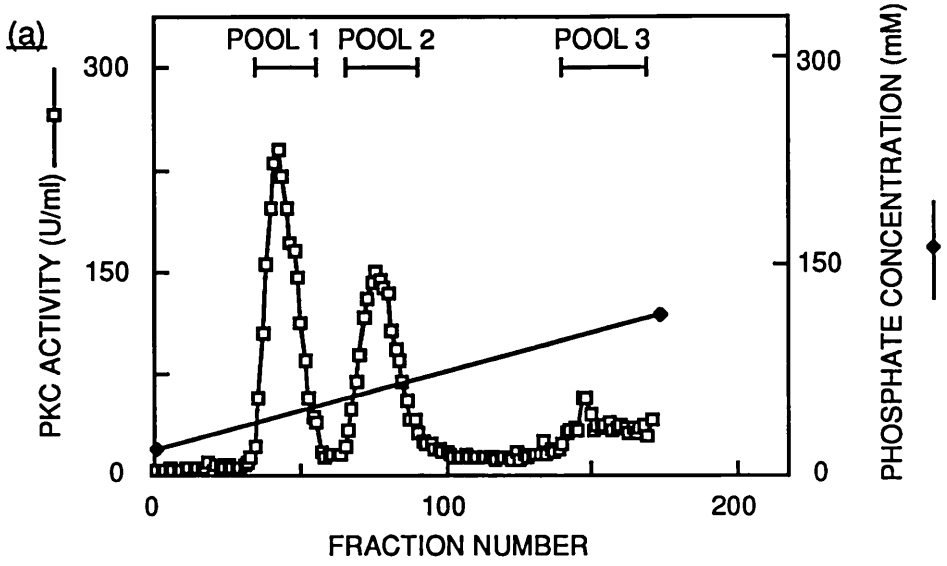
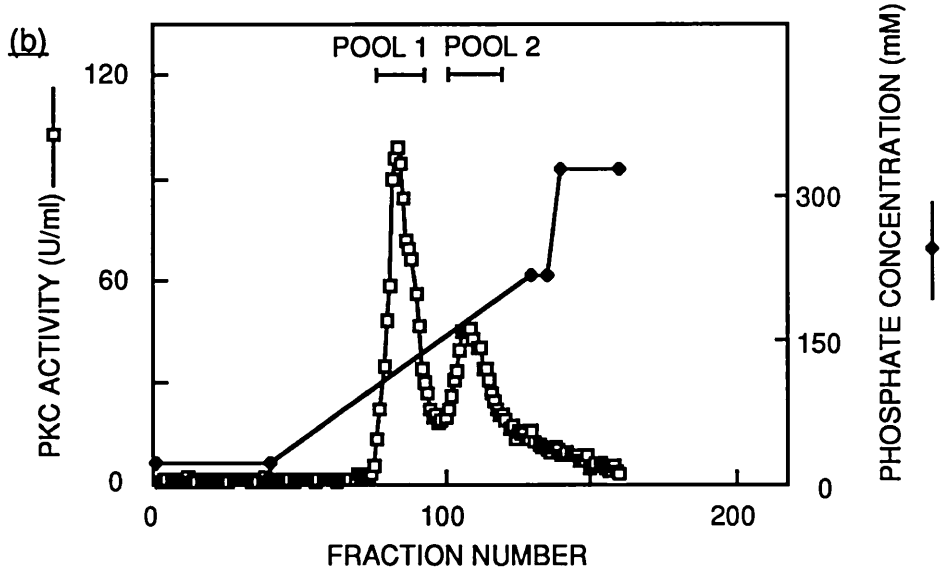

(c)
PKC ACTIVITY (U/ml)
300
150
0
0
100
200
FRACTION NUMBER
300
150
0
PHOSPHATE CONCENTRATION (mM)

Figure 4.2.15(a) Hydroxylapatite column chromatography
The column (made with DNA grade hydroxylapatite) was equilibrated in buffer H (see Figure 4.1.14). The PKC was mixed with an equal volume of equilibration buffer and loaded onto the column at a flow rate of 0.17 ml/min. The column was washed with 20ml buffer H and the PKC eluted with the gradient shown (♦-♦). 2ml fractions were collected and the activity (□—□) in every second fraction was determined. Three peaks of activity were detected although the third contained very low levels of PKC activity. Three pools were collected as shown by the bars. Immuno-blot analysis indicated that the first contained PKC-γ, the second PKC-β_1 and the third PKC-α.

Figure 4.2.15(b) Hydroxylapatite column chromatography
The conditions were as for Figure 4.2.15(a) but the PKC was eluted with a steeper gradient as shown (♦-♦). The PKC activity (□—□) in every second fraction was determined and two peaks of activity were detected. Two pools were collected as shown by the bars. Immuno-blot analysis revealed that pool 1 contained PKC-γ and -β, and pool 2 PKC-α.


(a)
POOL 1
POOL 2
POOL 3
300
150
0
PKC ACTIVITY (U/ml)
0
100
200
FRACTION NUMBER
300
150
0
PHOSPHATE CONCENTRATION (mM)


(b)
POOL 1
POOL 2
120
60
0
PKC ACTIVITY (U/ml)
0
100
200
FRACTION NUMBER
300
150
0
PHOSPHATE CONCENTRATION (mM)

designed to elute the -α isotype at concentrations similar to those at which the -β and -γ isotypes were eluted. This was achieved by a sharp increase in the gradient after the -β_1 isotype had eluted.

Attempts were also made to develop a separation protocol using an HPLC hydroxylapatite column (Bio-Rad). This column gave numerous peaks of activity, but the capacity of the column was low and therefore not enough material could be generated to yield reliable immuno-blotting data.

The use of different chromatographic conditions indicated that while it was possible to generate multiple peaks of activity from hydroxylapatite columns, these often consisted of mixtures of isotypes. If the column was overloaded, there was considerable cross-contamination between the pools although three peaks of activity could be identified (Figure 4.2.16). It is therefore not enough to simply assume (as did Jaken and Kiley, 1987 and Pelosin *et al*, 1987) that if three peaks of PKC activity are generated from hydroxylapatite columns, these represent the separated isotypes. Indeed, SDS-PAGE analysis of the pools collected by Jaken and Kiley (1987) shows that there are doublet bands in at least two of their pools. This suggests that their isotype samples were not efficiently separated. Hydroxylapatite columns have been used in this way to identify the PKC isotypes present in various tissues in the absence of other evidence (Pelosin *et al*, 1987; Shearman *et al*, 1987). The results from their studies may be misleading because extensive studies have not been done to show that the isotypes from different tissues or in different states of purity behave in the same way on hydroxylapatite columns. Further to this, if the -γ and -β isotype peaks are not baseline separated, the pools thus generated have been

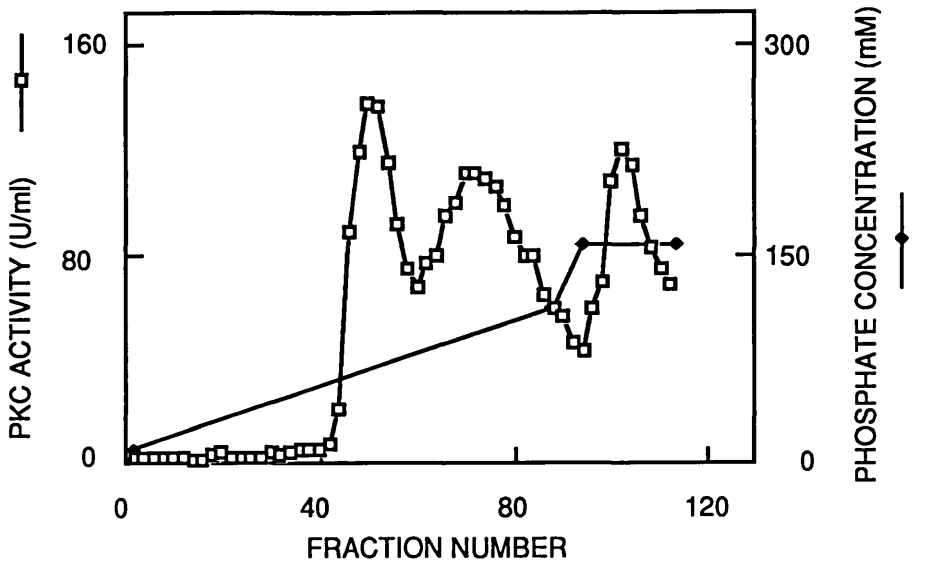


Figure 4.2.16 Hydroxylapatite column chromatography
The column was equilibrated in 20mM potassium phosphate pH 7.5, 0.5mM EDTA, 0.5mM EGTA, 0.067% v/v β-mercaptoethanol, 10% v/v glycerol (buffer H). 9mg PKC in buffer D (22ml) was mixed with an equal volume of buffer H and loaded onto the column. The column was washed with 20 ml buffer H and the PKC was eluted with the discontinious gradient shown (♦-♦). 2ml fractions were collected and the Ca^{++}/PtdSer dependent kinase activity (□—□) in these was determined. Three peaks of activity were detected which were pooled as indicated by the bars. Immuno-blot analysis of these indicated that pool 1 contained predominantly PKC-γ, pool 2 predominantly PKC-β_1 and pool 3 predominantly PKC-α. However, there was significant cross-contamination between the pools, separation being far from complete.

shown to contain significant amounts of cross-contamination with other isotypes. This may affect their individual biochemical properties. The monospecific antisera were therefore invaluable in these studies since they gave a direct indication of the efficiencies of the various separation protocols.

CHAPTER FIVE

BIOCHEMICAL ANALYSIS OF PKC -α, -β_1, AND -γ

This chapter describes the biochemical properties of the -α, -β_1 and -γ isotypes of PKC. The dependence of the isotypes on activating co-factors and their substrate specificities (using both protein and peptide substrates) were analysed. For the most part, the analyses were carried out in an assay system using PS presented in mixed micelles modified from Hannun *et al* (1985). Although mixed micelles are unlikely to be any more physiologically relevent than pure lipid micelles, the former were chosen because the activity of PKC in them is absolutely dependent on DG (Hannun *et al*, 1986) or TPA (Hannun and Bell, 1986). By contrast, with pure lipid micelles PKC can be activated at high concentrations of Ca^{++} in the absence of TPA or DG (Takai *et al*, 1979b). Thus, the dependence of PKC on Ca^{++}, DG and TPA can be more easily assessed with mixed micelles. Further, mixed micelles have the advantage of giving defined lipid vesicles in which the concentrations of the components can be expressed more exactly (Boni and Rando, 1985; Hannun *et al*, 1986). For most practical purposes, the activators and substrates were used at 5-10 times their K_m, K_a or $A_{0.5}$ concentrations to give optimal activation. Histone III-S was however an exception to this, since high concentrations caused inhibition of activity, apparently due to histone-induced aggregation of the lipid micelles. The assays were incubated for three minutes during which time phosphate incorporation was linear (Figure 5.1.1). Each data point was assayed twice in triplicate; the data shown constitute one such set of data for each parameter tested.

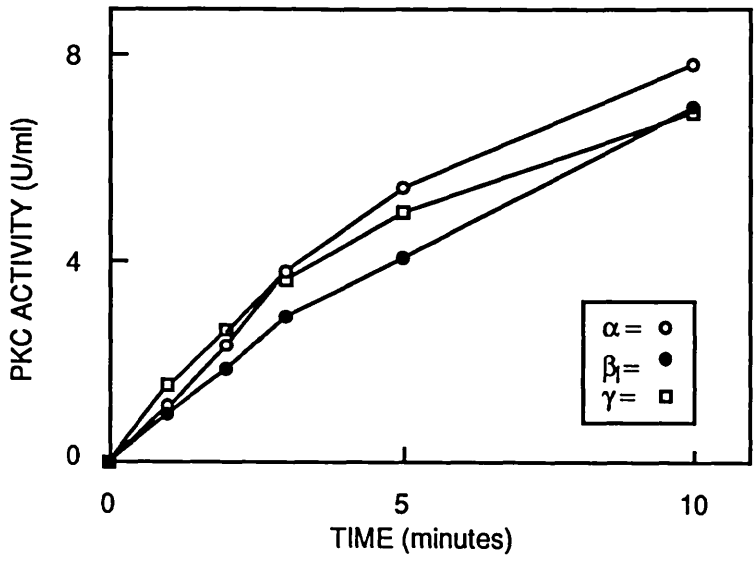


Figure 5.1.1 Incorporation of phosphate into histone III-S
The rate of phosphate incorporation into histone III-S by each of the PKC isotypes was measured in the standard assay for various times. The incorporation of phosphate under these conditions is linear for the first three minutes for each of the isotypes. The results are corrected for the incorporation of phosphate into histone III-S in the absence of PtdSer and TPA.

The results from these studies are expressed in terms of Michealis-Menton kinetics, by defining the V_{max} and K_m values for substrates and the K_a or $A_{0.5}$ values for activators. The V_{max} was defined as the maximal rate of incorporation of phosphate into substrate under optimal conditions. The K_m was defined as the concentration of substrate at which half V_{max} was achieved. The V_{max} gives an indication (but not a quantitativevalue) of the catalytic rate of the active site of the enzyme. The K_m gives an indication of the affinity of the active site for substrates. The K_a and $A_{0.5}$ values were defined as the concentration of activator required to give half maximal kinase activation (half V_{max}). The K_a and $A_{0.5}$ values are therefore only correct under the assumption that binding of co-factors leads to the complete activation of the enzyme. The K_m values were calculated from linear regression plots of the data using the programme 'Enzfitter' (Elselvier-Biosoft, England) with simple weighting parameters.

5.1. Activation kinetics of PKC -α, -β_1, and -γ

The dependence of each isotype on activating co-factors was defined *in vitro*. Thus the dependence of the isotypes on Ca^{++}, Mg^{++}, ATP, PtdSer, TPA and three types of diacylglycerol was defined. The results (table 5.1.1) show that while the three isotypes are similar in their dependence on Mg^{++}, ATP and PS, they differ in their dependencies on TPA, diacylglycerols and Ca^{++}. For the purposes of clarity, Mg^{++} and ATP (which can be considered to be substrates rather than activators) have been included in this section to distinguish them from protein and peptide substrates.

5.1.1. Dependence on magnesium, ATP and PtdSer The apparent K_m values of the three PKC isotypes for Mg^{++} were all between 1.5mM

Table 5.1.1 Summary of effector kinetics of PKC-α, -β_1 and -γ

	Mg^{++} K_m mM	ATP K_m µM	PtdSer $A_{0.5}$ mol%	Diolein $A_{0.5}$ mol%	diC_6 $A_{0.5}$ mol%	diC_8 $A_{0.5}$ mol%	TPA $A_{0.5}$ mol%X10^{-3}	Ca^{++} K_a nM
PKC-α	2.38±0.63	24.3±6.1	15.5±4.6	0.19±0.07	0.98±0.23	1.07±0.31	3.10±0.59	223±57
PKC-β_1	1.57±0.65	37.2±6.0	13.6±4.4	0.58±0.14	5.28±0.93	2.14±0.04	5.08±0.71	202±63
PKC-γ	1.96±0.25	36.0±7.6	12.6 ±5.6	0.59±0.16	5.37±0.78	2.18±0.35	13.3±2.5	116±28

The dependence of the three PKC isotypes for the effectors described was assayed using histone III-S as substrate. The K_m, K_a and $A_{0.5}$ values were calculated using the programme 'Enzfitter' with simple weighting parameters. For each parameter, two analyses were performed, each point being assayed in triplicate. The data represents one such set of analyses. The kinase in each assay was diluted to give a final concentration of 1 unit of activity per ml in the reaction mixture.

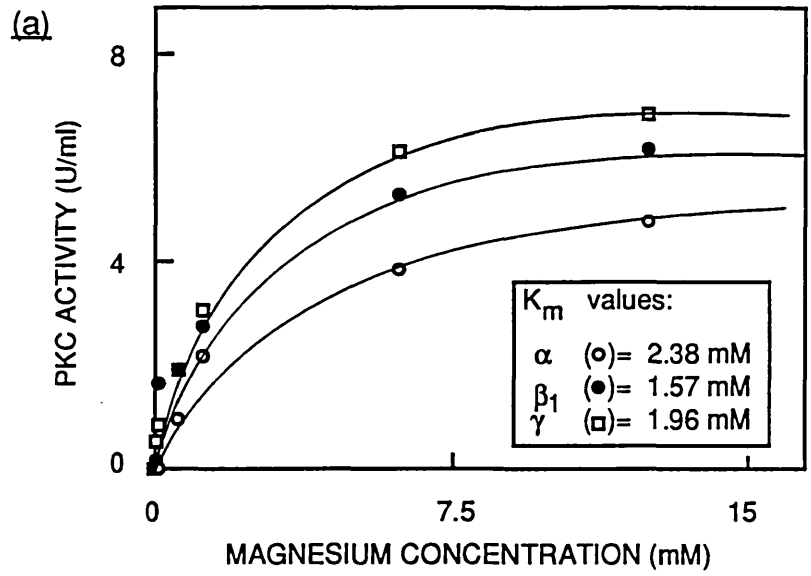
and 2.4mM {Figure 5.1.2(a)} and for ATP between 24μM and 38μM {Figure 5.1.2(b)}. The $A_{0.5}$ of the three isotypes for PtdSer were between 12 and 16 mol% {figure 5.1.2(c)}. These values for PtdSer are different from those found by Hannun and Bell (1986) who found that maximal activity was reached at 20 mol%. They did state however that the PtdSer dependence was affected by the concentration of TPA in the micelles and the results cannot therefore be compared directly. Huang *et al* (1988) also found that maximal activation of the PKC isotypes could be achieved at concentrations lower than those found in this study. However, they were using PBu_2 as an activator and therefore the two sets of results are not directly comparable.

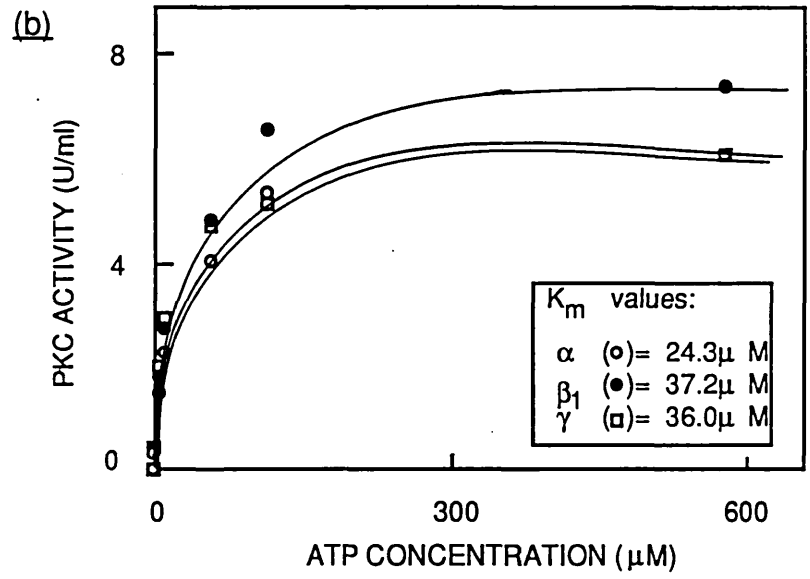
The similarity in the dependencies of the isotypes for PtdSer, Mg^{++} and ATP suggests that intracellular fluctuations in the concentrations of these compounds are unlikely to be used as physiological controls to activate differentially the three PKC isotypes. It is interesting to note that the K_m for Mg^{++} is about 100 fold higher than the K_m for ATP for all the isotypes, even though these compounds probably bind to PKC as a Mg^{++}.ATP complex as has been shown with other kinases (Knowles, 1980). Differences in the requirements of kinases for Mg^{++} and ATP have been observed previously (Palczewski *et al*, 1988; Miyamoto *et al*, 1969; Braun *et al*, 1986). With cA kinase, a similar result has prompted Armstrong *et al* (1979) to postulate that this kinase has two Mg^{++} binding sites, one of which is of low affinity and is inhibitory, while the other is located at the nucleotide binding site. PKC may also contain two Mg^{++} binding sites and these possibilities are discussed in Chapter Six.

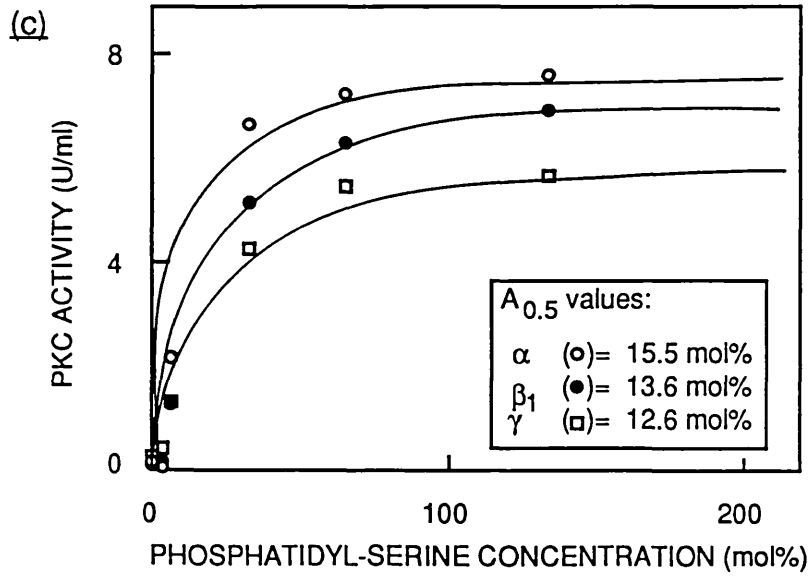
Figure 5.1.2(a) Magnesium dependence of the PKC isotypes
The protein kinase C activity was measured under the standard assay conditions at various concentrations of Mg^{++}. The activity shown is the activity in the presence of Ca^{++}/ PtdSer/ TPA compared to the activity in the absence of these activators. K_m values were calculated by the programme 'Enzfittter'.

Figure 5.1.2(b) ATP dependence of the PKC isotypes
The protein kinase C activity in the presence of various concentrations of ATP was measured. The conditions were as described in Section 2.8 and the Mg^{++} concentration was 12.5mM. The K_m values were calculated by the programme 'Enzfitter'.

Figure 5.1.2(c) The PtdSer dependence of the PKC isotypes
The effects of differing concentrations of PtdSer in the mixed micelle assay were determined. The micelles were prepared by evaporating to dryness the relevent amount of PtdSer with TPA and re-dissolving them back up in Triton X-100. In the absence of PtdSer, there was no detectable activity.


(a)
PKC ACTIVITY (U/ml)
8
4
0
0
7.5
15
MAGNESIUM CONCENTRATION (mM)
K_m values:
α (o)= 2.38 mM
β_1 (●)= 1.57 mM
γ (□)= 1.96 mM


(b)
PKC ACTIVITY (U/ml)
8
4
0
0
300
600
ATP CONCENTRATION (μM)
K_m values:
α (o)= 24.3μ M
β_1 (●)= 37.2μ M
γ (□)= 36.0μ M


(c)
PKC ACTIVITY (U/ml)
8
4
0
0
100
200
PHOSPHATIDYL-SERINE CONCENTRATION (mol%)
$A_{0.5}$ values:
α (o)= 15.5 mol%
β_1 (●)= 13.6 mol%
γ (□)= 12.6 mol%

5.1.2. Dependence on diacylglycerols Diacylglycerol (DG) is the natural second messenger activator of PKC and activates it by potentiating its activity at physiological Ca^{++} levels (see section 1.2.2). The $A_{0.5}$ of the three isotypes of PKC for the synthetic DG's diC_6, diC_8 and diolein were defined *in vitro*. DiC_6 and diC_8 are often used to stimulate PKC *in vivo* in preference to 'natural' or longer chain synthetic DG's because the shorter chained DG's can inter-chelate into cell membranes more efficiently than longer chained varieties (Davis *et al*, 1985a; Nishizuka, 1986).

The dependence of PKC-α, -β_1 and -γ on diC_6, diC_8 and diolein are shown in Table 5.1.1. PKC-α is more sensitive to these compounds than either -β_1 or -γ, as reflected in its lower $A_{0.5}$ values for these compounds. PKC-α had a 3 fold lower $A_{0.5}$ than -β_1 or -γ for diolein {Figure 5.1.3(a)}; a 5-6 fold lower $A_{0.5}$ for diC_6 {Figure 5.1.3(b)} and a 2 fold lower $A_{0.5}$ for diC_8 {Figure 5.1.3(c)}. PKC-β_1 and -γ had similar $A_{0.5}$ values to each other for all of these compounds. This consistently higher sensitivity of PKC-α over -β_1 and -γ suggests that the isotypes may be individually regulated by different DG species *in vivo*. It was observed that the -β_1 and -γ isotypes were more sensitive to diC_8 than diC_6 and had higher $A_{0.5}$ values for diC_6 than diC_8. The -α isotype by contrast had similar $A_{0.5}$ values for diC_6 and diC_8. Hannun *et al* (1986) observed a difference in activation characteristics for the diC_6 and diC_8 compounds, suggesting that their mixed isotype PKC sample may have contained predominantly -β and/ or -γ isotypes.

5.1.3. Dependence on TPA The activation of the PKC isotypes by TPA is shown in Table 5.1.1. PKC-α and -β_1 had $A_{0.5}$ values 3-4 fold lower than PKC-γ (Figure 5.1.4). This would suggest that at low

Figure 5.1.3(a) The effect of different concentrations of diolein on PKC activity

The PKC activity was measured in the standard assay in the absence of TPA at various concentrations of diolein. The $A_{0.5}$ values were defined as the concentration of diolein at which half maximal activity was achieved and were calculated with the programme 'Enzfitter'. In the absence of diolein, no activity could be detected. The assays were performed in the presence of 70mol% PtdSer and PKC at a final concentration of 1 unit/ml. The assays were incubated for 3 min.

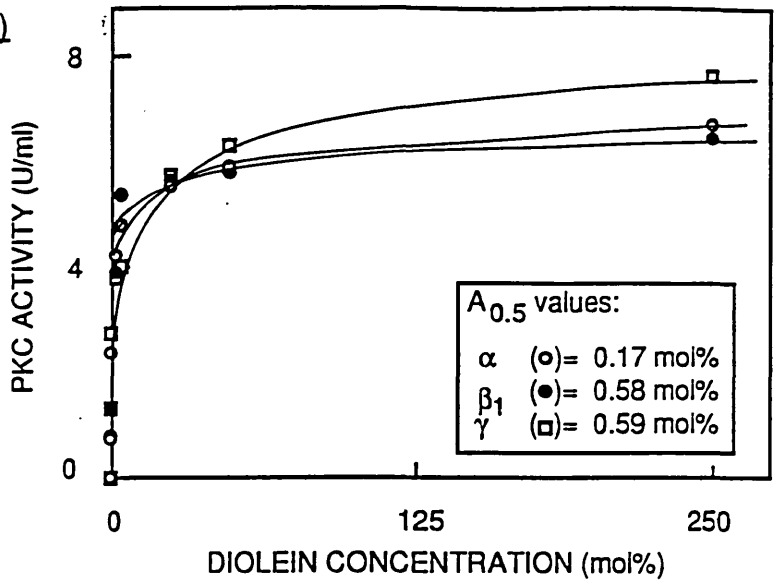
Figure 5.1.3(b) The effect of varying concentrations of diC_6 on the activity of the PKC isotypes

Short chain diacylglycerols are often used in cell studies to activate PKC. The dependence of the isotypes on diC_6 was determined in the absence of TPA. The $A_{0.5}$ value are defined as the concentration of diC_6 at which half maximal activity was achieved and were calculated with the programme 'Enzfitter'. In the absence of diC_6, no activity could be detected. The assay conditions were as for Figure 5.1.3(a) above.

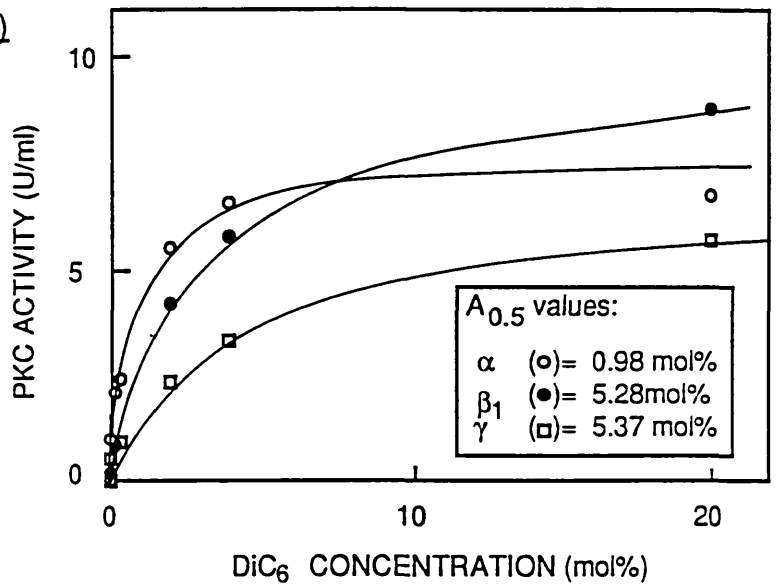
Figure 5.1.3(c) The effect of varying concentrations of diC_8 on the activity of the PKC isotypes

The activity of the PKC isotypes in the presence of varying concentrations of diC_8 was determined. The $A_{0.5}$ values were defined as the concentration of diC_8 at which half maximal activity was achieved and were calculated with the programme 'Enzfitter'. In the absence of diC_8, no activity could be detected. The assay conditions were as for Figure 5.1.3(a) above.

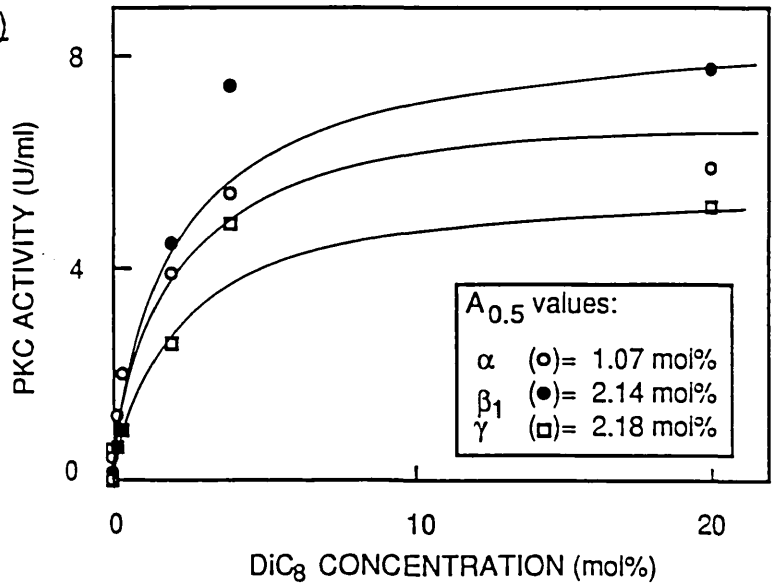
(a)


8
4
0
PKC ACTIVITY (U/ml)
$A_{0.5}$ values:
α (o)= 0.17 mol%
β_1 (●)= 0.58 mol%
γ (□)= 0.59 mol%
0
125
250
DIOLEIN CONCENTRATION (mol%)

(b)


10
5
0
PKC ACTIVITY (U/ml)
$A_{0.5}$ values:
α (o)= 0.98 mol%
β_1 (●)= 5.28mol%
γ (□)= 5.37 mol%
0
10
20
DiC_6 CONCENTRATION (mol%)

(c)


8
4
0
PKC ACTIVITY (U/ml)
$A_{0.5}$ values:
α (o)= 1.07 mol%
β_1 (●)= 2.14 mol%
γ (□)= 2.18 mol%
0
10
20
DiC_8 CONCENTRATION (mol%)

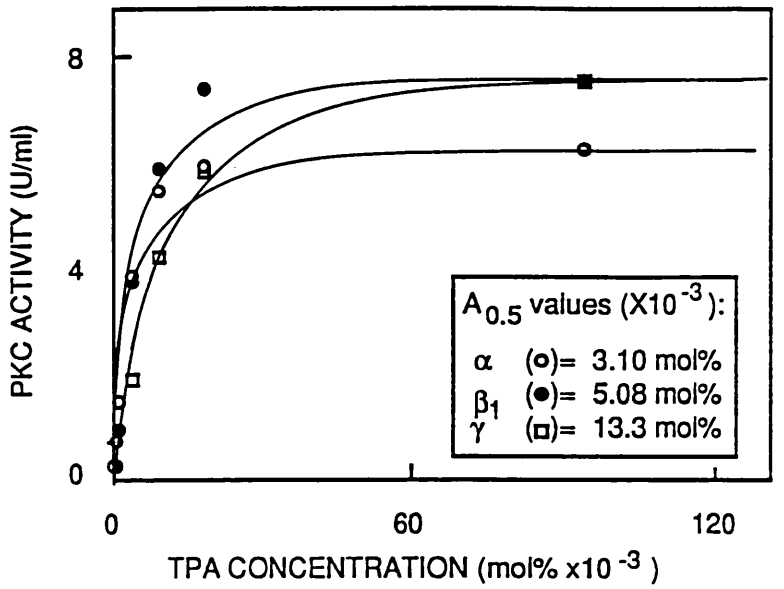


Figure 5.1.4 Dependence of the PKC isotypes on TPA
The effect of different concentrations of TPA on PKC isotype activity in the presence of 70 mol% was examined. The assays were performed under the standard conditions (section 2.8) and were incubated for 3 min. The final PKC concentration was 1 unit/ml. The $A_{0.5}$ values were calculated with the programme 'Enzfitter'.

concentrations of TPA *in vivo*, -α and -β_1 may be activated to a greater degree than -γ. A variety of phorbol esters have been shown to differ in their abilities to activate PKC (Castagna *et al*, 1982; Ashendel, 1985). It would be interesting to extend these observations to establish whether the various phorbol esters are able to activate the isotypes of PKC with differing potencies. If phorbol esters of this type did exist, they would prove extremely useful for *in vivo* studies.

5.1.4. Dependence on calcium PKC is dependent on Ca^{++} for activity and Majerus *et al* (1985) have suggested that Ca^{++} pulses may have a role in controlling the activity of PKC *in vivo*. The K_a of the isotypes for Ca^{++} were determined and were in the region of 100-200nM (Table 5.1.1). The K_a values of the isotypes for Ca^{++} are close to the values estimated to represent resting levels for Ca^{++} in cells (although estimates do vary from the low to the high nM range; see for example Streb *et al*, 1983; Berridge and Irvine, 1984; Johnson *et al*, 1985). The apparent K_a of PKC-γ for Ca^{++} is two fold lower than either -α or -β_1 when assayed in the mixed micelle assay system (Figure 5.1.5). PKC-γ therefore appears to be more sensitive to Ca^{++} than -α or -β_1, but this difference seems to depend on the method of lipid presentation and on the substrate. The sensitivity of the isotypes to EGTA for a particular batch of histone III-S is shown in Table 5.1.2. The lipids were presented in either mixed micelles or in pure lipid micelles. With the pure lipid micelles, PKC-γ is completely inhibited by EGTA, wheras PKC-β_1 was only inhibited by 15% and PKC-α by 47%. However, using PtdSer presented in mixed micelles all the isotypes are inhibited to similar degrees (between 55 and 75%). Attempts to repeat the experiment with different batches of histone III-S were unsuccessful and it was concluded that the

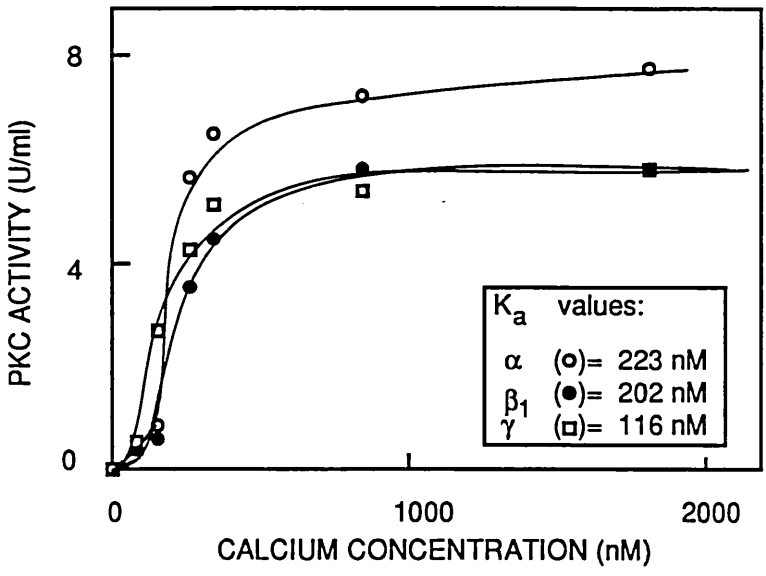


Figure 5.1.5 The dependence of the PKC isotypes on calcium

The activity of the PKC isotypes at various concentrations of Ca^{++} was determined. The concentration of Ca^{++} was buffered by a Ca^{++}/ EGTA/ EDTA/ Mg^{++} buffer system; the concentration of free calcium in this system was calculated by the programme 'Metlig'. The buffering capacity was 1mM and was calculated to give the free Ca^{++} concentrations required at 30°C and pH 7.5. The final PKC concentration was 1 unit/ml.

Table 5.1.2 Calcium dependence of the PKC isoforms

	% of inhibition of PKC activity with 2mM EGTA		
	PKC-α	PKC-β_1	PKC-γ
Mixed micelles	75	56	62
Pure lipid micelles	47	15	97

Assays were done under optimal conditions; PtdSer was presented either as mixed micelles or in pure lipid micelles (Section 2.8). The assays were incubated for 3min, in the presence of 2mM EGTA or 0.6mM Ca^{++}. The results are expressed as the amount of inhibition seen with EGTA compared to the activity seen in the presence of Ca^{++}. The PKC concentration in the assays was 1unit/ml.

difference in sensitivities was dependent not only on the method of presenting the PtdSer, but also on a peculiarity of that one batch of histone III-S. The reasons for these discrepencies were never established, but a similar difference in EGTA sensitivities between the isotypes were seen with the peptide [Ser^{159}]PKC-ε(149-164) (see section 5.2.1 for derivation and sequence). With this peptide as a PKC substrate in the mixed micelle assay system, 2mM EGTA inhibited PKC-β_1 by only 24% wheras -α and -γ were inhibited by 40.1% and 42.7% respectively. Further evidence of differences in response to Ca^{++} was seen during the development of the purification protocol for PKC (section 4.1.1). Under the conditions used on the PtdSer-acrylamide column, the isotypes bound with different affinities, suggesting that there may be differences between the isotypes in their response to Ca^{++}.

The K_a values shown in Figure 5.1.5 are in close agreement with the recent results from Huang *et al* (1988) who found that all the isotypes of PKC had K_a values for Ca^{++} of 200-400nM. They did not report any differences between the isotypes for Ca^{++} requirement in terms of K_a but in their analysis, PKC-γ (type I) was only activated to about 50% of the levels of -α (type III) and -β (type II) at high concentrations of Ca^{++}. Jaken and Kiley (1987) found that PKC-γ (type 1 PKC) was less dependent on Ca^{++} than either -α (type 2) or -β (type 3) in an assay system based on pure lipid micelles in the presence or absence of PDBu. Nishizuka (1988) reported that the -$\beta_{(1+2)}$ isotypes were less dependent on Ca^{++} than either -α or -γ when the lipids were presented in pure lipid micelles in the presence of diacylglycerol. Coussens *et al* (1986) have postulated that there may be differences in Ca^{++} dependencies from the conservation of the primary sequences of the isotypes.

While it is difficult to rationalize the discrepencies from the different laboratories in terms of Ca^{++} dependence, the isotypes do seem to respond differentially to Ca^{++} and this is reflected in their chromatographic behaviour on the PtdSer-acrylamide column. Although the precise details of the differences vary from study to study, from a comparative point of view it has been observed that under defined conditions, the isotypes respond to Ca^{++} in different ways and the discrepencies may merely reflect the different assay conditions used. This indicates that Ca^{++} fluxes or localised Ca^{++} concentrations *in vivo* may have a role in controlling the responses of particular PKC isotypes to extracellular signals. Hannun *et al* (1986) have shown that in the mixed micelle system, the activity of PKC is dependent on a complex function of the relative concentrations of PtdSer, DG and Ca^{++}. It is therefore difficult to relate the *in vitro* activation to the isotypes directly to their *in vivo* situation. However, to reiterate, it appears that under comparable conditions, the PKC isotypes behave differently with respect to Ca^{++} and this may have important physiological consequences.

5.1.5 Effect of arachadonic acid on the PKC isotypes Unsaturated fatty acids can activate PKC directly in the absence of PtdSer (McPhail *et al*, 1984; Murakami and Routtenberg, 1985). It has subsequently been shown that the PKC isotypes respond differentially to unsaturated fatty acids. In particular the response to arachidonic acid is different (Sekiguchi *et al*, 1987). In these studies, PKC -α and -$\beta_{(1+2)}$ were shown to be activated by arachidonic acid in a dose dependent manner and at high concentrations of this lipid, the activity of these isotypes approached the levels that could be reached with DG and PtdSer/

Ca^{++}. By contrast, PKC-γ was maximally activated by arachidonic acid to only 40 % of the levels obtained by DG/ PtdSer/ Ca^{++} and demonstrated a bi-phasic activation, becoming inactivated at higher lipid concentrations (>200μM) (Sekiguchi *et al*, 1987). This result has been repeated in the present studies (Figure 5.1.6). The results show the bi-phasic activation of PKC-γ but unlike the observations of Sekiguchi *et al* (1987), all the isotypes were all activated to levels which were similar to those attained by TPA/ PtdSer/ Ca^{++} at an arachidonic acid concentration of 100μM.

The activating potential of arachidonic acid is dependent upon the method of lipid presentation (Murikami and Routtenberg, 1985). This method of presentation of lipid would presumably result in the formation of 'detergent' like micelles, because arachidonic acid resembles ionic detergents in that it consists of a charged head group and an hydrophobic tail. Thus the activation of PKC by arachidonic acid may not have any physiological significance, unless it can be shown that the activation is reversible and not due to selective denaturation of the regulatory domain by the lipid micelles. In order to investigate this possibility, the reversibility of arachidonic acid pre-incubation was investigated. The results are presented in Table 5.1.3 and show that when the PKC isotypes are incubated with low concentrations of arachidonic acid (100μM), the activity of the isotypes was judged to be relatively stable. The amount of PtdSer/ DG dependent activity for all the isotypes was between 70 and 80% as compared to controls and no Ca^{++}/ PtdSer/ TPA independent PKC forms were generated. At higher concentrations of arachidonic acid (600μM), the recovery of activity for all the isotypes was reduced (Table 5.1.3). The -γ isotype however was significantly less stable than the -α or -β_1 isotypes;

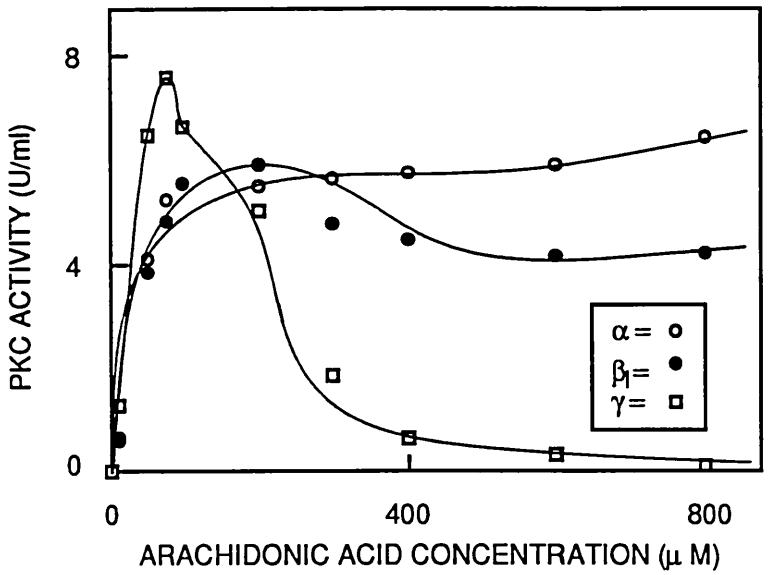


Figure 5.1.6 Effects of arachidonic acid on PKC isotypes
The activity of the PKC isotypes were determined at different concentrations of arachidonic acid. The assay conditions were modified from those stated in section 2.8. The basic reaction mixture was: 50mM Hepes pH 7.5, 0.25mM EDTA, 12.5mM magnesium acetate, 0.75mM calcium chloride, 1.25mg/ml histone H1, 0.125mM ATP (containing [γ-^{32}P] ATP at a specific activity of 150-200 cpm/pmol/min), PKC enzyme and various concentrations of arachidonic acid; total volume= 40μl. The assays were incubated for three minutes and terminated by spotting 25μl of reaction mix onto P81 paper, followed by immediate immersion into 30% acetic acid. The arachidonic acid was prepared by evaporating to dryness an appropriate amount down from solvent under a stream of nitrogen and dispersing into 20mM Hepes pH 7.5 by direct probe sonication. The final PKC concentration in the assays was 1 unit/ml.

Table 5.1.3 Effect of arachidonic acid pre-incubation on PKC-α, -β_1 and -γ

	% of PKC activity remaining after incubation		
	PKC-α	PKC-β_1	PKC-γ
100μM arachidonic acid	74	80	73
600μM arachidonic acid	67	71	16

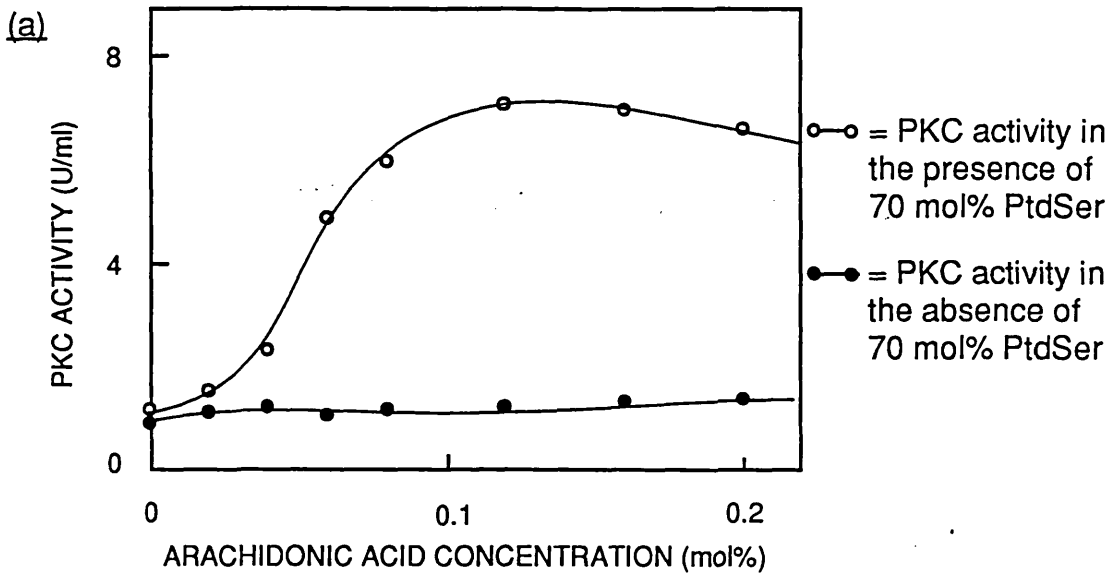
PKC-α, -β_1 and -γ were incubated (at a final concentration of 50units/ml) with arachidonic acid at 100μM or 600μM. The arachidonic acid was prepared by evaporating to dryness an appropriate amount from solvents and re-suspending in 20mM Hepes pH 7.5 by direct probe sonication. The PKC was incubated with the arachidonic acid at 4°C for 60 min and then diluted with 20mM Tris.HCl pH7.5, 2mM EDTA, 0.3% v/v β-mercaptoethanol, 0.02% v/v Triton X-100. The samples were assayed immediately after incubation for PtdSer/ TPA dependent activity. The final concentration of PKC in the assays was 1 unit/ ml. The final concentrations of arachidonic acid in the assays was 2μM and 12 μM for the samples incubated with low and high concentrations of arachidonic acid respectively. The assay conditions were as stated in section 2.8. The results are expressed as the amount of activity remaining after incubation compared to control assays incubated with 20mM hepes pH7.5 only. The amount of kinase independent activity did not increase under any of the incubation conditions tested.

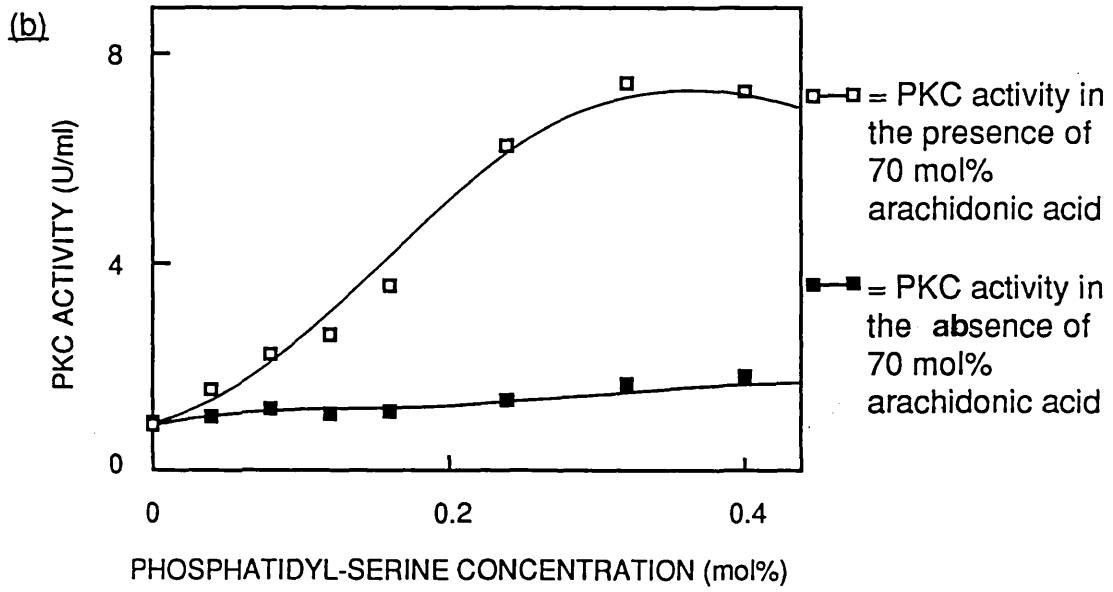
whereas the recovery of -α and -β_1 was in the region of 70%, only 16% of the -γ isotype activity was recovered. The loss of TPA/ PtdSer activity was not due to the generation of a co-factor independent form, but rather due to complete loss of activity (the independent activity was similar for all the assays). The biphasic response of PKC-γ to arachidonic acid would therefore appear to be due to an increased instability of this kinase at high concentrations of the lipid. This instability results in the irreversible inactivation of PKC-γ and is not a characteristic of the -α or -β_1 isotypes, although they were less stable at higher than at lower arachidonic acid concentrations.

To investigate further the activation of PKC by arachidonic acid, the effect of this fatty acid on the activation of PKC-α in mixed micelles was determined. In mixed micelles at 70 mol% PtdSer, the activity of PKC is absolutely dependent on the presence of either TPA or DG (see Figures 5.1.3 and 5.1.4). However, as shown in Figure 5.1.7 (a), at 70 mol% PtdSer, arachidonic acid is able to support the activity of PKC in a dose dependent manner. Full PKC activation was achieved at 70 mol% PtdSer and 0.12 mol% arachidonic acid. Thus arachidonic acid can activate PKC in pure lipid micelles and can also substitute for TPA/ DG in mixed micelles. Further investigation revealed that the mechanism of activation of PKC by arachidonic acid was more complex. Mixed micelles of 70 mol% arachidonic acid in Triton X-100 were unable to activate PKC, but the inclusion of low concentrations of PtdSer to the micelles activated PKC in a dose dependent manner as shown in Figure 5.1.7(b). Full activation was achieved at 70 mol% arachidonic acid and 0.3mol% PtdSer. This indicates that a combination of arachidonic acid and PtdSer is required for PKC activation, but that their relative concentrations

Figure 5.1.7(a) Activation of PKC-α by arachidonic acid and PtdSer
The activation of PKC-α in Triton X-100 mixed micelles in the presence of various concentrations of arachidonic acid was determined. The reaction mixture was: 50mM Hepes pH 7.5; 0.25 mM EDTA; 12.5 mM magnesium acetate; 0.75mM calcium chloride; 1.25mg/ml histone III-S; 0.125mM ATP (containing [γ-^{32}P]ATP at a specific activity of 150-200cpm/pmol; 0.25% v/v Triton X-100; 1.25mg/ml PtdSer; arachidonic acid; 1 unit/ml PKC-α; total volume= 40µl. The assays were incubated for three minutes and terminated by spotting 25µl of reaction mixture onto P81 paper followed by immediate immersion into 30% acetic acid. The lipids were prepared by evaporating to dryness appropriate amounts of each from solvents (in glass tubes) and re-dissolving them in 20mM Hepes pH 7.5, 1% Triton X-100 (30°C, 2min). The final PtdSer concentration was 70mol%. The results are representative of two experiments in which each point was assayed in triplicate.

Figure 5.1.7(b) Activation of PKC-α by PtdSer and arachidonic acid
The activation of PKC-α in Triton X-100/ arachidonic acid mixed micelles in the presence of various concentrations of PtdSer was determined. The reaction mixture was as stated in Figure 5.1.7(a) above, except that the concentration of PtdSer was varied as required and the arachidonic acid was added to a final concentration of 70 mol%.


(a)
8
4
0
PKC ACTIVITY (U/ml)
0
0.1
0.2
ARACHIDONIC ACID CONCENTRATION (mol%)
= PKC activity in the presence of 70 mol% PtdSer
= PKC activity in the absence of 70 mol% PtdSer


(b)
8
4
0
PKC ACTIVITY (U/ml)
0
0.2
0.4
PHOSPHATIDYL-SERINE CONCENTRATION (mol%)
= PKC activity in the presence of 70 mol% arachidonic acid
= PKC activity in the absence of 70 mol% arachidonic acid

are not important. The mechanism of PKC activation is not yet fully understood, but it is generally accepted that PKC must be able to associate with the micelles to enable it to become activated. If this model is accepted, it would appear that in mixed micelles at least two factors are required to allow this interaction to take place. These possibilities are discussed further in Chapter Six.

5.1.6 *In vivo* responses of the PKC isotypes to TPA The differential *in vitro* activation of the isotypes by TPA suggests that phorbol esters may elicit different physiological effects by selective isotype activation *in vivo*. In order to test this hypothesis, the effect of chronic TPA stimulation on a melanocyte cell line was investigated. This was a collaborative project with Professor L. Diamond of the Wistar Institute, Philadelphia. I would therefore like to extend my gratitude to Prof Diamond for organising the culture and TPA treatment of these cells.

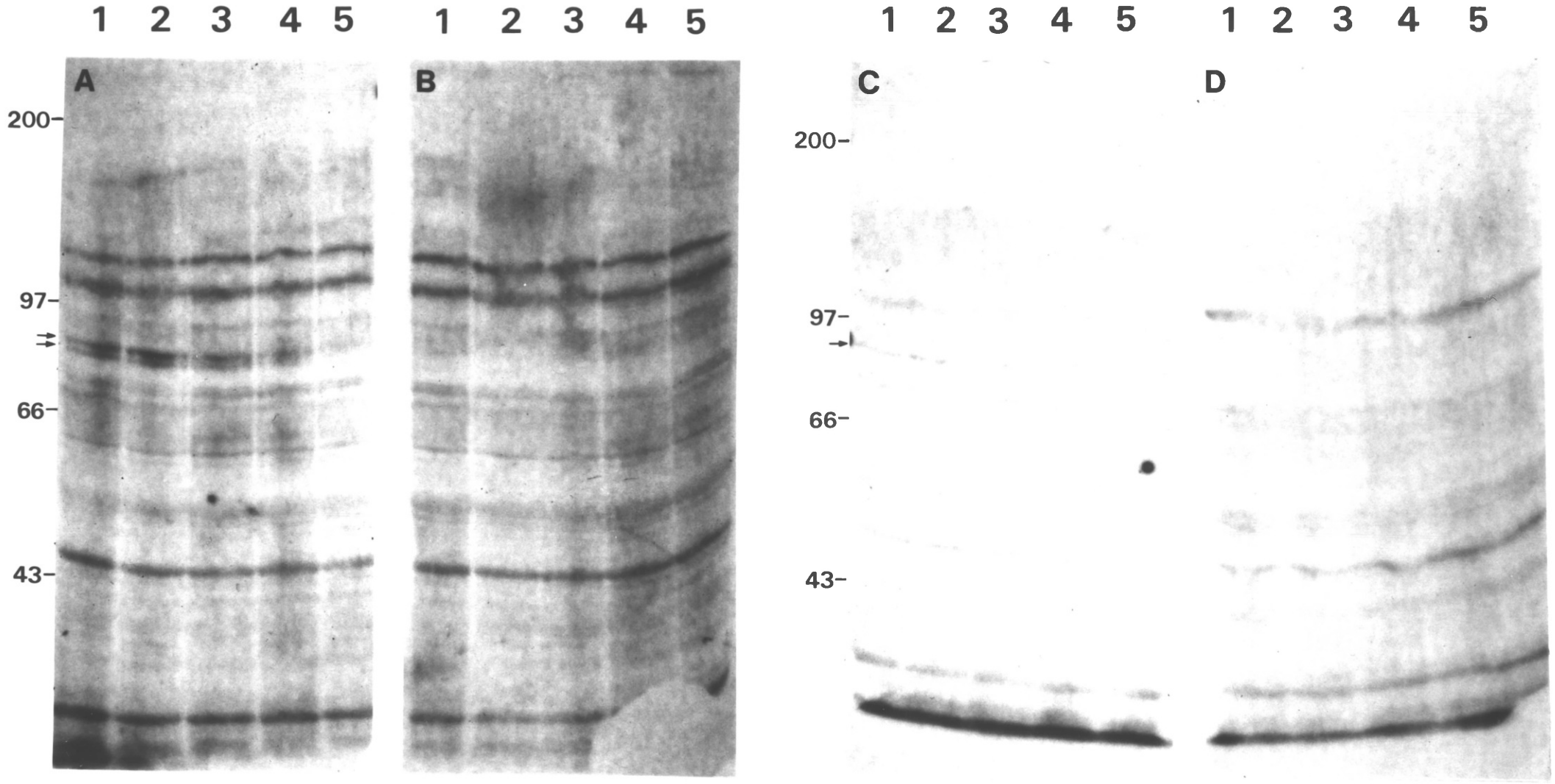
Melanocytes are neural crest derived cells that form a minor proportion of the cells of the epidermis. They produce melanin which they export to keratinocytes where it acts as a protective pigment against ultraviolet radiation (Melber *et al*, 1989). These cells do not normally undergo mitosis *in vivo* or *in vitro*, but they can be grown in culture in the presence of mitogenic compounds such as phorbol esters for which there is an absolute growth dependence (Melber *et al*, 1989). Cell lines have been established from human melanocytes which have been transformed with the SV40 T-antigen (Melber *et al*, 1989). These cell lines show altered rates of cell division when compared to the parental cell lines. They are also unlike the parental line in that they are anchorage independent and their growth is inhibited in the presence of TPA (Melber *et al*, 1989). Treatment of

one of these cell lines (FM516 SV3/3) with TPA results in a decrease in the PKC activity that can be assayed in triton X-100 lysates from these cells (L. Diamond, personal communication). However, the loss of activity is not complete and after 24hr of treatment, the amount of PKC activity that can be assayed in the lysates is about 30-50% of the pre-treatment levels. Further TPA treatment does not result in any more reduction in the levels of PKC activity in these cells (L. Diamond, personal communication).

FM516 SV3/3 cells were chosen as a model system to investigate the behaviour of the PKC isotypes in response to TPA because of these peculiar down-regulation characteristics. Cells were grown as published (Melber *et al*, 1989) and treated with TPA (10^{-8}M) for 1, 3, 12 and 24 hours. The cells were harvested, washed twice with PBS and then lysed directly into four times concentrated Laemmli sample buffer (Section 2.4) (2.5×10^7 cells/ 250µl). The lysates were immersed in a boiling water bath for 5 min, frozen and transported to the laboratory on dry ice. After thawing, the DNA was sheared by direct probe sonication (30 second burst) and the lysates were immuno-blotted. The results are shown in Figure 5.1.8. Only the $V_5\alpha$ and $V_5\beta_1$ monospecific antisera gave positive results. The $V_5\beta_2$ and $V_5\gamma$ antisera failed to give positive results even after two months of exposure to the autoradiographic film. The -α isotype was detected as a doublet with Mr values of approximately 78,000 and 80,000; the -β_1 isotype was detected as a single band with a Mr of approximately 79,000 (Figure 5.1.8). The physiological significance of the two forms of -α in these cells is not known. Parker *et al* (1987) have shown that PKC is post translationally modified *in vivo* and that the two forms can be disinguished from each other by their mobility on SDS-PAGE gels. The nature of this modification is not

Figure 5.1.8 The response of PKC to TPA in FM516 SV3/3 cells
Cells were grown as described (Melbar *et al*, 1989) and treated with TPA (10^{-8} M) for the times stated. The cells were harvested, lysed into four times concentrated Laemmli sample buffer (section 2.4) and frozen until immuno-blot analysis could be performed. For each lane, 10^6 cell equivalents were loaded and the proteins were resolved by SDS-PAGE (7% w/v acrylamide gels). The proteins were transferred to nitrocellulose and probed with the V5 region antisera. Only the $V_5\alpha$ and the $V_5\beta_1$ antisera gave positive identification of PKC bands. Two positive bands were seen with the $V_5\alpha$ antiserum and one with the $V_5\beta_1$ antiserum; these are indicated by appropriate arrows. The position of migration of 'Rainbow' molecular mass proteins is indicated (x 10^{-3}). Lane 1= cells which were not treated with TPA; lane 2= cells treated with TPA for 1hr; lane 3= cells treated with TPA for 3hr; lane 4= cells treated with TPA for 12hr; lane 5= cells treated with TPA for 24hr.
Antisera used:

panel A= $V_5\alpha$ panel B= $V_5\alpha$ with competing peptide
panel C= $V_5\beta_1$ panel D= $V_5\beta_1$ with competing peptide


1 2 3 4 5
A
200-
97-
66-
43-
1 2 3 4 5
B
1 2 3 4 5
C
200-
97-
66-
43-
1 2 3 4 5
D

known but it may be due to phosphorylation (Parker *et al*, 1987). The ratio of -α to -β_1 is estimated to be about 7:3 from the relative intensities of the autoradiographic signals. Treatment of these cells with TPA for 24 hr resulted in the down-regulation of PKC-α but not -β_1 (Figure 5.1.8). Both the -α isotypes down-regulated and could not be detected after the 24hr period of treatment. The faster migrating species appeared to down regulate slightly before the slower migrating band, suggesting that the slower migrating form is less stable or that this form has to become the slower migrating band in order to down-regulate. This observation remains to be confirmed. The -β_1 isotype could still be detected after 24hr and did not appear to down-regulate in response to TPA in these cells. The activation state of this isotype in these cells is not known. On the basis of these results, the PKC activity that can be measured in lysates from these cells after 24 hr is due to the persistence of the -β_1 isotype even after chronic TPA treatment.

In vitro, the -α and -β isotypes display similar dependencies on TPA as is indicated by their similar Ka values for this compound. However, PKC association with membranes does not always result in activation and Bazzi and Nelsesteun (1988) suggest that the association of PKC with membranes is a pre-requisite but not sufficient for activation. The association of PKC with membranes and the subsequent activation events may therefore be distinct, which may explain why the isotypes respond to TPA in different ways *in vivo*, but in similar ways *in vitro*. Alternatively, PKC-α and -β_1 may exist in different cellular compartments in FM516 SV3/3 cells which afford increased stability to the -β_1 isotype. Ase *et al* (1988) have provided evidence showing that the isotypes of PKC down-regulate differentially in KM3 cells, indicating that this may

not be a unique occurrence. However, these authors demonstrate only that the isotypes appear to down-regulate differentially but do not establish whether the isotypes are synthesised at the same rates. If the isotypes were synthesised at different rates then the differential down-regulation may be artefactual, because although the isotypes may be down-regulating at the same rate, one of the isotypes may get replaced more quickly than the other, resulting in an apparrent difference in down-regulation. With the FM516 SV3/3 cells by contrast, there is no loss of the $-\beta_1$ isotype and therefore the difference in response to TPA between the isotypes is clear.

These observations have important implications for both tumour promotion and for signal transduction since they demonstrate that under what appear to be similar conditions the isotypes of PKC respond differently to activators. This observation can be extrapolated to the physiological activation of PKC and begs the question whether the isotypes are activated differently by the various species of DG that can be produced from phospholipids. If this is the case then it may explain the reasons for the multiplicity of PKC and also why activation of PKC leads to such a large number of diverse responses in cells. These questions can be addressed by using the monospecific antisera to follow the activation of the PKC isotypes *in vivo* in response to natural agonists such as the growth factors and hormones.

5.2. Substrate specificity of PKC-α, β_1 and -γ using peptide substrates

Protein kinase substrate specificity appears to be principally dependent on the primary amino acid sequence around the target residue as judged by the ability of kinases to phosphorylate short peptides (see Sparks and Brautigan, 1986). The ability of kinases to phosphorylate particular cellular proteins determines the range of responses that they can control (Cohen, 1985). Therefore by understanding the requirements of kinases for substrate recognition, the ability of kinases to regulate particular pathways can be established. This knowledge can also be used to identify putative substrates by sequence comparisons. The substrate specificity requirements of many kinases have been defined by the use of synthetic peptides. These studies have revealed for example that cA kinase requires one or two basic residues N-terminal to the target amino acid (Kemp *et al*, 1977), whereas casein kinase II requires clusters of acidic residues around its target amino acid (Meggio *et al*, 1984). Other kinases which depend on basic amino acids for target recognition are cG kinase (Glass and Krebs, 1982), calmodulin dependent protein kinase (Pearson *et al*, 1985), myosin light chain kinase (Kemp *et al*, 1983), H4 specific protease activated kinase (Eckols *et al*, 1983) and PKC (Ferrari *et al*, 1985; Woodgett *et al*, 1986; House *et al*, 1987). Although all these kinases require basic residues for sequence recognition, there is limited overlap between the recognition sites. For example in $pp60^{src}$, PKC and cA kinase phosphorylate different residues with absolute specificity (Woodgett *et al*, 1986).

By the use of peptides, many groups have defined the sequence requirements of PKC for target recognition. PKC is a promiscuous kinase *in vitro*, phosphorylating many peptides and proteins (see Nishizuka, 1986; Woodgett *et al*, 1987) and peptide substrates specific for this kinase have been designed (Ferrari *et al*, 1985; Woodgett *et al*, 1986). All the substrates for PKC require basic residues in the vicinity of the target residue (Ferrari *et al*, 1985, Turner *et al*, 1985; Kishimoto *et al*, 1985; Woodgett *et al*, 1986; House *et al*, 1987), with an apparent preference for Arg over Lys (House *et al*, 1987). Turner *et al* (1985) concluded from studies with MBP based peptides that PKC preferred peptides with basic residues N-terminal to the target, whereas Kishimoto *et al* (1985) concluded that basic residues C-terminal to the target were important for recognition. However, Ferrari *et al*, (1985), Woodgett *et al* (1986) and House *et al* (1987) all concluded that while PKC could phosphorylate peptides with basic residues on either the N- or the C-terminal sides of the target residue, the best substrates were those flanked on both termini by basic residues.

In all previous studies with peptides involving PKC, the isotype content of the sample was not defined and were almost certainly mixtures. If the isotypes have different substrate specificities, this may in part explain why PKC has such a large range of substrates and it may also explain at least in part why the results from the specificity studies were sometimes contradictory. Peptides were therefore used to investigate the requirements of the isotypes for substrate recognition. Two classes of peptides were chosen for these studies; the first class was based on the 'pseudosubstrate prototope' sequence of PKC (see below); the second were peptides based on the sequences of targets in proteins that are known PKC

substrates. The results show that the isotypes have different substrate specificities suggesting that they may phosphorylate different physiological targets.

5.2.1. Pseudosubstrate prototope peptides PKC is a two domain protein and House and Kemp (1987) have postulated that the regulatory domain inhibits the catalytic domain by direct association. They have defined a region in the regulatory domain of PKC-α which they suggest has the ability to inhibit the catalytic domain. This 'pseudosubstrate prototope' is a region of primary sequence (defined as residues 19-31) that has a good theoretical structure as a PKC substrate. The prototope contains an Ala (residue 25) in the target position and was postulated to have high affinity for the catalytic site. House and Kemp (1987) suggest that this pseudosubstrate region binds tightly to the catalytic site of PKC and therefore inhibits the activity. When lipid cofactors and Ca^{++} bind to the regulatory domain, they cause a conformational change that removes the prototope from the catalytic cleft, thereby activating the kinase. Synthetic peptides corresponding to this region have been shown to be inhibitory for PKC at low concentrations and peptides substituted with Ser for Ala^{25} have been shown to be efficient PKC substrates (House and Kemp, 1987) . The prototopes are located at the V1/C1 junction in PKC-α and corresponding conserved regions can also be identified in PKC -β and -γ.

If the model proposed by House and Kemp is correct, then the affinity of each isotype for its own prototope sequence may be higher than its affinity for the other isotype prototopes, hence reflecting any substrate specificity differences that may exist between them. In order to investigate this, peptide substrates corresponding to the

prototope in PKC-α, -β_1 and -γ were synthesised and tested as substrates for each PKC isotype. The peptides were [Ser^{25}]PKC-α(15-31), (referred to as α-pep); [Ser^{25}]PKC-β(15-31), (referred to as β-pep); [Ser^{24}]PKC-γ(14-30), (referred to as γ-pep) and [Ser^{159}]PKC-ε(149-164), (referred to as ε-pep, as defined by Schaap *et al*, 1989) (See Table 5.2.1 for sequences). These peptides are extended at the N-termini compared to the peptides made by House and Kemp to include more of the region at which they diverge (the V1 region).

The kinetic analysis for the prototope substrate peptides is shown in Table 5.2.1. All the peptides are efficient substrates for each isotype, with K_m values in the μM range and V_{max} values in the μmol/min/mg range. These results are comparable to reported values for other peptide substrates (see Woodgett *et al*, 1986; House *et al*, 1987). The PKC isotypes did not show a preference for their particular prototope substrate, but the peptides were treated as a class of substrates by the isotypes and a pattern of preferences could be seen. The -β_1 isotype had the lowest K_m values for all the peptides; PKC-γ had the highest K_m values and PKC-α was intermediate between these two. PKC-β_1 had the lowest V_{max} values for these peptides and PKC-α the highest with PKC-γ between them (Table 5.2.1). These results indicate that PKC-β_1 had the highest apparent affinity for this class of peptides but that PKC-α had the highest catalytic activity towards them. The K_m values of the -α and -β isotypes for ε-pep were lower than for the other peptides and this peptide had the greatest proportion of basic residues. For PKC-γ, the γ-pep had the lowest K_m. The V_{max} values were all within about two fold of each other for a particular peptide and no patterns were seen to emerge from this data. This indicates that the catalytic rates of

Table 5.2.1 Prototope peptide substrate specificities for PKC-α, -β_1 and -γ

	Histone III-S		α-pep		β-pep		γ-pep		ε-pep	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
PKC-α	45±8.9	1.1±0.09	44±9.7	4.5±0.91	30±13	3.5±0.83	18±2.9	3.3±0.23	14±2.3	3.7±0.35
PKC-β_1	56±10	0.71±0.07	37±12	2.4±0.25	18±4.7	1.9±0.43	13±2.0	1.9±0.11	8.0±1.4	1.7±0.10
PKC-γ	33±9.6	1.6±0.23	95±14	3.9±0.25	45±11	2.9±0.86	24±5.5	2.3±0.26	30±3.7	3.6±0.24

The K_m and V_{max} values for the three PKC isotypes are indicated. The K_m units are μM, the V_{max} values are expressed as μmol/min/mg. The peptide sequences correspond to the sequence from the region of the pseudosubstrate prototope sequence defined by House and Kemp (1987) and are: α-pep= DVANRFARKGSLRQKNV, β-pep= ESTVRFARKGSLRQKNV, γ-pep= GPRPLFCRKGSLRQKVV and ε-pep= ERMRPRKRQGSVRRRV (the target residues are underlined). The published K_m and V_{max} values for [Ser25]PKC(19-31) are 0.2μM and 8μmol/min/mg respectively (House and Kemp, 1987). With these peptides the reaction mixture was as described in section 2.8 (lacking histone III-S). The assays were incubated for three minutes and terminated as described in section 2.8. For each assay, the final PKC concentration was approximately 1 unit/ml as determined with histone III-S as the substrate.

the isotypes for this class of peptides were approximately similar to each other.

The patterns that could be seen in the kinetic data from the prototope peptide studies indicates that residues 15-19 had an impact on the substrate-enzyme interaction since these are the only four amino acids different between α-pep and β-pep. Woodgett *et al* (1986) suggested that regions further than 6 residues from the target have only a minimal effect on substrate recognition. The data presented here suggests that with the prototope peptides, regions further than six residues from the target appeared to affect substrate-enzyme interaction. It is possible that there are secondary interactions between the enzymes and the N-terminal regions of the peptides due to catalytic site mimicry. With the β-pep for example, a secondary substrate site might exist composed of Ser^{16} or Thr^{17} with Arg^{19}. Similarly, in α-pep an inhibitory region could be composed of Ala^{17} with Arg^{19}. These secondary regions may be responsible for the differences seen in the kinetics data and this hypothesis can be tested by synthesizing truncated peptides, or by sequencing the phosphorylated residue(s) in β-pep to establish the extent of Ser^{16}/Thr^{17} phosphorylation.

The K_m values recorded here are higher than the values published by House and Kemp (1987) and the V_{max} values are lower than their values. With the α-pep as a substrate, the $K_m$values were 140-500 fold higher and the V_{max} values 2-4 fold lower than the values for the House and Kemp peptide [Ser^{25}]PKC(19-31). With the β-pep, γ–pep and ε-pep substrates, the K_m values were 40-225 fold higher and the V_{max} values 2-4 fold lower than the House and Kemp values for the [Ser^{25}]PKC(19-31) peptide. As mentioned, the peptides used in this

study were extended at their N-termini compared to the House and Kemp peptides and this may be the reason for the difference. In support of this suggestion is the observation made by House and Kemp (1987) that peptide length affects the inhibitory properties of the pseudosubstrate prototopes; this may also be true of the substrate peptides. Further to this the PKC used by House and Kemp was only partially purified and the isotype content not defined. However it is unlikely that the differences in assay system, peptide length and PKC sample would cause the large differences seen in the results from these two studies. The reason could have been due to impurities in the peptides used, which were inhibitory towards kinase activity. The peptides were therefore subjected to amino acid analysis (by R. Philp) and gas phase sequencing (by N. Totty). The results indicated that the peptides were > 95% w/w pure. The α-pep was purified by reverse-phase HPLC to remove contaminating salts and the peptide was identified by amino acid analysis. This purified peptide was used as a substrate for PKC-α and gave similar kinetics parameters to the un-purified peptide, suggesting that there were no salts or other impurities in the peptides that interfered with the kinase activity. Therefore the reasons for the differences in kinetic parameters between the results from this study and those from House and Kemp could not be explained.

Nevertheless, the prototope peptides have been useful for examining the substrate specificities of the PKC isotypes and for showing that there are differences. To investigate further this point, other pepides and proteins were compared as substrates.

5.2.2. Peptides based on PKC substrates As mentioned in the begining of this section, PKC has been shown to phosphorylate a

number of proteins both *in vivo* and *in vitro*. Some of the phosphorylation sites in these proteins have been mapped and synthetic peptides based on the sequences from these sites have been produced and used to examine the substrate specificity of PKC (Turner *et al*, 1985; Woodgett *et al*, 1986; House *et al*, 1987). Four such peptides were used to analyse the substrate specificity of the PKC isotypes. These peptides were obtained from Dr J. Woodgett.

The epidermal growth factor receptor (EGFR) is phosphorylated by PKC *in vitro* and *in vivo* at residue Thr^{654} and synthetic peptides to this region of EGFR have proved to be efficient substrates for PKC (Woodgett *et al*, 1986). The peptide EGFR(650-658) was used to probe the substrate specificities of the PKC isotypes. This peptide has the sequence: Val-Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu_{NH_2} (the target is underlined) and is referred to as EGF-R3. EGF-R3 is an efficient substrate for PKC, but not for other kinases, therefore making it a specific substrate for PKC (Woodgett *et al*, 1986).

The protein $pp60^{src}$ is a PKC substrate *in vivo* and *in vitro* and is phosphorylated on Ser^{12} by this enzyme (Gould *et al*, 1985). Ser^{12} is close to the plasma membrane *in vivo* and the peptide $pp60^{src}$(2-19) (referred to as p60-1) has been shown to be a good substrate for PKC (Gould *et al*, 1985). The peptide p60-1 is phosphorylated exclusively at Ser^{12} by PKC and at Ser^{17} by cA kinase (Woodgett *et al*, 1986). The sequence of p60-1 is Gly-Ser-Ser-Lys-Ser-Lys-Pro-Lys-Asp-Pro-Ser-Gln-Arg-Arg-Arg-Ser-Leu-Glu_{NH_2}.

Myelin basic protein (MBP) is phosphorylated on numerous residues by PKC *in vitro*, however mapping the sites has given controversial results. Turner *et al* (1984) claim that Ser^{115} is the major site, but

Kishimoto *et al* (1985) claim that Ser^{115} is only a minor phosphorylation site and that many other sites are phosphorylated to a greater degree. Nevertheless, Turner *et al* (1985) have defined a peptide based on the sequence around Ser^{115} of bovine MBP as a good peptide substrate for PKC. This peptide (MBP-1) has the structure [Ala^{105}]MBP(104-118) and the sequence: Gly-Ala-Gly-Arg-Gly-Leu-Ser-Leu-Ser-Arg-Phe-Ser-Trp-Gly-Ala (the target is underlined). In this peptide, Arg 105 has been substituted with Ala to block phosphorylation of $Ser^{110/112}$, therefore resulting in a peptide with a single PKC site that is an efficient PKC substrate (Turner *et al*, 1985).

The activity of glycogen synthase (GS) is controlled by phosphorylation on specific residues by many Ser/Thr kinases (Roach, 1981; Cohen, 1982). PKC has been reported to phosphorylate GS on at least two sites (Imazu *et al*, 1984; Ahmad *et al*, 1984). House *et al* (1987) found that a synthetic peptide representing the first ten residues of GS was a good PKC substrate and used analogues to this peptide to probe the substrate specificity requirements of PKC. Substitution of Ser^{9} and Ser^{10} for Ala residues does not affect the kinetics of phosphorylation overtly but reduces the number of potential sites, therefore making the analysis of the results more straightforward. The substituted peptide was phosphorylated on only Ser^{7} by PKC (House *et al*, 1987). This analogue of the first 10 residues of GS (referred to as GS1-10) has the structure [$Ala^{9,10}$]GS(1-10) and the sequence: Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala.

The results from the kinetic analyses of these peptides is shown in Table 5.2.2 and show that the isotypes have different affinities and

Table 5.2.2 Phosphorylation of physiological substrate peptides by PKC-α, -β_1 and -γ

	EGF-R3		p60-1		MBP-1		GS1-10	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
PKC-α	15.1±2.9	0.559±0.06	218±21	1.45±0.91	82.3±7.4	0.889±0.04	53.1±10	2.15±0.09
PKC-β_1	7.61±1.7	0.223±0.05	280±56	0.868±0.10	69.1±7.8	0.210±0.01	43.5±5.3	0.978±0.02
PKC-γ	15.3±3.3	0.230±0.03	264±16	0.746±0.02	146±14	0.371±0.06	552±110	1.16±0.10
Published values	48 [1*]	0.990	48 [1]	0.560	22 [2]	0.213	40.3 [3]	1.60

The K_m and V_{max} values for the three PKC isotypes are indicated. The K_m units are μM and the V_{max} values expressed as μmol/min/mg. The peptide sequences are given in the text. The assays with these peptides were performed in standard assay buffer (lacking histone; total volume= 20μl) and the reactions were terminated (after 3 min, 30°C) by the addition of 10μl of 4 times concentrated Laemmli sample buffer (section 2.4) followed by immediate immersion into a boiling water bath (5 min). The peptides were resolved by SDS-PAGE (25% w/v acrylamide gels), the electrophoresis being stopped after the ion front but before the dye front had eluted from the bottom of the gel. The gels were immersed in water containing 'Mixed bed resin AG501-X8(D)' for 5 min to remove unincorporated ATP, dried and exposed to autoradiographic film (-80°C). The phosphorylated peptides were identified, individually excised from the gels and the amount of ^{32}P incorporated into them was determined. *The published values come from: 1=Woodgett *et al*, 1986; 2= Turner *et al*, 1985; 3= House *et al*, 1987.

catalytic activities for these peptides. With EGF-R3 , the K_m of -β_1 is twice that of -α or -γ, but with p60-1, the K_m of the isotypes are similar to each other, suggesting that the isotypes have similar affinity for p60-1, but not for EGF-R3. With MBP-1, the K_m of -γ is about twice that of -α or -β_1 and with GS1-10, the K_m of the -γ isotype is about ten fold higher than -α or -β_1. The V_{max} data shows that PKC-α is the most active isotype towards all the peptides. The V_{max} of PKC-α is twice that of PKC-γ for EGF-R3, p60-1, MBP-1 and GS1-10. It is also twice that of PKC-β_1 for EGF-R3, p60-1 and GS1-10 and four times that of PKC-β_1 for MBP-1. This data indicates that although the K_m of the isotypes for the peptides is subject to quite large differences, the V_{max} values are less variable, suggesting that the major factor influencing substrate kinetics is affinity and not catalytic activity.

The K_m and V_{max} values seen in this study are lower than the published values for EGF-R3 but higher for p60-1 and MBP-1 (Table 5.2.2). The K_m values of the -α and -β_1 isotypes for GS1-10 are similar to published data, suggesting that the PKC sample of House *et al* (1987) contained predominantly these PKC isotypes. The published V_{max} for GS1-10 was 1.3 times lower than the V_{max} for -α but about 1.5 times higher than the -β_1 or -γ values. The differences seen between the results from this study and the published results are within experimental variation considering the variety of assay systems and the differences in the purity of the PKC sample used in the various investigations.

Alignment of the peptides substrates used in this study shows that the peptides all contain basic residues in the vicinity of the target (Figure 5.2.1). With the exception of p60-1, all the peptides also

Figure 5.2.1 Peptide substrates for the PKC isotypes

α-pep	DVAN**R**FA**R**K**G**S̲L**R**Q**K**NV
β-pep	EST**V**R**F**A**R**K**G**S̲L**R**Q**K**NV
γ-pep	GP**R**PL**F**C**R**K**G**S̲L**R**Q**K**NV
ε-pep	E**R**M**R**P**R**K**R**Q**G**S̲**V**R**R**R**V**
EGF-R3	**V**R**K**T̲L**R**R**L**$_{NH_2}$
p60-1	GS**S**K**S**K**P**K**D**P**S**̲Q**R**R**R**S**L** **E**$_{NH_2}$
MBP-1	GA**G**R**G**L**S**L**S**R**F**S̲**W**G**A**
GS1-10	P**L**S**R**T**L**S̲**V**A**A**

The peptide substrates are compared so that the target residues (underlined) are aligned. The basic residues are highlighted in bold. The amidated C-termini of EGF-R3 and p60-1 are also in bold, since these will be charged at neutral pH.

contain a hydrophobic residue on the C-terminal side of the target and this is consistent with the observation of House *et al* (1987), who found that a C-terminal hydrophobic residue was important for the effective phosphorylation of peptides by PKC. It is to be noted that p60-1 was consistently less efficient as a substrate for the PKC isotypes than the other peptides (with the exception of GS1-10; see below). Hydrophobic residues at this position are thought to have a role in the removal of water from the active site during catalysis, thus reducing the hydrolysis of ATP by water which would otherwise occur (Sparks and Brautigan, 1986).

All the peptides have basic residues within the region of the target residue (Figure 5.2.1). There is some correlation between the number of basic residues and their efficiency as substrates, although the differences in primary structure makes direct comparison difficult. However these results are consistent with previous reports stating the importance of basic residues in substrate recognition (Ferrari *et al*, 1985; Kishimoto *et al*, 1985; Ferrari *et al*, 1985; Woodgett *et al*, 1986; House *et al*, 1987).

The peptide that gave the greatest difference in K_m values was GS1-10, which was an efficient substrate for PKC-α and -β_1 but not for -γ. A similar pattern of preferences was seen with MBP-1 in which the K_m of PKC-γ was twice that of -α or -β_1. Both GS1-10 and MBP-1 have basic residues N-terminal, but not C-terminal to the target residue. This suggests that basic residues C-terminal to the target are permissive for -α, -β and -γ isotype substrates but N-terminal residues are selective for -α and -β isotype substrates. In the presence of both N- and C- terminal residues the selective effect of N-terminal residues appears to be overriden and these peptides are

efficient substrates for all the isotypes. The position of the N-terminal basic residue appears to be important to -γ selectivity. PKC-γ had a K_m for MBP-1 that was twice the K_m of -α or -β_1, but for GS1-10 the difference was ten fold. The basic residue in MBP-1 is two residues from the target, wheras in GS1-10, the basic is three residues from the target. It appears therefore that as the basic residue moves further from the target residue in the N-terminal direction, the selection against the -γ isotype becomes greater.

The precise environment of a potential target residue therefore appears to affect its potential as a PKC substrate. However all the studies with peptides and PKC that have been done to date have used undefined PKC samples. The importance of basic residues has been defined previously, but the results from this study indicate that basic residues may have a fundamental role in the selective phosphorylation of substrates by the PKC isotypes. In particular, N-terminal residues appear to select against PKC-γ phosphorylation in the absence of C-terminal residues. In order to answer the questions raised by these observations, peptides have been designed to incorporate the features which appear to be essentual to isotype substrate recognition. A rationale for the design of these types of peptides will be discussed in Chapter Six and the results will be presented elsewhere.

5.3 PROTEIN SUBSTRATES

Although peptides are useful for defining the primary sequence requirements of kinase targets, secondary and tertiary structure of proteins affect substrate specificity by limiting access to peptide sequences (see Sparks and Brautigan, 1986). Proteins known to be PKC substrates were employed therefore to investigate further the substrate specificity of the PKC isotypes. These studies were limited by the availability and the abundance of the substrate proteins. Nevertheless the results show that the isotypes do have different substrate specificities against protein substrates *in vitro*. Initially, commercially available proteins were used which are abundant enough to generate K_m and V_{max} data. These proteins were amongst the first proteins to be identified as PKC substrates and are often used for kinetic analysis studies.

5.3.1 Histone H1, MBP and protamine

Histones and protamine were amongst the first PKC substrates to be identified as PKC substrates (Takai *et al*, 1977). Protamine is unusual in that it is a substrate for PKC in the absence of PKC activation by either proteolysis (Takai *et al*, 1977) or by Ca^{++} and lipids (Takai *et al*, 1979a). Histone H1 is the most efficient histone substrate for PKC (Takai *et al*, 1979b) but not for PKM (Takai *et al*, 1977). Histone H1 is phosphorylated on a single major site (Ser^{103}) by PKC (Jakes *et al*, 1988), but it is probably not a PKC substrate *in vivo*. MBP is also an efficient substrate for PKC (Wise *et al*, 1982) and is phosphorylated on as many five sites *in vitro* (Turner *et al*, 1984; Kishimoto *et al*, 1985). Some of these phosphorylation sites on MBP have been mapped but different results have been reported from different groups (Turner *et al*, 1984; Kishimoto *et al*, 1985).

Phosphorylation of MBP by PKC *in vitro* is dependent on phospholipids and Ca^{++} (Wise *et al*, 1982) and the *in vivo* phosphorylation of this protein by PKC may be a signal for the formation of myelin in oligodendrocytes (Vartanian *et al*, 1988). The availability of histone H1, MBP and protamine makes them excellent candidates for the kinetic analysis of the PKC isotype substrates.

Histone H1 is available from the Sigma Chemical Company as a preparation called histone III-S (a Lys rich preparation of histone). This preparation is not particularly pure. It contains many PKC substrates which can be detected by SDS-PAGE analysis of the phosphorylated products (Figure 5.3.1) and is inexpensive and readily available. However, to avoid any variation that may occur due to batch to batch variation, a single batch of histone III-S was used in all the kinetic analyses presented in this thesis. A more purified preparation of histone H1 is available from Boerhinger Mannheim (calf thymus histone). Phosphorylation of this preparation by PKC results in two phosphoproteins which can be separated by SDS-PAGE on 15% w/v polyacrylamide gels (Figure 5.3.1). Both proteins in this preparation are histone H1, but the relationship between them is unknown.

The results from the biochemical analysis of histone III-S, histone H1, MBP and protamine are shown in Table 5.3.1. Histone III-S, histone H1 and MBP were all phosphorylated by the three PKC isotypes in a Ca^{++}, PtdSer and TPA dependent manner. Protamine was a substrate for all three isotypes, but in an activator independent manner. No patterns could be discerned in the phosphorylation of the substrate by the PKC isotypes. Each substrate was phosphorylated by the PKC isotypes with different kinetics and different preferences

Figure 5.3.1 Phosphorylation of histones by PKC

Histons III-S and histone H1 were both phosphorylated by PKC (-α, -β_1, -β_2 and -γ) and analysed by SDS-PAGE. The assay conditions were as stated in section 2.8 but the reactions were terminated by the addition of 20µl four times concentrated Laemmli sample buffer (section 2.4), followed by immediate immersion into a boiling water bath. The proteins were separated by SDS-PAGE on 15% w/v acrylamide gels. The gels were Coomassie blue stained, dried and the phosphorylated products were detected by autoradiographic analysis. The position of histone H1 is indicated by the arrow and the position of standard molecular mass markers is also indicated (x 10^{-3}). lane 1= 6.25 µg Histone III-S (Sigma chemical Co.) phosphorylated by PKC in the presence of Ca^{++}/ PtdSer/ TPA; lane 2= 0.63 µg histone III-S phosphorylated by PKC in the presence of Ca^{++}/ PtdSer/ TPA; lane 3= 6.25 µg histone III-S phosphorylated by PKC in the absence of activating co-factors; lane 4= 6.25 µg histone H1 (Boerhinger Mannheim) phosphorylated by PKC in the presence of Ca^{++}/ PtdSer/ TPA; lane 5= 6.25 µg histone H1 phosphorylated by PKC in the absence of activating co-factors. In histone III-S numerous phosphorylated products were detected; in histone H1, two phosphorylated products were detected.

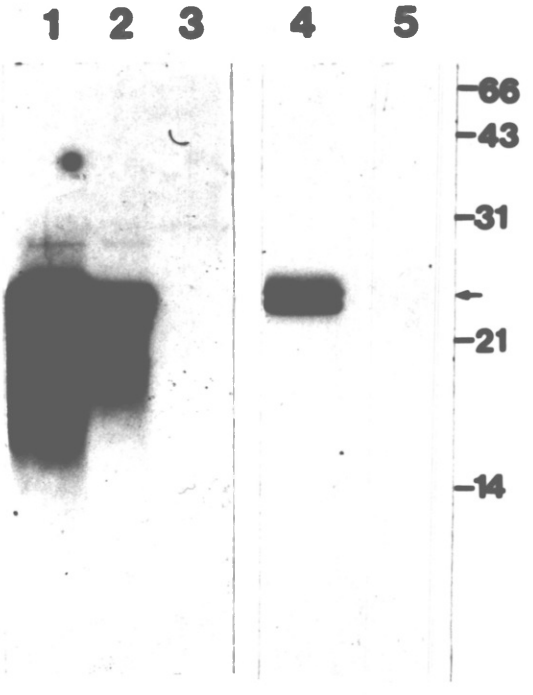

1
2
3
4
5
66
43
31
21
14

Table 5.3.1 Phosphorylation of protein substrates by the PKC isotypes

	Histone III-S		Histone H1		MBP		Protamine	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
PKC-α	45±8.9	1.1±0.09	18±1.6	0.49±0.03	12±1.3	0.54±0.02	0.92±0.19	0.36±0.02
PKC-β_1	56±10	0.71±0.07	14±1.1	0.21±0.04	6.9±1.5	0.23±0.01	1.1±0.13	0.32±0.01
PKC-γ	33±9.6	1.6±0.23	9.2±1.1	0.17±0.02	22±5.2	0.35±0.04	3.6±1.3	0.48±0.05

The K_m and V_{max} values for the three isotypes are indicated. The K_m values are in μM and the V_{max} values are in μmol/min/mg. The results were calculated using the programme 'Enzfitter'. The assays for histone III-S and histone H1 were performed as described in Section 2.8. Protamine was assayed under the same conditions but in the absence of Ca++/ TPA/ PtdSer and Triton X-100. The assays for MBP were performed in the standard reaction mixture (20μl, lacking histones) and the reactions were terminated by the addition of 10μl four times concentrated Laemmli sample buffer (Section 2.4). The phosphoproteins were resolved by SDS-PAGE on 15% w/v acrylamide gels. The gels were dried and the phosphoproteins identified by autoradiography. These were excised and the amount of phosphate that was incorporated into them was determined.

by the isotypes, so for example with histone III-S as substrate, the V_{max} of -β_1 was half that of of -γ but with histone H1, -β_1 had a higher V_{max} than -γ. Further, the K_m of -β_1 was lower than -α or -γ with MBP as substrate but with histone III-S as substrate, the K_m of -β_1 was higher than -α or -γ. These results indicate that the isotypes display different apparent affinities (K_m) and catalytic activities towards the protein substrates. There were no general traits of affinity for the isotypes. So for example, with protamine, the K_m of -α and -β were similar and the K_m of -γ was higher, indicating that the -γ isotype had the lowest affinity for this substrate; but with histone H1, the K_m of -γ was lower than either of the other isotypes. This was also true of the V_{max} data, where none of the isotypes was seen to have a generally higher catalytic activity towards all the substrates. The results suggest that the isotypes have differences in substrate specificity towards protein substrates. This may indicate that a fundamental difference between the isotypes is in their substrate specificities. The results for histone III-S and histone H1 are different from each other indicating that the contaminants in the histone III-S preparation significantly alter the apparent kinetic parameters of the isotypes for histone H1.

Despite the differences in kinetic parameters between the isotypes, comparison of the phosphopeptide maps for histone H1 and MBP phosphorylated by the isotypes shows that the isotypes appear to phosphorylate the same sites on these two substrates. In histone H1, the isotypes all phosphorylate one major and one minor peptide in both of the forms of histone H1 present in the preparation (Figure 5.3.2). The three isotypes all phosphorylate MBP on many sites in common (Figure 5.3.3). Comparison of the maps of MBP for each of the isotypes shows them to be similar and no unique phosphopeptides

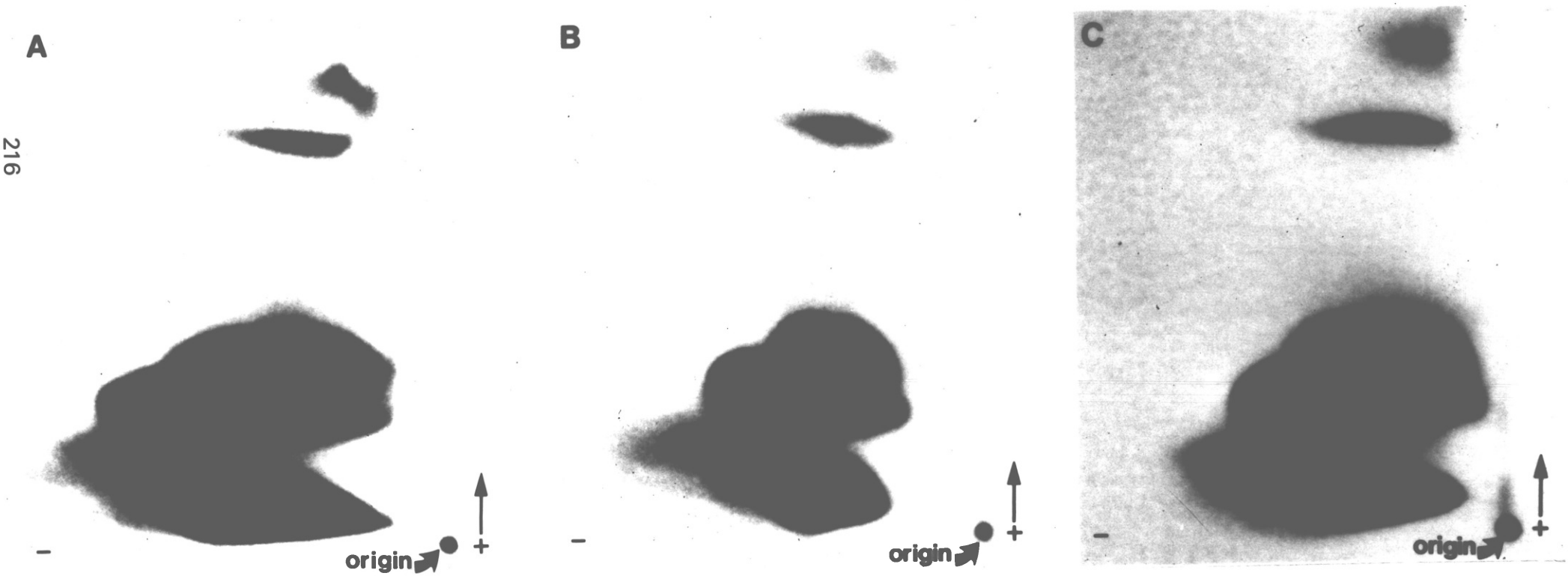
Figure 5.3.2 Phosphopeptide analysis of histone

Histone H1 (5μg) was phosphorylated by the PKC isotypes in the standard assay mixture (20μl) for three minutes and the reaction was terminated by the addition of 10μl four times concentrated Laemmli sample buffer, followed by immersion in a boiling water bath (5 min). The phosphorylated proteins were separated into the two forms of histone present in this preparation by SDS-PAGE analysis (see Figure 5.3.1). The gels were Coomassie Blue stained, dried and the phosphorylated products detected by autoradiographic analysis. The phosphorylated proteins were excised separately and will be referred to as the upper and lower bands (higher and lower Mr bands respectively). The gel pieces were extracted as described in Section 2.9 and the phosphopeptides were analysed in one dimension by electrophoresis at pH 1.9 (50mA, 45 min) (Section 2.9). The silica plates were dried and exposed to X-ray film (36 hr). The positions of the positive and negative electrodes are indicated and the position of the origin is shown by the arrow. Panels A and D are maps of the upper and lower histone bands respectively, phosphorylated by PKC-α; panels B and E are maps of the upper and lower histone bands respectively, phosphorylated by PKC-β_1; panels C and F are maps of the the upper and lower histone bands respectively, phosphorylated by PKC-γ.

A
B
C
D
E
F
−
+

Figure 5.3.3 Two dimensional phosphopeptide analysis of MBP

MBP was phosphorylated by PKC-α, -β_1 and -γ individually and the trypsin generated phosphopeptides were analysed. MBP (5μg) was phosphorylated in the standard reaction mixture (20μl) for 3 min and the reaction was terminated by the addition of 10μl four times concentrated Laemmli sample buffer (section 2.4). The phosphorylated proteins were analysed by SDS-PAGE (15% w/v acrylamide gels). The gels were Coomassie blue stained, dried and the phosphorylated proteins identified by autoradiographic analysis. The phosphoprotein containing gel pieces were excised and phosphopeptides were generated as described in Section 2.9. These were analysed in two dimensions; the first dimension was electrophoresis at pH 3.5 (50mA, 35 min) and the second was ascending chromatography as described in section 2.9. The positive and negative electrodes are shown and the direction of ascending chromatography is indicated by the straight arrow. The position of the origin is also indicated (curved arrow). Panel A = MBP phosphorylated by PKC-α; panel B= MBP phosphorylated by PKC-β_1; panel C= MBP phosphorylated by PKC-γ.


A
origin
+
−
B
origin
+
−
C
origin
+
−

could be detected for any of the isotypes. The results agree with the observations of Turner *et al* (1984) and Kishimoto *et al* (1985) that PKC phosphorylates numerous sites on MBP. The phosphopeptide maps show that the sites which are phosphorylated in histone H1 and MBP by the three PKC isotypes are the same. Therefore in spite of the differences that have been observed in K_m and V_{max} for these substrates *in vitro*, the sites of phosphorylation are the same. The differences in kinetic properties must therefore be due to different affinities and catalytic activities towards the same sites.

Huang *et al* (1988) have also shown that the K_m of the isotypes for histone III-S, protamine, MBP and poly(lysine, serine) (3:1) are different. However, the results of the study from Huang *et al* (1988) are different from the results of this study. They found that the -α isotype (type III) was on the whole the most active kinase and therefore gave the highest V_{max} values for all the substrates they tested. They also found that in general, the -γ isotype had the lowest K_m values for the substrates tested, suggesting that the affinity of this isotype for the substrates was greater than -α or -$\beta_{(1+2)}$. Although the details from their results are different from those described in this study, the conclusions that they reached were the same; namely that under comparable conditions the isotypes behave differently towards substrates, both in terms of affinities and catalytic rate. The differences seen between the results presented here and those of Huang *et al* (1988) may reflect the different assay systems used. Pelosin *et al* (1987) have separated PKC into four peaks of activity by hydroxylapatite chromatography. They have not identified the isotype content of each peak, but they have shown that the PKC in these peaks have different affinities for at least two protein substrates.

In order to investigate further the different kinetic properties of the isotypes for protein substrates, proteins that have been reported to be PKC substrates *in vivo* were tested as *in vitro* substrates. The proteins tested were glycogen synthase (GS), vinculin and the '80K' substrate of PKC that has been extensively charaterised by the laboratory of E. Rozengurt (see Rozengurt, 1985). None of these proteins are commercially available and I am therefore extremely grateful to Dr. J. Woodgett (Ludwig Inst, London) for supplying the GS; to Dr. D. Critchley (Leicester University) for supplying the vinculin and to Drs. C. Morris and E. Rozengurt (ICRF, London) for supplying the 80K.

5.3.2 80K

80K is a protein that was first identified as the major PKC substrate in 3T3 cells stimulated with TPA or DG; it has a Mr of 80,000 and a pI of 5 (Rozengurt, 1985). Phosphorylation of this protein can be detected in 3T3 cells within 15 seconds of stimulation of PKC and reaches a peak within one minute (Rodriguez-Pena *et al*, 1986). The function of this protein is unknown but its identification as a PKC substrate has resulted in its extensive characterization and subsequent purification from rat brain (Morris and Rozengurt, 1988). 80K can only be produced in limited amounts and so K_m and V_{max} values could not be generated. The comparison of the phosphorylation by the PKC isotypes was therefore restricted to a comparison of time course of phosphate incorporation, a crude titration and two dimensional phospho-peptide map analysis.

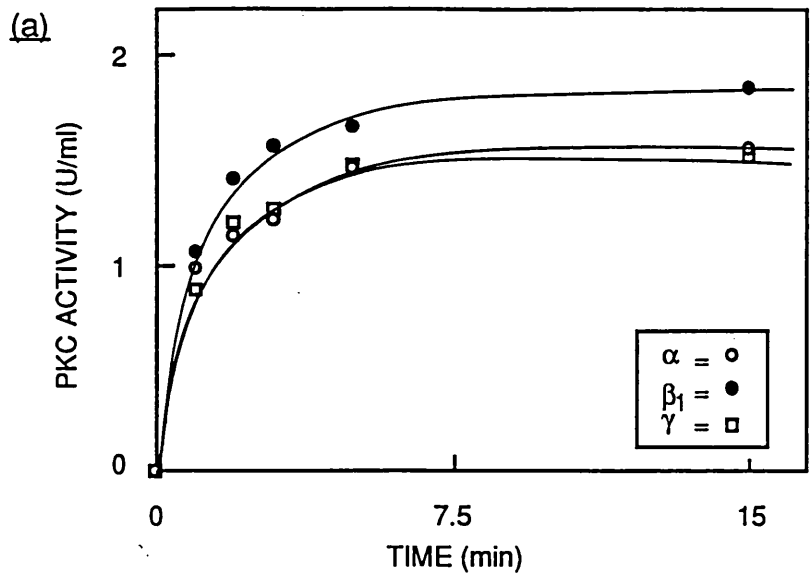
The rate of incorporation of phosphate into 80K substrate is similar for each isotype {Figure 5.3.4(a)}. The incorporation of phosphate

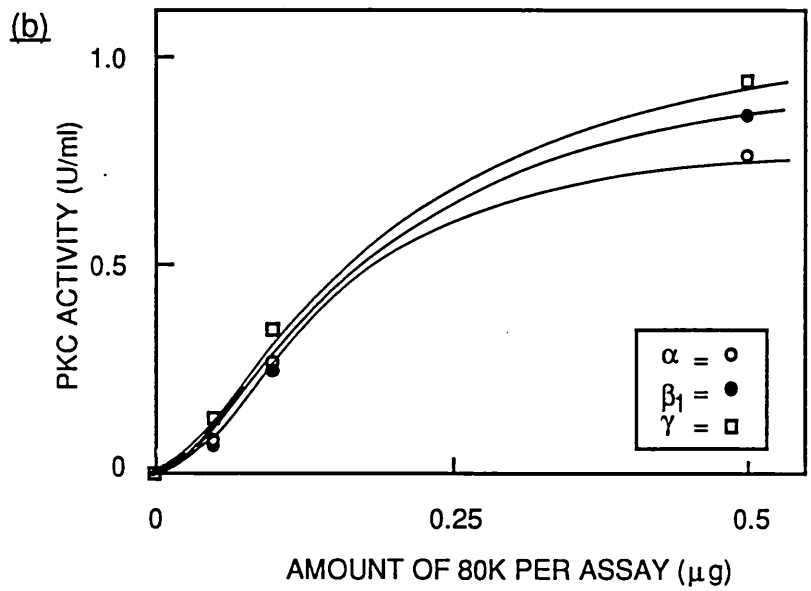
Figure 5.3.4(a) Time dependent phosphorylation of 80K substrate by PKC

The 80K substrate (1μg) was phosphorylated by the PKC isotypes in 10μl of standard assay mixture lacking histones (section 2.8) for various times. The reactions were terminated by the addition of 5μl four times concentrated Laemmli sample buffer (section 2.4) and the 80K was resolved by SDS-PAGE (10% w/v acrylamide gels). No phosphorylation was detected in the absence of PtdSer/ TPA. The gels were Coomassie blue stained, dried and the phosphoproteins detected by autoradiographic analysis. The radioactive bands were excised and the amount of incorporated ^{32}P was determined.

Figure 5.3.4(b) Phosphorylation of 80K substrate by PKC

Different amounts of 80K sample were phosphorylated in 10μl of standard reaction mixture, lacking histones (section 2.8). The reactions were incubated for 45 sec at 30°C and the phosphoproteins were analysed as described in Figure 5.3.4(a) above.


(a)
PKC ACTIVITY (U/ml)
2
1
0
0
7.5
15
TIME (min)
α =
β1 =
γ =


(b)
PKC ACTIVITY (U/ml)
1.0
0.5
0
0
0.25
0.5
AMOUNT OF 80K PER ASSAY (μg)
α =
β1 =
γ =

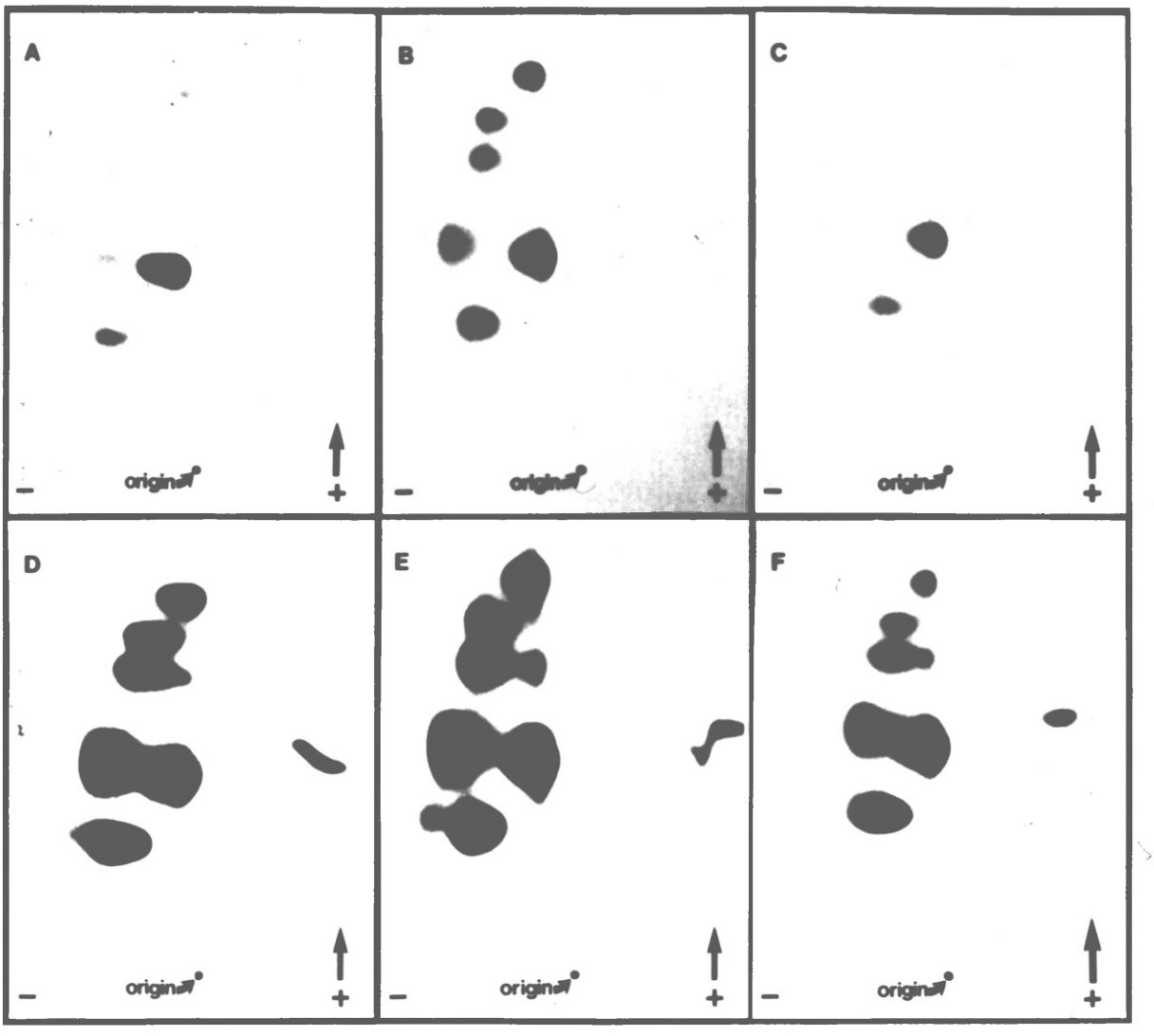
into 80K under these condidtions was extremely rapid, probably due to the low concentrations of 80K used. The amount of phosphate incorporated into three concentrations of 80K under limiting time conditions was also shown to be similar {Figure 5.3.4(b)}. These results indicate that there is no selective phosphorylation of the 80K substrate by any of the PKC isotypes. This suggests that *in vivo* 80K would be a substrate for PKC-α, -β_1 and -γ with similar kinetic parameters. The isotypes therefore appear to phosphorylate this protein with similar kinetic properties. Comparison of the two dimensional maps of 80K phosphorylated by the PKC isotypes shows that the isotypes appear to phosphorylate the same sites on this protein (Figure 5.3.5). Each phosphorylates one major site and a similar array of minor sites on this substrate; the precise site(s) of phosphorylation of the 80K substrate by the PKC isotypes remain to be mapped to confirm whether the sites are the same. Thus, activation of any one of these isotypes in vivo is predicted to result in the phosphorylation of 80K. This would indicate that this substrate does not have a role in the selection of a particular physiological pathway which the individual isotypes may control, but suggests that it has a more basic role in the responses of cells to PKC activating agonists. What this role is remains to be established.

5.3.3 Glycogen synthase

GS plays a central role in the control of carbohydrate storage in the liver and skeletal muscle. GS is itself controlled in a negative fashion by phosphorylations carried out by numerous kinases (see Roach, 1981; Cohen, 1982). PKC has been reported to phosphorylate GS at least two sites (Imazu *et al*, 1984; Wang *et al*, 1986) and House *et al* (1987) used synthetic peptides analogues based on GS(1-10) to probe the substrate requirements of PKC. As described in

Figure 5.3.5 Phosphorylation of 80K by PKC isotypes

80K substrate (1μg) was phosphorylated by each of the isotypes in 10μl of standard reaction mixture, lacking histones (section 2.8). The reactions were incubated for 45 sec and terminated by the addition of 5μl four times concentrated Laemmli sample buffer (section 2.4). The 80K was resolved by SDS-PAGE (10% w/v polyacrylamide). The gels were Coomassie blue stained, dried and the 80K was identified by autoradiographic analysis. The 80K samples were excised from the gels and treated as described in Section 2.9. The phosphopeptides thus generated were resolved by two dimensional phosphopeptide analysis. The first dimension was by electrophoresis at pH 3.5 (50 mA, 30 min) and the second by ascending chromatography as described (Section 2.9). The position of the positive and negative electrodes are shown and the direction of ascending chromatography is indicated by the large arrow. The origin is also indicated (curved arrow). The phosphopeptides were detected by autoradiographic analysis. Panels A and D= 80K phosphorylated by PKC-α; panels B and E= 80K phosphorylated by PKC-β_1; panels C and F= PKC phosphorylated by PKC-γ. Panels A, B and C were exposed to autoradiographic film for 12 hr; panels D,E and F are the same maps exposed for 72 hr.


A
origin
–
+
B
origin
–
+
C
origin
–
+
D
origin
–
+
E
origin
–
+
F
origin
–
+

section 5.2.2 one of these peptides was a good substrate for PKC-α and -β_1, but not for -γ. Purified GS was therefore compared as a substrate for the three isotypes of PKC. Under the assay conditions used (Table 5.3.2), [^{32}P] was incorporated into GS in a Ca^{++}/TPA/PtdSer dependent manner. Surprisingly however, the incorporation of phosphate was independent of exogenously added PKC and the amount of phosphate incorporated into GS did not increase in the presence of added PKC isotypes or unseparated PKC mixtures (Table 5.3.2). Heat treatment of GS (65°C, 20min) inativated the endogenous PtdSer/ TPA dependent kinase, but the resultant GS sample was not a PKC substrate and no phosphate incorporation could be detected. This data suggests that purified PKC-α, -β_1 or -γ do not phosphorylate GS, but the results are consistent with the observation that a PtdSer/TPA dependent GS kinase activity exists. This activity appears to co-purify with GS under some conditions but has not been identified (it should be noted that PKC defined by the standard histone H1 assay is very low in skeletal muscle, the source of the GS).

5.3.4 Vinculin

Vinculin is a protein found associated with adhesion plaques, where it is believed to anchor microfilaments to the cell membrane (Werth *et al*, 1983). Interest in this protein stems from the fact that transformed cells undergo numerous morphological changes and alterations in adhesion properties. Vinculin is a phosphoprotein and is phosphorylated by at least one transforming kinase (Sefton and Hunter, 1984). Vinculin has been reported to be a PKC substrate (Werth *et al*, 1983) and the *in vivo* phosphorylation of vinculin in response to TPA has been reported (Werth and Pastan, 1984). Purified vinculin (>95% homogenous) was tested as a substrate for

Table 5.3.2 The PtdSer and TPA dependent phosphorylation of GS.

PKC added	Glycogen synthase		Histone III-S	
	+PtdSer/TPA	-PtdSer/TPA	+PtdSer/TPA	-PtdSer/TPA
PKC-α	22,657	13,032	11,230	1,234
PKC-β_1	23,655	13,688	12,876	1,876
PKC-γ	25,430	12,866	11,873	2,156
PKC-mix	23,873	11,345	8,792	1,020
NONE	24,853	13,345	1,435	1,657

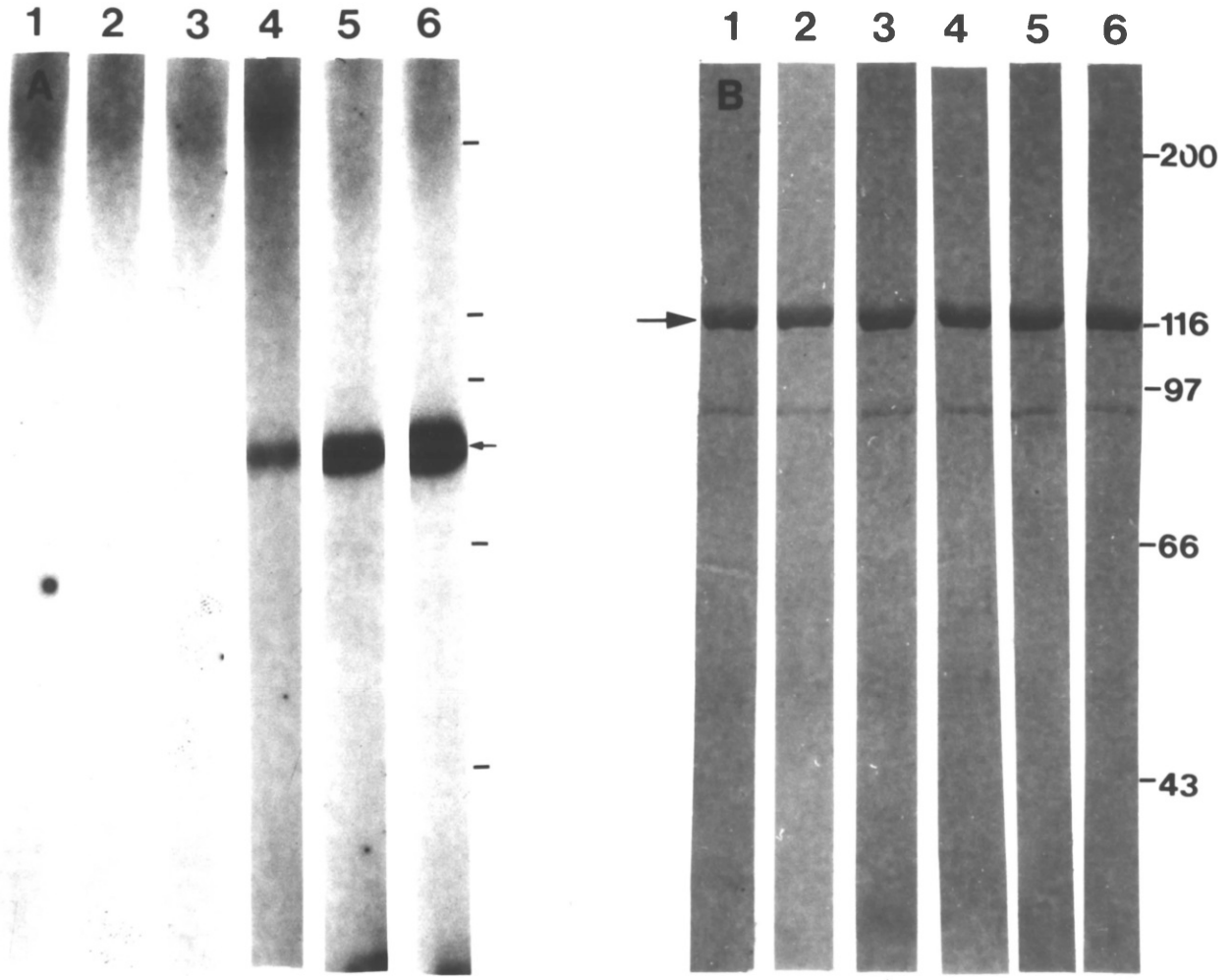
GS (6.25μg) was incubated with PKC in the standard reaction mixture (20 μl, lacking histone) at 30°C for 15 min. The reaction was terminated by the addition of TCA to 20% w/v and the mixture was incubated on ice for 10min. The precipitated proteins were collected by centrifugation (10,000 rpm, 10min) and the pellet was washed three times with 5% w/v TCA. The amount of [^{32}P] incorporated into the precipitates was determined. In a parallel experiment the incorporation of phosphate into histone III-S was measured. Histone III-S was treated in the same way as GS. The PKC isotypes were added as indicated. The 'mix' fraction was a threonine-Sepharose pool (Section 4.1.2) and as such contained PKC -α, -β_1, -β_2 and -γ. NONE indicates no PKC was added. The specific activity of the ATP was 120 cpm/pmol/min.

the PKC isotypes. Under the assay conditions used (Figure 5.3.6), vinculin was not phosphorylated by PKC-α, -β_1 or -γ, although the autophosphorylation of PKC could be detected (Figure 5.3.6). Incubation of freshly purified vinculin with PKC for up to one hour in the presence of [γ-^{32}P]ATP with a specific activity of 2,500 cpm/pmol did not result in detectable phosphorylation of vinculin. The presence of the vinculin was confirmed by the Coomassie blue staining (Fig 5.3.6).

The kinetic parameters of phosphorylation observed by Werth *et al* (1983) indicate that in their assay system vinculin was a poor substrate for PKC, with a V_{max} of 24nmol/min/mg. The preparation of vinculin used by these authors appeared to contain an endogenous Ca^{++}/PtdSer dependent kinase that phosphorylated vinculin. Phosphorylation of vinculin was enhanced by the addition of partially purified PKC. In subsequent work from this laboratory, the authors were able to demonstrate that the level of phosphate in vinculin purified from 3T3 cells and chick fibroblasts increased after TPA treatment (Werth and Pastan, 1984). However, there were details in their publication that were inconsistent with the phosphorylation being a direct result of PKC activation. First, the time course of phosphorylation was slow, with half maximal phosphorylation being detected after about 20 minutes and maximal activation after one hour. The work from Rodriguez-Pena *et al* (1986) indicates that incorporation of phosphate into the 80K substrate is extremely rapid after PKC activation, with maximal incorporation being detected within one minute. This suggests that vinculin may be phosphorylated by another kinase which is itself activated by PKC. Second, the addition of Ca^{++} to intact cells resulted in increased phosphorylation of vinculin. This the authors suggest was possibly

Figure 5.3.6 The phosphorylation of vinculin by PKC

Freshly purified vinculin (5μg) was incubated with PKC in the standard assay buffer (20μl) with [γ-^{32}P] ATP at a specific activity of 2,500 cpm/pmol. The reactions were incubated for 60 min and terminated by addition of 10μl 4 times concentrated Laemmli sample buffer (section 2.4). The vinculin was resolved by SDS-PAGE (7% w/v polyacrylamide gels). The gels were Coomassie blue staind, dried and exposed to autoradiographic film for 7 days. Lanes 1, 2 and 3= vinculin phosphorylated by PKC-α, -β_1 and -γ respectively in the absence of activating co-factors; lanes 4, 5 and 6= vinculin phosphorylated by PKC-α, -β_1 and -γ in the presence of activating co-factors (Ca^{++}/ PtdSer/ TPA). Panel A shows the autoradiographic analysis of the gels and panel B shows the Coomassie stained gels themselves. The position of PKC is indicated by the smaller arrow; the position of vinculin is indicated by the larger arrow. The position of migration of standard molecular mass proteins are also indicated ($x10^{-3}$). A minor contamination present in the vinculin preparation can be detected in the Coomassie stained gels and has an apparent Mr of 90,000. This is a proteolytic fragment of vinculin (D. Critchley, personal communication). The autophosphorylation of PKC under these conditions is clearly visible.


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2
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4
5
6
A
B
200
116
97
66
43

due to the Ca^{++} dependent activation of PKC in the absence of TPA or DG as had been shown to occur *in vitro* at high concentrations of Ca^{++} (Takai *et al*, 1979a). However, Kaibuchi *et al* (1983) have demonstrated that in human platelets the mobilisation of Ca^{++} in the absence of DG or TPA was not sufficient to activate PKC (defined by 40K phosphorylation). This argues against the conclusions of Pastan and Werth that Ca^{++} causes direct activation of PKC with subsequent phosphorylation of vinculin.

The results presented here suggest that vinculin is not a substrate for PKC-α, -β_1 or -γ, but it does not exclude the possibility that the phosphorylation is due to another isoform of PKC. The -δ and -ε isotypes are present in brain, heart and lung (Ono *et al*, 1988; Ohno *et al*, 1988; Schaap *et al*, 1989) but until the cellular distribution of these is known, it is not possible to predict whether either of these isotypes is responsible for the phosphorylation of vinculin. The results could be due to PKC activating a cascade which contains a vinculin kinase. Thus activation of PKC by TPA would result in increased phosphorylation of vinculin in vivo. This is a possibiltiy that Werth and Pastan (1984) have suggested.

The results from the protein substrates shows that the PKC isotypes display differences in both the apparent catalytic rate and the apparent affinity for some protein substrates. Some substrates appear to be efficeintly phosphorylated by all three PKC isotypes with similar kinetic parameters. The isotypes therefore may selectively phosphorylate different as well as similar proteins *in vivo*. The consequences of these observations is discussed in Chapter Six.

CHAPTER SIX

DISCUSSION

The aim of this project was to investigate whether the isotypes of PKC had different biochemical properties that would indicate the reason(s) for the multiplicity of this kinase family. To this end, the native PKC-α, -β_1 and -γ were purified from bovine brain and separated by hydroxylapatite chromatography. As a prerequisite to the purification and separation, it was necessary to develop antibodies which would distinguish between the isotypes in order to monitor their behaviour on chromatographic columns. In Chapter Three a method for producing high titre antibodies specific for PKC-α, -β_1, -β_2 and -γ is described. Peptides corresponding to the variable domains of the PKC isotypes were synthesised and used to generate polyclonal antisera in rabbits. The peptides from the C-termini were the most antigenic and have resulted in the production of antisera with high titres which are exquisitely selective for the relevent isotypes. These antisera are able to recognise the native and the denatured PKC isotypes in both purified and crude preparations of PKC and were used primarily to monitor the co-purification of the isotypes from bovine brain.

A purification protocol was attempted but did not co-purify the PKC isotypes efficiently as described in section 4.1.1. A new procedure was developed through which PKC-α, -β_1, -β_2 and -γ co-purified. The mono-specific antisera were essential in allowing the co-purification of the isotypes to be monitored. The purification protocol developed produces milligram amounts of PKC-α, -β_1, -β_2 and -γ in a highly purified state. This sample of PKC has been used to generate monoclonal antibodies against PKC-γ as described by

Cazaubon *et al* (see ref 2, appendix). It has also been used in conjunction with the monospecific antisera to demonstrate that PKC-α, -β_1, -β_2 and -γ are all substrates for calpain as described in Chapter Three.

The purified PKC has been separated into three fractions of activity, containing PKC-α, -β_1 and -γ by hydroxylapatite chromatography. These pools have been analysed by SDS-PAGE and have been judged to be pure. The antisera have been used to identify the isotype content of each pool and to show that the separation was efficient. These pools do not contain any histone III-S kinases, other than those identified by the immunoblotting technique and do not contain any Ca^{++}/ PtdSer/ TPA(DG) dependent kinases. Thus these pools have been subjected to detailed biochemical analysis and *in vitro* characterisation. The pools were ideally suited for this purpose since they were pure, well separated and did not contain any Ca^{++}/ PtdSer/ TPA independent kinase activity. The results indicate that the isotypes are different with respect to activation by DG and phorbol esters *in vitro*. They also have different substrate specificities and these results suggest that they may serve different functions *in vivo*. The monospecific antisera are being used to assess the individual behaviour of the isotypes *in vivo*. Studies so far indicate that there do appear to be differences in the responses of the isotypes to TPA in some systems.

During the course of these studies, other workers have presented data in which they have analysed different fractions generated by hydroxylapatite column chromatography. However in all cases, the systems employed have been poorly defined. None of the studies have addressed the issue of PKC isotype co-purification, neither have

they convincing data to show that the subsequent separation was efficient (see for example Jaken and Kiley, 1987; Pelson *et al*, 1987; Shearman *et al*, 1987). During the development of the separation protocol, it became apparent that multiple peaks of PKC activity could be produced by hydroxylapatite chromatography and chromatography on other column matrices. However, analysis with the monospecific antisera of the pools thus generated has shown that they often contain multiple isotypes of PKC. The monospecific antisera have therefore proven invaluable for the development of efficient separation protocols. Studies involving separation of the isotypes which have been published from other laboratories have not had the benefit of similar antisera and have therefore had to make assumptions about the separated products. However, it is apparent from the results presented in this thesis that these assumptions may not be valid, since there is no evidence that the isotypes from different sources or in different states of purity behave in the same manner.

Previous studies have been further hampered in their studies by the lack of suitably characterised antibodies with which to monitor the *in vivo* characteristics of the individual isotypes. Some studies have been published in which it is claimed that mono-specific antisera were used to investigate the *in vivo* distribution of the PKC isotypes, but suitable evidence is not available to corroborate the results. In the studies of Ase *et al* (1988) for example, these authors do not provide immuno-blot evidence to show that the only antigen which is recognised by their antisera is PKC. The antisera described here should therefore prove to be extremely useful for studies involving PKC isotype behaviour *in vivo*.

6.1 CONCLUSIONS

The kinetic analyses have revealed that the isotypes have the potential to be individually regulated *in vivo*, because their *in vitro* dependencies on Ca^{++}, DG and TPA are different. The isotypes also have different substrate specificities which suggests that they may phosphorylate unique cellular targets. The *in vitro* dependence of PKC on PtdSer, Ca^{++} and TPA/ DG is complex and depends on the relative concentrations of these activators (Hannun *et al*, 1985; Hannun and Bell, 1985). Nonetheless, these authors have proposed a model for the activation of PKC. In their model they postulate that PKC associates with the membrane in a Ca^{++} dependent manner but remains inactive until DG (or TPA) associates with the complex (Ganong *et al*, 1987). They suggest that PKC requires four molecules of acidic phospholipid (PtdSer) and one molecule of DG (TPA) to orientate to the divalent metal ion (Me^{++}), forming a Ca^{++} bridge between enzyme and lipid co-factors.

Bazzi and Nelsestuen (1988) oppose this model and have suggested an alternative. Like Ganong *et al* (1987), they suggest that the association of PKC with membranes is necessary but not sufficient for activation. They have shown that PKC is able to associate with membranes and remain inactive and that a second event is required for activation. This second event appears to involve the insertion of a hydrophobic region of the PKC molecule into the membrane bilayer. The inserted form of the kinase becomes constitutively active and does not appear to require activating co-factors for maintenance of the active state. This hypothesis is partially supported by the observations of Walker and Sando (1988) who have shown that short chain neutral lipids can activate PKC in the presence of DG and Ca^{++}.

Their results suggest that the potential of lipids to activate PKC depends on their ability to form micelles, rather than on the structure of the lipid head group as was suggested by Ganong *et al* (1987). Thus it appears that it is the hydrophobic lipid environment and not the type of lipid from which it is composed which is important for PKC activation. This view would certainly be supported by the observations of arachidonic acid activation of PKC as described in section 5.1.5 of this thesis. In mixed micelles of Triton X-100 and PtdSer, arachidonic acid can support the activity of PKC-α at low concentrations; and in mixed micelles of Triton X-100 and arachidonic acid, low concentrations of PtdSer can support PKC-α activity. Arachidonic acid/ Triton X-100 mixed micelles could not support the activity of the kinase in the absence of PtdSer and PtdSer/ Triton X-100 mixed micelles could not support the activity of PKC-α in the absence of arachidonic acid. These observations imply that a hydrophobic environment with which PKC can associate is required for activation. The membrane constituents therefore appear to be important only in allowing the interaction between enzyme and hydrophobic environment to take place. It would appear that in mixed micelles, at least two types of interactions are necessary for association to occur and that these interactions can be fulfilled by a combination of PtdSer and TPA/ DG, or by a combination of PtdSer and arachadonic acid.

The existence of two separate activator binding sites has been suggested by the studies of Cazaubon *et al* (see ref 2, appendix). In these studies, monoclonal antibodies were raised to PKC-γ. The epitopes for these antibodies were localised to the regulatory domain of the kinase by proteolytic studies. Two of the antibodies were found to be inhibitory towards PKC activity in the presence of

Ca^{++}/ PtdSer/ TPA. In order to discover where these antibodies were binding, PKC was incubated with activating co-factors and then immunoprecipitation by the antibodies. The binding of one of the antibodies (5A2) was inhibited by Ca^{++}/ PtdSer/ TPA or by Ca^{++}/ PtdSer in the absence of TPA. The binding of the other (36G9) was only inhibited by Ca^{++}/ PtdSer/ TPA and not by Ca^{++}/ PtdSer alone. Thus it appears that these two monoclonals bind to different epitopes of PKC-γ which are both within the region of the lipid and/or TPA binding sites. They also suggest that binding of PKC to membranes occurs in the absence of TPA (DG) as proposed by Bazzi and Nelsestuen (1988) and that the binding of lipids and TPA (DG) occur at distinct sites. The effects of arachidonic acid on the binding of the monoclonal antibodies has not been investigated, but it would be useful to establish if there is any competition between arachidonic acid (at low and high concentrations of PtdSer) and the binding of the monoclonals to try to establish whether arachidonic acid binds to the PtdSer, the TPA site or to an unique third site (it should be remembered that the responses of PKC-γ to arachidonic acid are different from those of PKC-α).

The features which are important for activation of PKC by phorbol ester and DG's indicate that both the polar portions of these activators and the hydrophobic regions are required. The most potent phorbol ester is TPA and comparison of this compound with other classes of PKC activating tumour promotors has suggested important structural features which appear to be necessary for activity (Jeffrey and Liskamp, 1986; Wender *et al*, 1986; Itai *et al*, 1988). While not all in agreement, the important features include conserved hydrophobic moieties and at least three hydrophilic groups. These observations are consistent with the notion that PKC must associate

with lipid membranes in a polar (Me^{++}) dependent manner and that subsequently hydrophobic interactions become important. Similar conclusions have been reached with studies on DG dependent activity. Derivatisation of DG molecules has shown that both the hydrophobic chain length and the 3' hydroxyl are important for activity *in vivo* and *in vitro* (Davis *et al*, 1985b). Further evidence has shown that both of the carbonyl groups and the hydroxyl group are required for activity (Ganong *et al*, 1985). There are precise steriochemical requirements at the glycerol back bone for activity and a strict requirement for the lipophilic portion of the molecule (Bonser *et al*, 1988).

The ac tvation of PKC is dependent on the presence of Me^{++} ions in most situations. In section 5.1.1, it was observed that the Mg^{++} requirement of the PKC isotypes was greater than their requirement for ATP, even though these compounds are thought to bind as a Mg.ATP complex. This suggests that Mg^{++} may bind to the enzyme in a second site which is distinct from the catalytic site. Binding of phorbol esters to soluble PKC has been shown to be optimum in the presence of free Mg^{++} and Ca^{++} (Sando and Young, 1983; Ashendel *et al*, 1983) and this may explain why high concentrations of Mg^{++} are required for the *in vitro* activity of PKC. It has been shown that zinc (Zn^{++}) is able to potentiate the activity of PKC in the presence of arachidonic acid at low concentrations of Ca^{++}, but at high Ca^{++} concentrations, Zn^{++} is inhibitory towards this enzyme (Murakami *et al*, 1987; Csermely *et al*, 1988a). Phorbol ester binding to PKC has also been shown to be potentiated by Zn^{++} (Csermely *et al*, 1988b). Murakami *et al* (1987) have suggested that PKC has two Ca^{++} binding sites and a third Me^{++} binding site which can be occupied by Zn^{++} (Murakami *et al*, 1987). One question raised by these observations is

whether the high concentrations of Mg^{++} that are required for the *in vitro* activation of PKC can be satisfied by Zn^{++}. If this is so, then it may indicate that the activation of PKC is dependent upon more than one Me^{++} ion. The precise function of each in directing the association of PKC with membranes and its subsequent activation could be investigated by combinations of Me^{++} ions. There have been suggestions that the Zn^{++} requirement of PKC is involved in DNA binding (see Murakami *et al*, 1987 and Testori *et al*, 1988), however to date there is no firm evidence for this.

These observations indicate that PKC association with lipid vesicles and membranes is complex and can be initiated by particular combinations of divalent metal ions, phospholipids and activators which can be either DG, fatty acids or hydrophobic tumour promotors. Once PKC is activated, the role of the Me^{++} ions and activators in maintaining this state is questionable (Bazzi and Nesestuen, 1988). The consequences of these observations in activating the isotypes are complex. As discussed in Chapter Five, the isotypes respond to DG, arachidonic acid and TPA with different characteristics. This probably reflects differences in affinity of the isotypes for the activators tested. Further to this, the cell studies in section 5.1.6 indicate that in some cells particular isotypes respond differently to TPA. It is not yet known whether the differential activation of PKC isotypes by TPA *in vivo* can be extrapolated to the activation by DG but it would appear that they have different affinities for some classes of DG as well as for TPA. In all the studies with activators of PKC that have been performed to date, the isotype content of the PKC sample has not been defined, but it would be interesting to establish what structural features are required for activation of each PKC isotype. It would then be possible to predict whether the

30 possible forms of DG that can be produced from PIP_2 (Holub, 1970) would differentially regulate the PKC isotypes. The production of DG in cells is extremely rapid and transient. In platelets stimulated by thrombin, DG production peaks within 30-40 sec and returns to normal levels after 2-3 min (Kaibuchi *et al*, 1983 and Prescott and Majerus ,1983). If the isotypes do respond to particular DG molecules differently, then the transient nature of DG production would enhance the effects on isotype activation, because the DG would not accumulate in high enough levels to activate all the isotypes present. Further, the subsequent production of arachidonic acid from DG may prolong the activation of particular isotypes, or may even activate isotypes which were not activated in the initial DG pulse. This may form the basis of selective responses directed by the PKC isotypes.

The results presented in Chapter Five also indicate that the isotypes show differences in substrate specificity towards both peptide and protein substrates. This suggests that the isotypes can phosphorylate unique cellular targets although some substrates (eg 80K) appear to be efficient substrates for all the isotypes. In order to investigate further the requirements of each isotype for substrate recognition, it is convenient to use peptide substrates, since these can be manipulated to reveal precise structures. A rationale for the design of peptides in order to investigate the substrate specificity of the isotypes is described below. The results from this study are not yet available.

The greatest difference in substrate specificity was observed with peptide GS1-10, which was an efficient substrate for PKC-α and -β_1 but a poor substrate for PKC-γ (see section 5.2.2). Alignment of the

peptides as shown in Figure 6.1 indicates the importance of basic residues in substrate recognition of PKC. As mentioned in section 5.2, basic residues are known to be important in the recognition of substrates for PKC and in particular, the importance of Arg^4 in GS1-10 is established. Replacement of this residue with a Lys has been shown to increase the K_m of this peptide for PKC by 20 fold (House *et al*, 1987). Addition of basic residues to the C-terminus of this peptide or substitution of Ser^3 or Thr^5 reduced the K_m of the peptide for PKC by 2-3 and 10 fold respectively: Val^8 is also necessary for PKC recognition of this peptide (House *et al*, 1987). Work with other peptides has shown how the position of basic residues is extremely important for target recognition. With the peptides S6(229-239), substitution of Arg^{238} for Ala switched the site of preferential phosphorylation from Ser^{236} to Ser^{235} (House *et al*, 1987). Similar conclusions about the importance of position of basic residues in PKC substrate recognition were reached by Turner *et al* (1985) when using peptides based on MBP sequences.

Figure 6.1 Peptide substrates for the PKC isotypes

α-pep	DVAN**R**FA**RK**G<u>S</u>L**R**Q**K**NV
β-pep	EST**VR**FA**RK**G<u>S</u>L**R**Q**K**NV
γ-pep	GP**R**PL FC**RK**G<u>S</u>L**R**Q**K**NV
ε-pep	E**RMRPRK**R**Q**G<u>S</u>V**RRR**V
EGF-R3	V**RK**<u>T</u>L**RRL**$_{NH_2}$
p60-1	GSS**K**S**K**P**K**DP<u>S</u>Q**RRR**SL **E**$_{NH_2}$
MBP-1	GA**GR**GL SL S**R**F<u>S</u>WGA
GS1-10	PL S**R**TL<u>S</u>VAA

The peptide substrates are compared so that the target residues (underlined) are aligned. The basic residues are highlighted in bold. The amidated C-termini of EGF-R3 and p60-1 are also in bold, since these will be charged at neutral pH.

MBP-1 is similar to GS1-10 in that it does not have any C-terminal basic residues, but it does contains N-terminal Arg residues two and eight residues from the target. This peptide was also a preferable substrate for PKC-α and -β_1 over -γ, although the differential was only two and not ten fold as with GS1-10. With these observations in mind, peptides were designed based on the peptide GS1-10 to probe the requirement of the isotypes for substrate recognition. Before the peptides were designed, the site of phosphorylation by PKC on this peptide was determined. The results are in agreement with House *et al* (1987) and show that for PKC-α, phosphorylation was only detected on Ser^7 (Figure 6.2). In order to make the analysis of the results easier, Ser^3 and Thr^5 were replaced by Ala residues, thus resulting in a single target residue. The peptides are designed so that the importance of the position of the basic residues in PKC isotype substrate recognition may be determined (Figure 6.3). The only basic residue in GS1-10 is Arg^4, although the N-terminus will be charged at neutral pH and therefore may contribute to peptide recognition, even though it is seven residues from the target. The peptides are extended at their N-termini by two residues and the Pro was replaced by Gly or Arg residues to investigate how important basic residues at this position are for isotype substrate recognition. The C-terminus was extended one residue and combinations of Arg and Ala residues were placed 1 and/ or 2 residues C-terminal to the target in order to investigate the importance of basic residues thus placed in isotype substrate selectivity (Figure 6.3). In MBP-1, the Arg was two residues from the target Ser, whereas in GS1-10, it was three residues from the target (Figure 6.1) and the selectivity against the peptides seemed to increase as the basic residue moved further from the target. The peptides were therefore designed to

Figure 6.2 Phosphorylation of GS1-10 by PKC-α

Peptide GS1-10 (10μM) was phosphorylated by PKC-α in 40μl standard reaction mixture (15min). The reaction was terminated by the adition of 10μl 100% trichloroacetic acid and the mixture was incubated on ice for 10 min. The phosphopeptides were derivatised as described in section 2.10 and the derivatised peptides sequenced.

The table shows the yield of the residues at each sequencing cycle. The sequence of the peptide is confirmed but the yield drops off dramatically due to loss of peptide from the polybrene disc. No residue could be identified at cycle 7. Shown in panels A and B are the chromatographs from cycles 3 and 7 respectively. Ser residues are found in both these positions in GS1-10. The position of Ser in cycle 3 is shown and the expected position of Ser in cycle 7 is also indicated. However, little Ser could be detected at residue 7 but a novel peak was found to elute just before the diphenylthiourea (DPTU) peak on this chromatogram (indicated by the ?). This is the expected position of elution for the *S*-ethylcysteine according to Holmes (1987). The lack of Ser at this cycle suggests that a high stoichiometry of phosphorylation of the peptide occured under these conditions; however this may have been due to the separation of the unphosphorylatedpeptide from the phosphopeptide on the G25 column.

Residues identified at each cycle

Cycle number	Residue	Amount of residue (pmol)
1	Proline	115
2	Leucine	130
3	Serine	65
4	Arginine	28
5	Threonine	72
6	Leucine	65
7	S-ethylcysteine	ND
8	Valine	30
9	Alanine	22
10	Alanine	18

ND= not determined

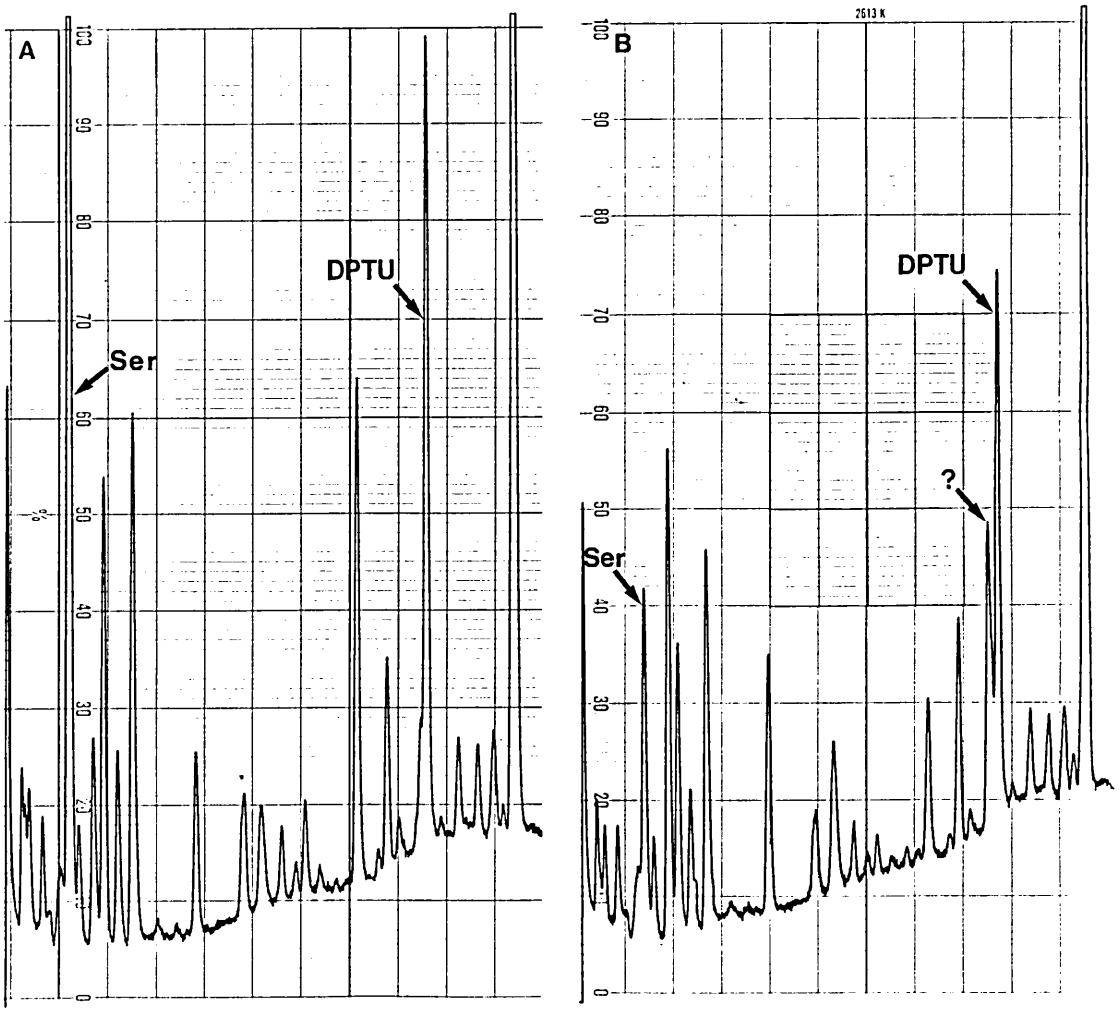


Figure 6.3 Peptides designed to probe substrates requirements of PKC-α, -β_1 and -γ.

	Peptide code GS1-10
NH_2-P L S **R** T L S V A A-COOH	
NH_2-G G G L A **R** A L S V A A G-COOH	1.1
NH_2-G G G L A **R R** L S V A A G-COOH	1.2
NH_2-G G G L **R R** A L S V A A G-COOH	1.3
NH_2-G G **R** L A **R** A L S V A A G-COOH	1.4
NH_2-G G **R** L A **R R** L S V A A G-COOH	1.5
NH_2-G G **R** L **R R** A L S V A A G-COOH	1.6
NH_2-G G G L **R** A A L S V A A G-COOH	1.7
NH_2-G G G L A A A L S V A A G-COOH	1.8
NH_2-G G G L A **R** A L S V A **R** G-COOH	2.1
NH_2-G G G L A **R R** L S V A **R** G-COOH	2.2
NH_2-G G G L **R R** A L S V A **R** G-COOH	2.3
NH_2-G G **R** L A **R** A L S V A **R** G-COOH	2.4
NH_2-G G **R** L A **R R** L S V A **R** G-COOH	2.5
NH_2-G G **R** L **R R** A L S V A **R** G-COOH	2.6
NH_2-G G G L **R** A A L S V A **R** G-COOH	2.7
NH_2-G G G L A A A L S V A **R** G-COOH	2.8
NH_2-G G G L A **R** A L S V **R R** G-COOH	3.1
NH_2-G G G L A **R R** L S V **R R** G-COOH	3.2
NH_2-G G G L **R R** A L S V **R R** G-COOH	3.3
NH_2-G G **R** L A **R** A L S V **R R** G-COOH	3.4
NH_2-G G **R** L A **R R** L S V **R R** G-COOH	3.5
NH_2-G G **R** L **R R** A L S V **R R** G-COOH	3.6
NH_2-G G G L **R** A A L S V **R R** G-COOH	3.7
NH_2-G G G L A A A L S V **R R** G-COOH	3.8

The peptide sequences are based on the sequence of GS1-10. The basic residues are highlighted in bold; the targets are underlined. The peptide which most resembles GS1-10 is 1.1. Ser^9 in 1.1 corresponds to Ser^7 in GS1-10. The positions in 1.1 which correspond to Ser^3 and Thr^5 have been replaced by Ala residues. The position in 1.1 which corresponds to Pro^1 in GS1-10 has been replaced by a Gly and the N-terminus of this peptide has been extended by two residues. The C-terminus of 1.1 has also been extended by one residue compared to GS1-10. All the other peptides in the series are based on the sequence of 1.1 with the positions of the Arg residues being altered to investigate the importance of these residues in PKC isotype substrate recognition.

investigate how Arg or pairs of Arg residues 2, 3 and 4 amino acids from the target affected isotype selectivity (Figure 6.3). These peptides are currently being synthesised. It is hoped that this series of petides will reveal the features which are essential for isotype substrate recognition. It is anticipated that this study may result in the identification of, or subsequent design of, peptides that are specific for individual PKC isotypes and the results may aid in the identification of unique PKC isotype substrates.

Differential phosphorylation of particular proteins is likely to direct particular physiological responses in cells. In addition to this, the rate of turnover of phosphate in at least one PKC substrate has been shown to be extremely rapid *in vivo* (Rodriguez-Pena *et al*, 1986). In this case, the phosphate is lost from the substrate after removal of the agonist responsible for PKC activation with a half life of about 2 minutes. The immediate effects of PKC activation are therefore very short lived although other more permanent changes may follow. The effects of activation of a particular PKC isotype may therefore occur very rapidly *in vivo* so that even if other isotypes are activated subsequently, the biochemical consequences of the initial events may already have been established. The sequential activation of particular isotypes may also be a mechanism by which physiological responses are directed, with temporal activation of each isotype in turn directing particular parts of the overall response.

The analysis of the purified isotypes has shown them to differ in two fundamental ways. First, they respond to activating co-factors differently and second, they have different substrate specificities. This suggests that the isotypes can be activated by unique signals

and could then phosphorylate unique cellular proteins; support for this comes from studies on the EGF receptor (Iso *et al*, 1987). This indicates a degree of selectivity in cellular responses. It may indicate that in particular cells, hormones can activate the isotypes individually and therefore can tailor the physioloigical responses of cells to extracellular signals. This 'one receptor, one kinase' model is problably too simplistic, because the isotypes also have common activator characteristics (eg the response of -α and -β_1 to Ca^{++}) and subtrates (eg 80K). Thus the isotypes may direct similar physiological responses of the growth factors in some cells but may control unique and divergent responses in others. These possibilities are shown in Figure 6.4. One final consequence of the results from this study concerns the question of previous studies with PKC. All of the early studies and most of the later studies have been performed with undefined PKC isotype pools (*in vitro*) or species (*in vivo*). Since it appears that the isotypes have different biochemical properties, the question of which biochemical property (activator dependence/ substrate specificity) belongs to which isotype should be addressed. Further, the activation of PKC *in vivo* by addition of exogenous activators or particular external messengers (growth factors/ hormones) may have different physiological consequences, depending on which PKC isotype(s) are present. The results from these types of studies should be re-examined as should the physiological importance of PKC down-regulation in view of the fact that in some systems the isotypes behave differentially *in vivo* in response to TPA (section 5.1.6).

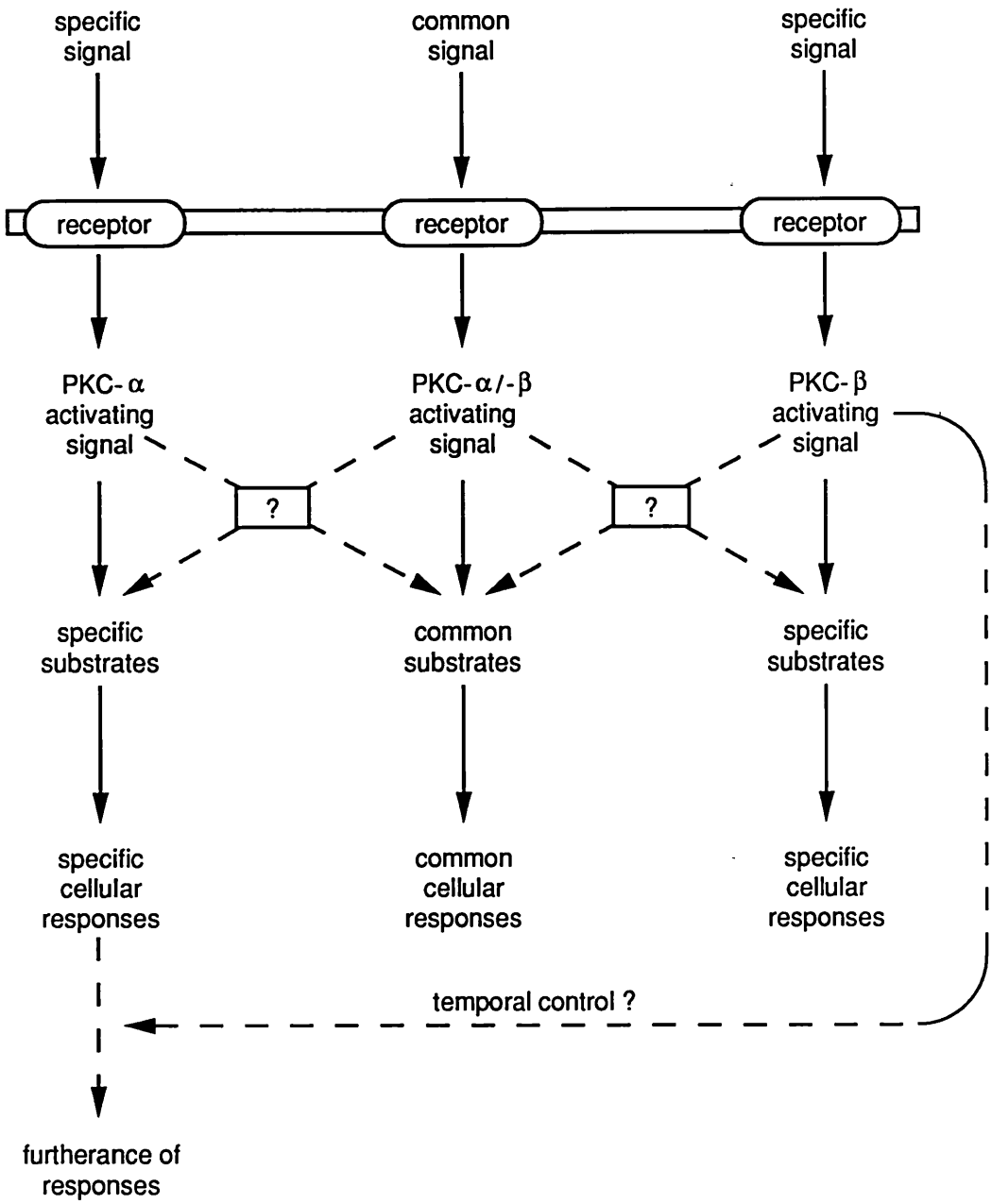


Figure 6.4 Cellular significance of PKC isotypes

The possibilities for differential activation of PKC-α and -β in a putative cell containing both is depicted. Specific signals may activate individual PKC isotypes through particular receptors. The activated kinases then phosphorylate specific (and common) substrates and through these the potential for particular cellular responses is realised. The possibility of temporal control of the isotypes is indicated in which PKC-β activation is delayed but necessary for complete activation. A pathway in which both PKC -α and -β are activated, resulting in a common cellular response.

6.2 PERSPECTIVES

Signal transduction is a ubiquitous mechanism by which cells are able to respond to external signals. PKC is one of a number of signal transducing pathways and traditionally these pathways were viewed merely as coupling responses, not responsible for any of the diversity of the plethora of responses that cells have to external signals. The specificity for these was believed to reside almost entirely at the receptor level, with individual cell responses being dictated by the receptor repetoire which they expressed. However, this concept may be wrong in view of the results presented in this thesis. These results suggest that there is diversity within the signal transducing pathways which is responsible for some of the diversity of responsiveness of cells to external stimuli. The cloning of multiple forms of cA kinase (Edelman *et al*, 1987) suggests that in this system too, the signal transducing pathways may contain an element of diversity. The existence of multiple isotypes which have been conserved through evolution to such a high degree (Parker *et al*, 1989) and their carefully controlled cellular expression (see for example Coussens *et al*, 1986; Brandt *et al*, 1987; Huang *et al*, 1987a; Shearman *et al*, 1987; Yoshida *et al*, 1988; Nishizuka, 1988) suggests that the isotypes serve particular functions in individual cells. Further, the expression of multiple isotypes in individual cells (Brandt *et al*, 1987; Nishizuka, 1988) and their localisation into particular cellular compartments (Nishizuka, 1988) supports the idea that they serve unique functions and that in some cells multiple PKC isotypes are required for a complete repetoire of cellular responses to external messengers.

More information on the function of the isotype in individual cells will be gained with the identification of particular isotype substrates and activating signals. Synthetic peptides will increase our understanding of the substrate issue, but the design of activators specific for each isotype will await further structural studies on individual isotypes. However, effects on the behaviour of the isotypes can be followed by the use of the mono-specific antisera in order to establish how the individual proteins translocate, become phosphorylated (see for example ref 3, appendix) or down-regulate in response to particular activating signals. Thus it will become possible to establish the role of each isotype in the responses of cells to extracellular signals and explain how such diversity is generated. This will lead to a greater understanding of the role of PKC in cells and the effect that this kinase has on other signal transducing pathways.

REFERENCES

Ahmad, Z., Lee, F.-T., DePaoli-Roach, A. and Roach, P. J. (1984) *J Biol Chem* **259**, 8743-8747.

Anderson, W. B., Estival, A., Tapiovaara, H. and Goopalakristna, R. (1985) *Adv Cyc Nuc Res* **19**, 287-306.

Ansorge, W. (1985)*J. Biochem. and Biophys. Methods*,**11** 13-20.

Armstrong, R. A., Kondo, H., Granot, J., Kaiser, E. T. and Mildvan, A. S. (1979) *Biochemistry* **18**, 1230-1238.

Ase, K., Berry, N., Kikkawa, U., Kishimoto, A. and Nishizuka, Y. (1988) *FEBS Lett* **236**, 396-400.

Ashendel, C. L., Staller, J. M. and Boutwell, R. K. (1983) *Cancer Res* **43**, 4327-4332.

Ashendel, C. L. (1985) *Biochim Biophys Acta* **822**, 219-242.

Bazzi, M. D. and Nelsestuen, G. L. (1987) *Biochemistry* **26**, 5002-5008.

Bazzi, M. D. and Nelsestuen, G. L. (1988) *Biochemistry* **27**, 7589-7593.

Berenblum, I. (1941) *Cancer Res* **1**, 807-814.

Berg, J. M. (1986) *Science* **232**, 485-487.

Berridge, M.J. and Irvine, R. F. (1984) *Nature* **312**, 315-321.

Boni, L. T. and Rando, R. R. (1985) *J Biol Chem* **260**, 10619-10625.

Bonser, R. W., Thompson, N. T., Hodson, H. F. Beams, R. M. and Garland, L. G. (1988) *FEBS Lett.* **234**, 341-344.

Bradford, M.M. (1976) *Anal Biochem* **72**, 248-254.

Brandt, S. J., Neidel, J. E., Bell, R. M. and Young, W. S. III. (1987) *Cell* **49**, 57-63.

Bruan, S., Ghany, M. A., Lettieri, J. A. and Racker, E. (1986) *Archiv Biochem Biophys* **247**, 424-432.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) *J Biol Chem* **257**, 7847-7851.

Cabral, F., Gottesman, M. M. and Yuspa, S. H. (1981) *Canc Res* **41**, 2025-2031.

Carpenter, D., jackson, T., and Hanley, M. R. (1987) *Nature* **325**, 107-108.

Cohen, P. (1982) *Nature* **296**, 613-620.

Cohen, P. (1985) *Eur J Biochem* **151**, 439-448.

Cori, G. T. (1945) *J Biol Chem* **158**, 333-339.

Cori, G. T. and Cori, C. F. (1945) *J Biol Chem* **158**, 321-332.

Cori, G. T. and Green, A. A. (1943) *J Biol Chem* **151**, 31-38.

Coussens, L., Parker, P.J., Rhee, L., Yang-Feng, T.L., Chen, E., Waterfield, M.D., Francke, U. & Ullrich, A. (1986) *Science* **233**, 859-866.

Coussens, L., Rhee, L., Parker, P. J. and Ullrich, A. (1987) *DNA* **6**, 389-394.

Csermely, P., Szamel, M., Resch, K. and Somogyi, J. (1988a) *J Biol Chem* **263**, 6487-6490.

Csermely, P., Szamel, M., Resch, K. and Somogyi, J. (1988b) *Biochem Biophys Res Commun* **154**, 578-583.

Davis, R. J., Ganong, B. R., bell, R. M. and Czech, M. P. (1985a) *J Biol Chem* **260**, 1562-1566.

Davis, R. J., Ganong, B. R., bell, R. M. and Czech, M. P. (1985b) *J Biol Chem* **260**, 5315-5322.

Diamond, L., O'Brian, T. G. and Baird, W. M. (1980) *Adv Canc Res* **32**, 1-74.

Driedger, P. E. and Blumberg, P. M. (1980) *Proc Natl Acad Sci USA* **77**, 567-571.

Durell, J., Garland, J. T. and Friedel, R. O. (1969) *Science* **165**, 862-866.

Eckols T. K., Thompson, R. E. and Masaracchia R. A. (1983) *Eur J Biochem* **134**, 249-254.

Edelman, A. M., Blumenthal, D. K. and Krebs, E. G. (1987) *Ann Rev Biochem* **56**, 567-613.

Eggman, L. D. and Hokin, L. E. (1960) *J Biol Chem* **235**, 2569-2571.

Ferrari, S., Marchiori, F., Borin, G. and Pinna, L. A. (1985) *FEBS Lett.* **184**, 72-77.

Fischer, E. H. and Krebs, E. G. (1955) *J Biol Chem* **216**, 121-132.

Foulkes, J. G. and Rosner, M. R. (1985) in *Molecular mechanisms of transmembrane signalling* Cohen P. and Houslay, M. D., Eds (Else vier Science Publishers Ltd, New York), pp 217-252.

Fung, B. K.-K. (1985) in *Molecular mechanisms of transmembrane signalling* Cohen P. and Houslay, M. D., Eds (Elselvier Science Publishers Ltd, New York), pp 185-214.

Ganong, B. R., Loomis, C. R. Hannun, Y. A. and Bell, R. M. (1987) in *Cell Membranes, Methods and Reviews*, Elson, E., Frazier, W. & Glaser, L., Eds (Plenum Press, New York), Vol **3**, pp 183-214.

Gilman, A.G. (1987) *Ann Rev Biochem* **56**, 615-649.

Glass, D. B. and Krebs E. G. (1982) *J Biol Chem* **257**, 1196-1200.

Gould, K. L., Woodgett, J. R., Cooper, J. A., Buss, J. E., Shalloway, D. and Hunter, T. (1985) *Cell* **42**, 849-857.

Gullick, W. J. (1987) in *Methods in Molecular Biology*, Walker, J., Ed (Humara Press, New Gersey), vol **3**, pp341-354.

Hanks, S. K., Quinn, A. M. and Hunter, T. (1988) *Science* **241**, 42-52.

Hannun, Y. A., Loomis, C. R. and Bell, R. M. (1985) *J Biol Chem* **260**, 10039-10043.

Hannun, Y. A. and Bell, R. A. (1986) *J Biol Chem* **261**, 9341-9347.

Hannun, Y. A., Loomis, C. R. and Bell, R. M. (1986) *J Biol Chem* **261**, 7184-7190.

Hesketh, J.E., McKenzie, N. and Campbell (1988) *FEBS Lett* **241**, 115-118.

Hess, F. J., Bourret, R. B. and Simon, M. I. (1988) *Nature* **336**, 139-143.

Hokin, L. E. (1985) *Ann Rev Biochem* **54**, 205-235.

Hokin, L. E. and Hokin, M. R. (1958) *J Biol Chem* **233**, 805-810.

Hokin, L. E. and Hokin, M. R. (1960) *J Gen Physiol* **44**, 61-85.

Hokin, M. R. and Hokin, L. E. (1953) *J Biol Chem* **203**, 967-977.

Hokin-Neaverson, M. (1974) *Biochem Biophys Res Commun* **58**, 763-768.

Holmes, C. F. B. (1987) *FEBS Lett* **215**, 21-24.

Holub, B. J., Kuksis, A. and Thompson, W. (1970) *J Lipid Res* **11**,558-564.

House, C. Wettenhall, R. E. H. and Kemp B. E. (1987) *J Biol Chem* **262**, 772-777.

House, C. and Kemp, B. E. (1987) *Science* **238**, 1726-1728.

Huang, F. L., Yoshida, Y., Nakabayashi, H. and Huang, K.-P. (1987a) *J Biol Chem* **262**, 15724-15720.

Huang, F. L., Yoshida, Y., Nakabayashi, H., Knopf, J. L., Young, W. S. and Huang, K.-P. (1987b) *Biochem Biophys Res Commun* **149**, 946-952.

Huang, K.-P., Nakabayashi, H. and Huang, F. L. (1986) *Proc Natl Acad Sci USA* **83**, 8535-8539.

Huang, K.-P., Huang, F. L., Nakabayashi, H, and Yoshida, Y. (1988) *J Biol Chem* **263**, 14839-14845.

Huberman, E. and Callaham, M. F. (1979) *Proc Natl Acad Sci USA* **76**, 1293-1297.

Hunter, T. (1987) *Cell* **50**, 823-829.

Hjelmeland, L. M. (1980) *Proc Natl Acad Sci USA* **77**, 6368-6370.

Imazu, M., Strickland, W. G., Chrisman, T. D. and Exton, J. H. (1984) *J Biol Chem* **259**, 1813-1821.

Ido, M., Sekiguchi, K., Kikkawa, U. and Nishizuka, Y. (1987) *FEBS Lett* **219**, 215-218.

Inoue M., Kishimoto, A., Takai, Y. and Nishizuka, Y. (1977) *J Biol Chem* **252**, 7610-7616.

Itai, A., Kato, Y., Tomioka, N., Iitaka, Y., Endo, Y., Hasegawa, M., Shudo, K., Fujiki, H. and Sakai, S.-I. (1988) *Proc Natl Acad Sci USA* **85**, 3688-3692.

Jaken, S. and Kiley, S. C. (1987) *Proc Natl Acad Sci USA* **84**, 4418-4422.

Jeffrey, A. M. and liskemp, R. M. J. (1986) *Proc Natl Acad Sci USA* **83**, 241-245.

Jeng, A. Y., Sharkey, N. A. and Blumberg, P.M. (1986) *Cancer Res* **46**, 1966-1971.

Johnson, P. C., Ware, J. A., Cliveden, P. B., Smith, M., Dborak, A. M. and Salzman, E. W. (1985) *J Biol Chem* **260**, 2069-2076.

Kaibuchi, K., Takai, Y., Sawamura, M., Hoshijima, M., Fujikura, T. and Nishizuka, Y. (1983) *J Biol Chem* **258**, 6701-6704.

Kawahara, Y., takai, Y., Minakuchi, R., Sano, K. and Nishizuka (1980) *Biochem Biophys Res Commun* **97**, 309-317.

Keller D. L. and Johnson, G. L. (1985) *J Cyclic Nucleotide Protein Phosphorylation Res* **10**, 579-591.

Kemp, B. E., Graves, D. J., Benjamini E. and Krebs E. G. (1977) *J Biol Chem* **252**, 4888-4894

Kemp B. E., Pearson, R. B. and House, C. (1983) *Proc Natl Acad Sci USA* **80**, 7471-7475.

Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S. and Nishizuka, Y. (1982) *J Biol Chem* **257**, 13341-13348.

Kikkawa, U., Go, M., Koumoto, J. and Nishizuka, Y. (1986) *Biochem Biophys Res Commun* **135**, 636-643.

Kikkawa, U., Ono, Y., Ogita, K., Fujii, T., Asaoka, Y., Sekiguchi, K., Koasaka, Y., Igarashi, K. and Nishizuka, N. (1987) *FEBS Lett* **217**, 227-231.

Kishimoto, A., Takai, Y. and Nishizuka,Y. (1977) *J Biol Chem* **252**, 7449-7452.

Kishimoto, A., Mori, T., Takai, Y., and Nishizuka, Y. (1978) *J. Biochem* **84**, 47-53.

Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y. (1980) *J Biol Chem* **255**, 2273-2276.

Kishimoto, A., Nishiyama, K., Nakanishi, H., Uratsuji, Y., Nomura, H., Takeyama, Y. and Nishizuka, Y. (1985) *J Biol Chem* **260**, 12492-12499.

Knowles, J. R. (1980) *Ann Rev Biochem* **49**, 877-919.

Krebs, E. G. (1985) *Biochem Soc Transactions* **13**, 813-820.

Krebs, E. G. and Beavo, J. A. (1979) *Ann Rev Biochem* **48**, 923-959.

Krebs, E. G., Graves, D. J. and Fischer E. H. (1959) *J Biol Chem* **234**, 2867-2873.

Kris, R.M., Lax, I., Sasson, I., Copf, B., Werlin, S., Gullick, W., Waterfield, M.D., Ullrich, A., Fridkin, M. and Schlessinger, J. (1985) *Biochemie* **67**, 1095-1101.

Laemmli, U.K. (1970) *Nature* **227**, 680-685.

Lee, L.-S. and Weinstein I. B. (1979) *J. Cell Physiol* **99**, 451-460.

Lerner, R. A., Green, N., Alexander, H., Liu, F.-T., Sutcliffe, J. G. and Shinnick, T. M. (1981) *Proc Natl Acad Sci USA* **78**, 3404-3407.

Levitski, A. (1988) *Science* **241**, 800-806.

Macnab, R. M. (1985) in *Molecular mechanisms of transmembrane signalling* Cohen P. and Houslay, M. D., Eds (Elsevier Science Publishers Ltd, New York), pp 455-487.

Makowske M., Ballester, R., Cayre, Y. and Rosen, O. M. (1988) *J Biol Chem* **263**, 3402-3420.

McPhail, L. C., Clayton, C. C. and Snyderman, R. (1984) *Science* **224**, 622-625.

Meggio, F., Marchiori, F., Borin, G., Chessa, G and Pinna L. A. (1984) *J Biol Chem* **259**, 14576-14579.

Melber, K., Zhu, G. and Diamond, L. (1989) *Cancer Res* in press.

Michell, R. H. (1975) *Biochim Biophys Acta* **415**, 81-147.

Miyamoto, E., Kuo, J. F. and Greengard, P. (1969) *J Biol Chem* **244**, 6395-6402.

Morris, C. Rice, P and Rozengurt, E. (1988) *Biochem Biophys Res Commun* **155**, 561-568.

Morris, C. and Rozengurt, E. (1988) *FEBS Lett* **231**, 311-316.

Murakami, K. and Pouttenberg, A. (1985) *FEBS Lett* **192**, 189-193.

Murakami, K. Whitley, M. K. and Routtenberg A. (1987) *J Biol Chem* **262**, 13902-13906.

Nishizuka, Y. (1984) *Nature* **308**, 693-698.

Nishizuka, Y. (1986) *Science* **233**, 305-312.

Nishizuka, Y. (1988) *Nature* **334**, 661-665.

Ohno, S., akita, Y., Kunno, Y., Imajoh, S. and Suzuki, K. (1988) *Cell*, **53**, 731-741.

Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Koichi, I. and Nishizuka, Y. (1988) *J Biol Chem* **263**, 6927-6932.

Patel, G. and Stabel, S. (1988) *Cellular Signalling* **0**, 00-00.

Palczewski, K., McDowell, J. H. and Hargrave, P. A. (1988) *Biochemistry* **27**, 2306-2313.

Palfreyman, J. W., Aitcheson, T. C. and Taylor, P. (1984) *J Immunol Meth* **75**, 383-393.

Parker, P. J., Stabel, S. and Waterfield, M. D. (1984) *EMBO J* **3**, 953-959.

Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. D. and Ullrich, A. (1986) *Science* **233**, 853-859.

Parker, P. J. and Ullrich, A. (1986) in *Oncogenes and growth control* Kahn, P. and Graf, T. Eds (Springer-Verlag, Heidelberg), pp 154-162.

Parker, P. J., Mitchell, F., Stabel, S., Marais, R., Ullrich, A. and Goris, J. (1987) *Adv Prot Phosphatases* **4**, 363-373.

Parker, P. J., Stabel, S. and Ullrich, A. (1989) in *Genes and signal transduction in multistage carcinogenesis* Colburn, N. H. Ed (Marcel Dekker Inc, New York) pp 263-278.

Pearson R. B., Woodgett, J. R., Cohen, P. and Kemp, B. E. (1985) *J Biol Chem* **260**, 14471-14476.

Pelech, S. L. and Vance, D. E. (1989) *Trends Bioch Sci* **14**, 28-30.

Pelson, J.-M., Vilgrain, I. and Chambez, E. M. (1987) *Biochem Biophys Res Commun* **147**, 382-391.

Prescott, S. M. and Majerus, P. W. (1983) *J Biol Chem* **258**, 764-769.

Rhee, S. G., Suh, P.-G., Ryu, S.-H. and Lee, S. Y. (1989) *Science* in Press.

Roach, P. J. (1981) *Curr Topics Cell Regul* **20**, 45-105.

Rodriguez-Pena, A., Zachary, I and Rozengurt, E. (1986) *Biochem Biophys Res Commun* **140**, 379-385.

Rothbard, J. B., Fernandez, R. and Schoolnik, G.K. (1984) *J Exp Med* **160**, 208-221.

Rozengurt, E. (1985) in *Molecular mechanisms of transmembrane signalling* Cohen P. and Houslay, M. D., Eds (Elsevier Science Publishers Ltd, New York), pp 429-452.

Sando, J. J. and Young, M. C. (1983) *Proc Natl Acad Sci USA* **80**, 2642-2646.

Schaap, D., Parker, P. J., Bristol, A., Kris, R., Knopf, J. (1989) *FEBS Lett* **243**, 351-357.

Sekiguchi, K., Tsukuda, M., Ogita, K., Kikkawa, U. and Nishizuka, Y. (1987) *Biochem Biophys Res Commun* **145**, 797-802.

Sefton, B. M. and Hunter, T. (1984) *Adv Cyc Nuc Res* **18**, 195-226.

Sefton, B. M. (1985) *Trends in Genetics* **1**, 306-308.

Sharkey, N. A., Leach, K. L. and Blumberg, P. M. (1984) *Proc Natl Acad Sci USA* **81**, 607-610.

Shearman, M. S., Noar, Z., Kikkawa, U. and Nishizuka, U. (1987) *Bioch Biophys Res Commun* **147**, 911-919.

Shinohara, O., Knecht, M. and Catt, K. J. (1985) *Biochem Biophys Res Commun* **133**, 468-474.

Sparks, J. W. and Brautigan, D. L. (1986) *Int J Biochem* **18**, 497-504.

Stabel, S., Rodriguez-Pena, A., Young, S., Rozengurt, E. and Parker, P. J. (1987) *J Cell Physiol* **130**, 111-117.

Streb, H., Irvine, R. F., Berridge, M. J. and Schultz, I. (1983) *Nature* **306**, 67-69.

Stryer, L. (1988) in *Biochemistry* Stryer, L. Ed (Freeman Press, New York).

Sutcliffe, J. G., Shinnick, T. M., Green, N., Liu, F.-T., Niman, H. L. and Lerner, R. A. (1980) *Nature* **287**, 801-805.

Sutcliffe, J. G., Shinnick, T. M., Green, N. and Lerner, R. A. (1983) *Science* **219**, 660-666.

Sutherland, E. W. and Wosilait W. D. (1955) *Nature* **175**, 169-170.

Takai, Y., Kishimoto, A., Inoue, M., and Nishizuka, Y., (1977a) *J Biol Chem* **252**, 7603-7609.

Takai, Y., Yamamoto, M., Inoue, M., Kishimoto, A., and Nishizuka, Y. (1977b) *Biochem Biophys Res Commun* **77**, 542-550.

Takai, Y., Kishimoto, A., Iwasa, Y., kawahara, Y., Mori, T. and Nishizuka, Y. (1979a) *J Biol Chem* **254**, 3692-3695.

Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T., and Nishizuka, Y. (1979b) *Biochem Biophys Res Commun* **91**, 1218-1224.

Tanaka, T., Slamon, D. J. and Cline, M. J. (1985) *Proc Natl Acad Sci USA* **82**, 3400-3404.

Testori, A., Hii, C. S. T., Fournier, A., Burgoyne, L. A. and Murray, A. W. (1988) *Biochem Biophys Res Commun* **156**, 222-227.

Turner, R. S., Chou, C.-H. J., Mazzei, G. J., Dembure, P. and Kuo, J. F. (1984) *J Neuroscience* **43**, 1257-1264.

Turner, R. S., Kemp, B. E., Su, H. and Kuo J. F. (1985) *J Biol Chem* **260**, 11503-11507.

Uchida, T. and Filburn, C. R. (1984) *J Biol Chem* **259**, 12311-12314.

Walker, J. M. and Sando, J. J. (1988) *J Biol Chem* **263**, 4537-4540.

Walsh, D. A., Perkins, J. P. and Krebs, E. G. (1969) *J Biol Chem* **243**, 3763-3774.

Walter, G., Scheidtmann, K.-H., Carbone, A., Laudano, A. P. and Doolittle, R. F. (1980) *Proc Natl Acad Sci USA* **77**, 5197-5200.

Walton, G. M., Bertics, P. J., Hudson, L. G., Vedvick, T. S. and Gill, G. (1987) *Analyt Biochem* **161**, 425-437.

Wang, J. H., Pallen, C. J., Sharma, R. K., Adachi, A.-M. and Adachi, K. (1985) *Curr Topics Cell Regul* **27**, 419-436.

Wender, P. A., Koehler, K. F., Sharkey, N. A., Dell'Aquila, M. L. and Blumberg, P. M. (1986) *Proc Natl Acad Sci USA* **83**, 4214-4218.

Werth, D. K., Neidel, J. E. and Pastan, I. (1983) *J Biol Chem* **258**, 11423-11426.

Werth, D. K. and Pastan, I. (1984) *J Biol Chem* **259**, 5264-5270.

Wise, B. C., Raynor, R. L. and Kuo, J. F. (1982a) *J Biol Chem* **257**, 8481-8488.

Wise, B. C., Glass, D. B., Chou, C.-H. J., Raynor, R. L., Katoh, N., Schatzman, R. C., Turner, R. S., Kilber, R. F. and Kuo, J. F. (1982b) *J Biol Chem* **257**, 8489-8495.

Woodgett, J. R., Gould, K. L. and Hunter, T. (1986) *Eur J Biochem* **161**, 177-184.

Woodgett, J .R., Hunter, T. & Gould,K .L. (1987) in *Cell Membranes, Methods and Reviews*, Elson, E., Frazier, W. & Glaser, L., Eds (Plenum Press, New York), Vol **3**, pp 215-340.

Wooten, M. W., Vanderplas, M. and Nel, A. E. (1987) *Eur J Biochem* **164**, 461-467.

Yamamoto M., Takai, Y., Inoue, M., Kishimoto, A. and Nishixuka, Y. (1987) *J Biochem* **83**, 207-212.

Yeng, A. Y., Sharkey, N. A. and Blumberg, P. M. (1986) *Canc Res* **46**, 1966-1971.

Yoshida, y., Huang, F. L., Nakabayashi, H., and Huang, K.-P. (1988) *J Biol Chem* **263**, 9868-9873.

Young, S., Rothbard, J. and Parker, P. J. (1987) *Eur J Biochem* **173**, 247-252.

Young, S., Parker, P. J., Ullrich, A. and Stabel, S. (1987) *Biochem J* **244**, 775-779.

Yuspa, S. H., Lichti, U., Ben, T., Patterson, E., Hennings, H., Slaga, T. J., Colburn, N. and Kelsey, W. (1976) *Nature* **262**, 402-404.

APPENDIX

Publications from this thesis:

1. Marais, R. M. and Parker, P. J. (1989) 'Purification and characterisation of bovine brain protein kinase C isotypes α, β and γ' *European J Biochem* in Press.

2. Cazaubon, S., Marais, R. M., Parker, P. and Strosberg, D. (1989) 'Monoclonal antibodies to protein kinase Cγ: functional relationship between epitopes and cofactor binding' *European J Biochem* In Press.

3. Mitchell, F. E., Marais, R. M. and Parker, P. J. (1989) 'The phosphorylation of protein kinase C as a potential measure of activation' *Biochem J* in Press.

ABBREVIATIONS

BSA	bovine serum albumin
DG	diacylglycerol
cA kinase	cAMP dependent kinase
cG kinase	cGMP dependent kinase
cAMP	cyclic 3',5'-AMP
cGMP	cyclic 3',5'-GMP
CaM	calmodulin
CANP	calcium activated neutral protease
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
GS	glycogen synthase
Ins1,4,5P_3	inositol-1,4,5-trisphosphate
'M' form	see PKM
MBP	myelin basic protein
MLC	myosin light chain
MLCK	myosin light chain kinase
4,5-PIP_2	phosphatidyl 4,5 bisphosphate
PKC	protein kinase C
PKM	the 'M' form of PKC (see section1.2.1)
PLC	phospholipase C
PtdSer	phosphatidylserine
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TPA	12-*O*-tetradecanoylphorbol-13-acetate

For amino acids, the three letter code is used.
The definitive names of chemical compounds is given in section 2.1 (page 66).