INTERACTIONS BETWEEN PROTEIN KINASE C AND ARGININE-RICH PEPTIDES

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ABSTRACT

Protein kinase C (PKC) is translocated to a phospholipid bilayer by calcium. Once at the membrane protein kinase C undergoes a conformational change which results in the removal of the pseudosubstrate domain from the active site. The enzyme then phosphorylates Ser/Thr residues on positively charged substrates. Certain substrates, however, can undergo cofactor independent phosphorylation by producing a conformational change in the enzyme in the absence of phospholipid and calcium. Studying the conformational change in PKC by physical techniques is difficult to perform with a phospholipid bilayer present. To study the conformational change in PKC in the absence of a membrane, the interactions between an Arginine-rich peptide (ARP), which underwent cofactor independent phosphorylation, and PKC was investigated. The K_m and k_{cat} of the enzyme for ARP, in the absence of cofactors, was around 10 μM and 0.38 s^{-1}, respectively. The K_m did not significantly change upon the addition of phospholipid and calcium. However, the k_{cat} increased 2-3 fold in the presence of phospholipid and calcium. In the absence of phospholipid and calcium, ARP induced the exposure of hydrophobic site(s) on the enzyme. Additionally, ARP was able to promote the translocation of PKC to the membrane in the absence of calcium. PKC translocated to the membrane by ARP displayed the same susceptibility as the calcium membrane bound enzyme to limited proteolytic cleavage. Therefore, both ARP and calcium induce a similar

membrane bound conformation in PKC. Additionally, the binding of ARP to PKC seems to occur through at least one high affinity site apart for the active site. These results demonstrate new insight into cofactor independent phosphorylation by PKC as well as illustrate a novel mechanism by which a substrate can promote the translocation of PKC in the absence of calcium.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
ARP	arginine rich peptide, $[Arg]_4$-Tyr-Gly-Ser-$[Arg]_5$-Tyr
BAR	biotinylated analog ARP, N-biotinyl-$[Arg]_4$-Tyr-Gly-Cys-$[Arg]_5$-Tyr
BSA	bovine serum albumin
bis-ANS	1,1'-bi(4-anilino)-naphthalene-5,5'-disulfonic acid
dansyl PE	N-(5-dimethylaminonaphalene-1-sulfonyl)-L-α-phosphatidylethanolamine
DG	*sn*-1,2-diacylglycerol
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid (disodium salt)
EGTA	ethylene glycol-bis(β-aminoethylester) N,N,N',N'-tetraacetic acid
LUV(s)	large unilamellar vesicle(s)
MARCKS	myristoylated alanine-rich C kinase substrate
MARCKS-K	Ac-Phe-Lys-Lys-Ser-Phe-Lys-Leu-NH_2
MARCKS-R	Ac-Phe-Arg-Arg-Ser-Phe-Lys-Leu-NH_2
PA	phosphatidic acid
PC	phosphatidylcholine

PE	phosphatidylethanolaime
PG	phosphatidylglycerol
PKA	cAMP dependent protein kinase
PKC	protein kinase C
aPKC	atypical PKC
cPKC	classical PKC
nPKC	novel PKC
PS	phosphatidylserine
PMSF	phenylmethylsulfenyl fluoride
PO	1-palmitoyl-2-oleoyl
SAA	substrate analog A, N-biotinyl-Arg-Arg-Arg-Cys-Leu-Arg-Arg-Leu
SAB	substrate analog B, N-biotinyl-Arg-Lys-Arg-Cys-Leu-Arg-Arg-Leu
SAC	substrate analog C, N-biotinyl-Lys-Lys-Lys-Cys-Leu-Lys-Lys-Leu
SAS	substrate analog S, N-biotinyl-Arg-Arg-Arg-Thr-Leu-Arg-Arg-Leu
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
TPA	12-O-tetradecanoylphorbol 13-acetate
TRIS	tris (hydroxymethyl) aminomethane

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1 INTRODUCTION

1.1 General Introduction

Protein kinase C (PKC) was initially found as an inactive enzyme which could be activated by limited proteolysis (Takai et al., 1977; Inoue et al., 1977). Soon after, it was elucidated that the enzyme could be activated by phospholipid and calcium (Takai et al., 1979a; Takai et al., 1979b). In the cell, protein kinase C has been found to be important in cellular signalling (Nishizuka, 1986; Nishizuka, 1992; Haller et al., 1994; Nakamura and Nishizuka, 1994; Tanaka and Nishizuka, 1994; Nishizuka, 1995; Buchner,1995). The cellular signalling ascribed to PKC is not due to only one enzyme but a family of PKC isoforms (Azzi et al., 1992; Parker, 1992; Dekker et al., 1995). This family of PKC isoforms has been divided into three classes according to their primary structure and mechanism of activation (Figure 1.1). The classical PKC isoforms α, β_I, β_{II}, and γ are activated by calcium and phospholipid. The novel PKC isoforms δ, ϵ, θ, and η lack the calcium binding domain in their primary structure and can be activated by phospholipid alone. Lastly, the atypical PKC isoforms ζ, λ,τ, and μ do not have a calcium binding domain as well as having only one Cys rich domain in their primary structure, with their regulation not yet fully elucidated.

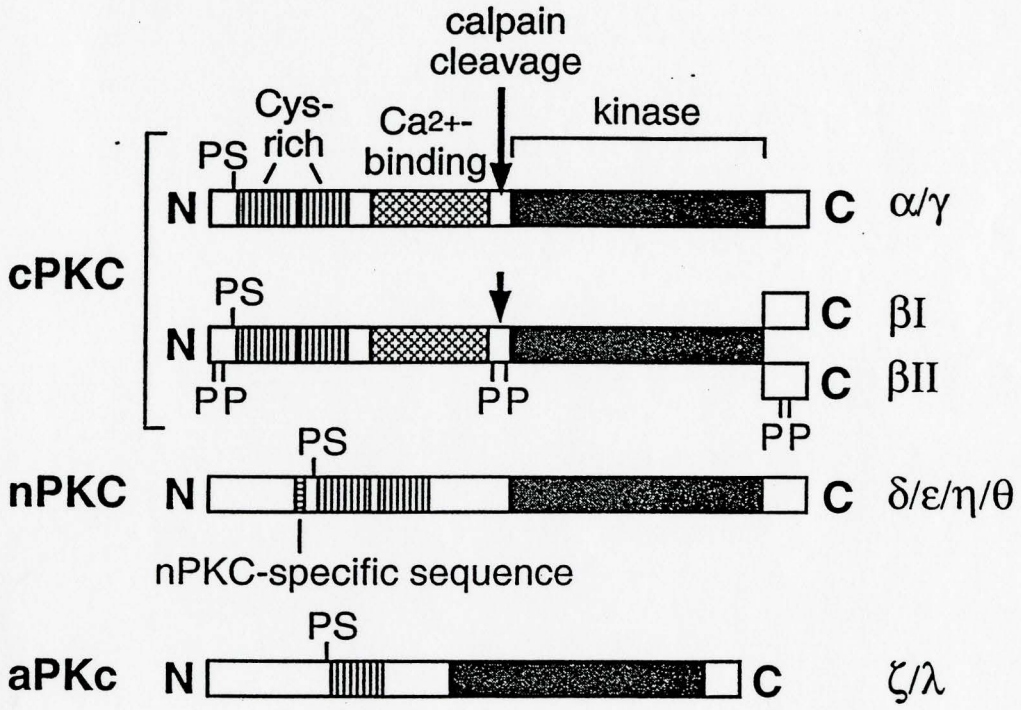


Figure 1.1 The domain structure of protein kinase C isoforms. PS represents the pseudosubstrate domain, Cys-rich region is the C1 domain, Ca^{2+}-binding region is the C2 domain, while the kinase domain consists of the C3 and C4 domains. Reproduced from Ohno and Suzuki, 1995.

1.2 Structural domains of protein kinase C

All PKC isoforms consist of a single polypeptide chain with the calculated molecular masses from the primary sequences between 67-83 kDa (Ohno and Suzuki, 1995). The calcium and phospholipid dependent enzyme purified from rat brain, displays a molecular mass of 82 kDa estimated by SDS-PAGE, 77 kDa estimated from it's sedimentation coefficient and 87 kDa estimated from gel filtration analysis (Kikkawa et al., 1983). The primary structure of PKC can be divided into 2 regions; the regulatory and the kinase domains (Ohno and Suzuki, 1995). The regulatory domain of approximately 30 kDa comprises a number of the differences found among the PKC isoforms. The regulatory region includes the pseudosubstrate domain, the Cys rich region (C1), the phospholipid/calcium binding site (C2), as well as three variable domains. The kinase domain of approximately 50 kDa is highly homologous to other protein kinases (Hanks et al., 1988; Hanks and Hunter, 1995). Data from an electron microscopic study of PKCβ_I microcrystals show that the enzyme is in a kidney bean shape (Newman et al., 1994). By superimposing the crystal structure of the catalytic domain of PKA onto the 2-D density, it was suggested that the regulatory domain of PKCβ_I lies parallel to its catalytic domain. However, with the lack of 3-D data, this is only a best guess model.

1.2.1 Pseudosubstrate domain

A 20 amino acid peptide of the C-terminal region was initially found to be a potent inhibitor of protein kinase C substrate phosphorylation. The same peptide with an alanine to serine substitution was found to be an excellent PKC substrate (House and Kemp, 1987). An antibody against this peptide allows the activation of PKC in the absence of phospholipid and calcium (Makowske and Rosen, 1989). These results indicated that this region could act as a pseudosubstrate and retain the enzyme in an inactive state in the absence of calcium and phospholipid. All PKC isoforms contain a homologous pseudosubstrate domain. This domain has conserved positively charge amino acids with an absolutely conserved alanine residue (Parker, 1992).

Multiple protein kinases contain a pseudosubstrate domain (Kemp et al., 1994) including a protein kinase highly homologous to PKC, cAMP-dependent protein kinase (PKA). The crystallographic data of PKA with a bound pseudosubstrate peptide has been used to model catalytic domain of PKC with the pseudosubstrate present (Orr and Newton, 1994; Srinivasan et al., 1996). These models predict important stabilizing interactions between the active site and the pseudosubstrate. The Ala, which is in place of a phosphorylatable residue, has hydrophobic contacts. The positively charged Arg residues of the pseudosubstrate are interacting with invariant Asp residues in the active site of the enzyme. Of interest, Arg^{19} of the pseudosubstrate of $PKC\beta_{II}$, which is masked and

coordinated by acidic residues in the enzymes inactive state, becomes exposed to proteolytic cleavage when the enzyme becomes active, either in the presence or in the absence of phospholipid and calcium (Orr et al., 1992; Orr and Newton, 1994).

The pseudosubstrate domain has additional functions apart from maintaining the enzyme in an inactive conformation. When the pseudosubstrate domain of PKCη was replaced with the similar sequence from PKCα, an enzyme with altered substrate selectivity was produced (Dekker et al., 1993a; Dekker et al., 1993b). A pseudosubstrate peptide binds to negatively charged lipids with a binding energy of 6 kcal/mol (Mosior and McLaughlin, 1991). This interaction may help stabilize the active membrane bound form of the enzyme. Additionally, the pseudosubstrate domain of PKCε has been implicated in the localization of the enzyme to the plasma membrane and to cytoskeletal components (Lehel et al., 1995).

1.2.2 The C1 domain

The C1 domain in cPKCs and nPKCs contains two Cys rich domains, while in aPKCs, only one Cys rich domain is present (Parker, 1992). The potent activators, diacylglycerol (DG) and phorbol esters, bind in this region (Ono et al., 1989; Burns and Bell, 1991). Each Cys rich region binds two zinc ions and forms a zinc finger motif (Hubbard et al., 1991; Quest et al., 1992; Quest et al., 1994b). A single Cys rich domain alone is able to retain the ability to bind phorbol esters in a stereospecific manor and translocate to membranes and

thereby displays similar characteristics to the whole enzyme (Quest and Bell, 1994; Quest et al., 1994a; Kazanietz et al., 1995a). This led to the elucidation of the solution structure of a single Cys rich domain of PKCα by NMR (Ichikawa et al., 1995). Also, the crystal structure of a single Cys rich domain of PKCδ complexed with a phorbol ester has been determined (Zhang et al., 1995).

These structures along with site-directed mutagenesis studies (Kazanietz et al., 1995b; Kazanietz and Blumberg, 1996; Wang et al., 1996) have shed light on the mechanism by which phorbol esters and protein kinase C interact. The binding site for phorbol esters is a cavity formed by the "unzipping" of β strands in the top half of the domain. This cavity is lined with hydrogen bonded water molecules which become displaced upon phorbol ester binding. Once the phorbol ester fills this hydrophilic pocket, the top portion of this domain forms a continuous hydrophobic surface. This surface could bury about 6-8 Å into a phospholipid membrane. The middle portion of this domain has a number of positively charged residues on the surface. These most likely interact with negatively charged phospholipid headgroups. The bottom portion of the domain has each the of zinc ions coordinated by 3 cysteine residues and 1 histidine residue. Interestingly, the conformation of this domain does not significantly change upon phorbol ester binding. However, the binding of phorbol esters may increase the conformational order of this domain (Wender et al., 1995).

A recent study has shown that Cys regions in PKCδ, a novel PKC isoform, are not

functionally equivalent (Hunn and Quest, 1997). They have shown that Cys1 region lacked the ability to bind phorbol esters while the Cys2 region bound phorbol esters with high affinity. This is in contrast to the classical PKC isoform, PKCγ, where both Cys rich regions can bind phorbol esters with high affinity (Burns and Bell, 1991; Quest and Bell, 1994). The functional role of the Cys1 region of PKCδ is not known yet but the authors are beginning to investigate it.

1.2.3 The C2 domain

The C2 domain has been termed the calcium and phospholipid binding (CaLB) region. Although, the CaLB domain is capable of binding Ca^{2+} in the presence of phospholipid (Shao et al., 1996) it is not the only region in the regulatory domain of PKC which binds Ca^{2+} (Luo and Weinstein, 1993; Luo et al., 1993). The C2 domain does not contain a typical calcium binding motif. However, there are a number of proteins with homologous C2 domains (Ponting and Parker, 1996).

The structures of two of these C2 domain containing proteins, synaptotagmin I (Sutton et al., 1995) and phospholipase C-δ1 (Essen et al., 1996) have been solved by x-ray crystallography. These structure are similar with some interesting differences. Both structures include an antiparallel eight-stranded β sandwich. A "C2 key" motif is formed by four of the strands with the Ca^{2+} binding site between two different loops at one end of this

motif. The topology of the sandwich in synaptotagmin I (s-variant) and phospholipase C-δ1 (p-variant) varies by a circular permutation. However, the calcium binding still occurs at the same end. When the C2 domains of PKCs are aligned with other C2 domains, the cPKCs are classified into the s-variant while the nPKCs are classified into the p-variant (Essen et al., 1997). Since the C1 and C2 domains are in different linear order in cPKCs and nPKCs, this variation would allow for the same interdomain packing between the C1 and C2 domains in both cPKCs and nPKCs.

Using the structure of synaptotagmin, a model for the C2 domain of PKC interaction with the bilayer has been proposed (Newton, 1995). Briefly, upon binding calcium, the C2 domain of cPKCs would undergo a conformational change producing protruding aromatic residues to serve as membrane anchors. A positively charged region may also obtain a preferable orientation for membrane interaction by this conformational change. A proposed binding motif for phosphatidylserine (Igarashi et al., 1995) is located between these two regions and thereby adds support to the model. Additionally, the author noted a interesting difference between the cPKCs and the nPKCs. Only two of the five conserved Asp residues are present in the nPKCs. At one of these changed Asp sites, an Arg residue is present. This led the author to suggest that this Arg residue may serve as a mimic for Ca^{2+} and thereby remove the need for Ca^{2+} in the nPKCs.

1.2.4 The C3 and C4 domains

The C3 and C4 domains of PKC comprise the kinase domain. As stated earlier, this domain is highly homologous to other protein kinases (Hanks et al., 1988; Hanks and Hunter, 1995). The crystal structure of PKA has been used as a model for the kinase domain of PKC since there is almost 40 % identity between the two sequences. A good review of protein kinase domains has been done by Hanks and Hunter (1995) while two models of the kinase domain of PKC have been proposed (Orr and Newton, 1994; Srinivasan et al., 1996). The kinase domain can be viewed as two lobes. The C3 domain is a smaller lobe which binds ATP while the C4 domain is a larger lobe that has the substrate recognition determinants along with phosphotransferase initiation machinery.

Briefly, the C3 domain contains the consensus ATP-binding motif GXGXXGXV in a β-strand, turn, β-strand structure. Two other highly conserved residues are a lysine which interacts with the α and β phosphates and a alanine which helps form a hydrophobic pocket for the adenine ring. These residues help bind and align ATP.

The C4 domain forms the groove where the substrate can bind. From the PKC models, this groove is highly negatively charged with a hydrophobic pocket. The mechanism of phosphotransfer has been proposed to be through a direct in-line nucleophilic attack mechanism (Madhusudan et al., 1994). The reaction would be initiated by an active site Asp residue which would act as a catalytic base.

1.3 Protein kinase C translocation and activation

Protein kinase C functions at the phospholipid bilayer (Epand, 1992; Zidovetzki and Lester, 1992; Newton, 1993). cPKCs are mainly located in the cytosol and upon increased intracellular Ca^{2+}, are translocated to the membrane (Wolf et al., 1985). Once at the membrane, PKC is activated by diacylglycerol and undergoes a conformational change which involves the insertion of a portion of the enzyme into the membrane (Brumfeld and Lester, 1990; Lester et al., 1990). This conformational change leads to the removal of the pseudosubstrate domain from the active site of the enzyme (Orr et al., 1992; Orr and Newton, 1994) (Figure 1.2).

1.3.1 Protein kinase C and Ca^{2+}

The affinity of protein kinase C for Ca^{2+} in the absence of phospholipid is low (Bazzi and Nelsestuen, 1990). However, in the presence of anionic phospholipid, PKC binds one Ca^{2+} and translocates to the membrane (Mosior and Epand, 1994). The affinity of PKC for Ca^{2+} increases 3.5 fold with a phospholipid bilayer present (Mosior and Epand, 1994). Once at the membrane, PKC may be associated with at least eight Ca^{2+} (Bazzi and Nelsestuen, 1990; Bazzi and Nelsestuen, 1993). The association of multiple Ca^{2+}, once the enzyme is membrane bound, may reflect the presence of a number of lower affinity sites on PKC. These

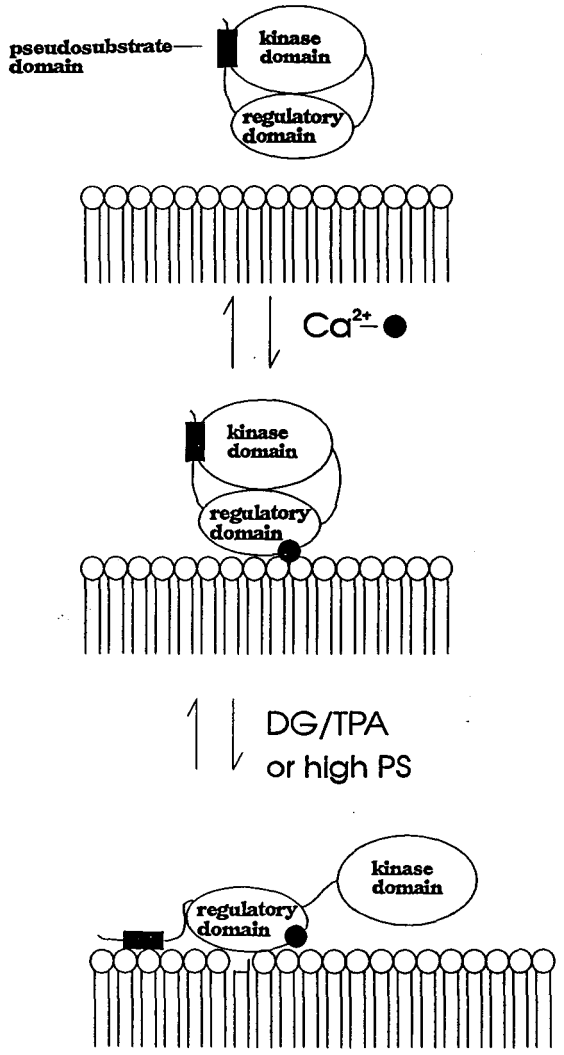


Figure 1.2 The translocation and activation of protein kinase C. Protein kinase C binds to one Ca^{2+} and translocates to the membrane. Once at the membrane, protein kinase C is activated by diacylglycerol (DG), phorbol esters (TPA), or high mol % of phosphatidylserine (PS).

sites would require the higher concentration of the cation as provided by the electrostatic potential of the membrane. The apparent dissociation constant for Ca^{2+} from membrane-bound PKC is reported to be 700 nM *in vitro* (Mosior and Epand, 1994). Additionally, the binding site on PKC for Ca^{2+} has been shown to be at 0.3 nm from the membrane (Mosior and Epand, 1994). The *in situ* concentration of Ca^{2+} require for translocation has been reported to be 198 nM (Khalil et al., 1994).

In the absence of phospholipid, the binding of Ca^{2+} to PKC exposes a hydrophobic site(s) on the enzyme (Walsh et al., 1984). This suggestion was made since PKC could be purified by hydrophobic-interaction chromatography in a Ca^{2+}-dependent manner. This is in agreement with the proposed exposure of hydrophobic residues from the C2 domain upon binding Ca^{2+} (Newton, 1995). Additionally, a conformational change in PKC can be detected by circular dichroism when Ca^{2+} binds the enzyme in the absence of phospholipid (Lester and Brumfeld, 1991; Shah and Shipley, 1992; Bosca and Moran, 1993). This change in the secondary structure of the enzyme was observed to increase upon the addition of membranes. Also, PKC undergoes a free energy change of approximately 5 kcal/mol upon binding Ca^{2+} and PS (Mosior and Epand, 1994). Interestingly, the C2 domain of synaptotagmin does change its secondary structure upon binding Ca^{2+} in the crystal structure (Sutton et al., 1995). However, the authors did indicate that the Ca^{2+} binding site in the crystal might not be fully formed and that a further conformational change may be required. This is supported by the increased sensitivity of the first C2 domain of synaptotagmin to

proteolytic cleavage upon binding Ca^{2+} (Davletov and Sudhof, 1994).

1.3.2 Protein kinase C and anionic phospholipid

Protein kinase C requires anionic phospholipid for membrane binding (Bazzi and Nelsestuen, 1987a). Early studies have suggested that phosphatidyl-L-serine (PS) is essential for PKC binding to membranes (Lee and Bell, 1989; Orr and Newton, 1992; Newton and Keranen, 1994). These studies also implied that PKC had a specific binding site for the PS headgroup. This binding site was stereospecific with necessary functional groups of PS interacting with PKC. It has been noted that other anionic phospholipids could be a partial supplement for PS (Lee and Bell, 1992).

However, a recent study demonstrates that PKC lacks a distinctive PS binding site (Mosior et al., 1996). By examining the differences between the anionic headgroups of PS and N-dansyl-phosphatidylethanolamine (PE), this group illustrated that the PKC membrane association constants with these dissimilar anionic phospholipids were almost identical. This is in spite of the dansyl- PE headgroup lacking two functional groups that had previously been reported to be important in the specific binding site for PS. The membrane-bound activity of PKC with dansyl-PE was between 50-75 % of that seen with PS in vesicles containing diacylglycerol (DG) depending on the substrate. However, the membrane-bound activity of PKC in the absence of DG, although lower, was the same with these two

headgroups. This may implicate anionic phospholipids in regulating the maximal rate of phosphorylation of the enzyme in the presence of DG. The headgroups, phosphatidic acid (PA) and phosphatidylglycerol (PG), display lower association constants for PKC, than PS or PE (Mosior et al , 1996). This observation prompted the authors to suggested other attributes of the bilayer may be necessary for PKC association and activation. Interestingly, the earlier studies on the interactions of PKC and PS were performed with mixed micelles, while the later studies have been done with model membranes in the form of unilamellar vesicles. The use of these different systems may explain the discrepancies between the observations.

PKC binding to PS was found to be cooperative with at least 4 molecules of PS required per enzyme (Hannun et al., 1986). Also, PKC has been found to be in association with multiple acidic phospholipids once the enzyme is bound to the membrane (Bazzi and Nelsestuen, 1991). However, the apparent cooperativity of PS binding to PKC seems to be due in part, to a reduction in dimensionality when PKC binds to phospholipid membranes (Mosior and Epand, 1993). Membrane domains of PS have been found to be formed by Ca^{2+} as well as by substrates (Yang and Glasser, 1995). Therefore these domains were suggested to be important for PKC activation as well as for the binding of substrate in the same domain.

The modulation of the membrane properties on the association and activity of PKC have been observed in a number of previous publications (reviewed recently by Stubbs and

Slater, 1996). Phospholipids with the tendency to promote the hexagonal phase in membranes increase the membrane binding and activity of PKC (Epand, 1992). Two similarly structured phospholipids, but with opposite effects on the bilayer to hexagonal phase transition temperature, produced the opposite effects on PKC activity (Epand et al. 1991). These results suggested that the effect on PKC activity was a result of changing the propensity of the bilayer to form hexagonal phase rather than a result of directly binding to and altering PKC activity. Also, an increase in the membrane affinity and activity of PKC is produced by substituting acyl chains with a greater tendency for negative curvature. The role of unsaturation of the phospholipid acyl chains on PKC activity has been analyzed in multiple studies (Bolen and Sando, 1992; Slater et al., 1994a; Slater et al., 1996a). All these studies demonstrate that the degree of unsaturation of the acyl chains does affect PKC activity. Interestingly, it was noted that vesicles of with anionic phospholipid and diacylglycerol but with totally saturated acyl chains did not support PKC activity (Bolen and Sando, 1992). Also, certain phospholipid mixtures can bind PKC in the presence of EGTA (Epand et al., 1992). The authors suggest that binding of PKC to these mixtures was due to the less-tightly packed headgroups. All these observations demonstrate the importance not only of the anionic headgroup but the phospholipids overall effect on the bilayer structure.

1.3.3 Protein kinase C and DG/phorbol esters

DG and phorbol esters will also promote membrane partitioning of PKC (Bell and Burns, 1991). DG and inositol 1,4,5 triphosphate (IP_3) are produced from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C. Additionally, the hydrolysis of phosphotidylcholine (PC) by phospholipase D generates phosphatidic acid (PA) which can be converted to DG by the removal of the phosphate by a phosphomonoesterase (Nishizuka, 1995). Phorbol esters are naturally occurring tumour promoters such as 12-O-tetradecanoylphorbol 13-acetate (TPA) which is from a medicinal plant called *Croton tiglium* (Rouhi, 1995).

As stated earlier, cPKCs and nPKCs have two potential sites in the C1 domain for the binding of DG/phorbol esters. Although, the nPKCs have only one site for the binding of DG/phorbol esters (Hunn and Quest, 1997). However, DG and phorbol esters seem to bind to cPKCs at distinct sites. Initial indications for the distinct sites came from observations that inhibitors of phorbol ester binding did not affect the binding of DG to PKC and *visa versa* (Slater et al., 1994b). Further evidence was obtained with the use of a novel fluorescent phorbol ester (Slater et al., 1996b). It was ascertained that this phorbol ester bound to PKC at two sites, a high and a low affinity site. Additionally, the low affinity site for phorbol esters on PKC bound DG with high affinity and thereby supporting the notion for distinct binding sites for these activators on PKC.

The role of DG and phorbol esters binding to PKC is to increase the affinity of the enzyme for the membrane and to increase the activity of the enzyme (Mosior and Epand, 1993; Mosior and Newton, 1996). With a bilayer composed of PS:PC, 1:3, DG increases membrane affinity of PKC by 500 times. Additionally, 1 mol% DG was found to double the rate of phosphorylation of the enzyme (Mosior and Epand, 1993). Interestingly, the potency of DG can be related to the position of the unsaturation and the length of the sn-2 acyl chain (Marignani et al., 1996). Phorbol esters are more potent as they increase the membrane affinity of PKC by two orders of magnitude more than DG, with a dissociation constant from PKC of 1.5×10^{-5} mol % (Mosior and Newton, 1996). Phorbol esters can even induce the binding of PKC to neutral membranes (Mosior and Newton, 1996). Contrary to early studies which suggested synergism between the binding of Ca^{2+} and phorbol esters to PKC (Wolf et al., 1985), the binding of DG and phorbol esters to PKC is independent of the binding of Ca^{2+} (Mosior and Epand, 1994; Mosior and Newton, 1996). Additionally, the binding of DG and phorbol esters to PKC is reversible and once bound the enzyme interacts mainly with the membrane interface (Mosior and Newton, 1995). Phorbol ester binding to PKC increases the motional freedom of the phorbol esters acyl chain and suggests that PKC binds to less ordered and less tightly packed regions of the membrane (Svetek et al., 1995).

1.4 Protein kinase C and substrate phosphorylation

Protein kinase C is a Ser/Thr kinase. A recent review article lists 110 currently known substrates for PKC (Liu, 1996). These substrates include receptors, G proteins, enzymes, cytoskeleton proteins, proto-oncogene products, nuclear proteins and a number of others. The study of the phosphorylation of these substrates and others by PKC has been done in a variety of areas including the mechanism of phosphotransfer, specific requirements of the substrate, and the cofactor requirements for phosphorylation.

1.4.1 The mechanism of phosphotransfer by protein kinase C

Once activated, PKC transfers the γ-P of adenosine triphosphate (ATP) to a Ser/Thr residue in the consensus site. The pathway for this reaction has been investigate by kinetic analysis. Additionally, mechanistic information can be hypothesized from the highly homologous Ser/Thr protein kinase, PKA.

Initial kinetic analysis was performed by Hannun and Bell, 1990. Using the catalytic domain of PKC, it was ascertained that the K_m of histone and MgATP were 117 μg/ml and 4.3 μM, respectively. Mg^{2+} was found to be an activator of the catalytic domain of the enzyme. Interestingly, Mn^{2+} could also activate the catalytic domain but lowered the V_{max} to 25 % of that seen with Mg^{2+}. Free ATP was neither a substrate or an inhibitor. Also from

their studies with the catalytic domain, it was suggested that substrate binding to the enzyme was sequential but randomly ordered. Then by using the whole enzyme, this group analyzed the contribution of the cofactors to the kinetic parameters. PS and Ca^{2+} increase the K_m of the enzyme for histone without affecting the K_m for MgATP. The K_m for these substrates was not affected by DG but did increase the k_{cat} of the enzyme substantially. Another study expanded these observations by elucidating the order of binding of the reactants and products (Leventhal and Bertics, 1991). Briefly, the reaction mechanism of PKC follows that of a sequential bi bi mechanism. MgATP binds the enzyme first followed by histone. After phosphotransfer, the phosphorylated histone would be released followed by MgADP. Interestingly, the binding of histone to PKC is accompanied by the binding of approximately 2300 water molecules to the enzyme, with approximately 1300 of these water molecules being removed as the enzyme goes into the transition state. However, the binding of protamine sulfate, a cofactor independent substrate, to the enzyme is accompanied by a dehydration of the enzyme-substrate complex (Giorgione and Epand, 1997).

The mechanism of the phosphotransfer by PKA has been established (Madhusudan et al., 1994) and provides possible insight for the mechanism of PKC. The reaction proceeds along an Sn_2 mechanism. An active site catalytic base, an Asp residue (invariant in PKA and PKC), would remove a proton from the hydroxyl of the target Ser/Thr. This oxygen would then perform a direct in-line nucleophilic attack on the γ-P of the ATP stabilized by an active site acid, a Lys residue, and Mg^{2+}. The subsequent products would then be released from the

active site.

1.4.2 Specific requirements of substrates of protein kinase C

All protein kinases have specific substrates which the enyzmes recognize as the target. The specificity of these enzymes is categorized by a consensus sequence. The consensus sequence is derived from a tabulation of invariant residues present in the substrates of the enzyme. The consensus sites for PKC elucidated from this method are S/T-X-K/R, K/R-X-X-S/T, K/R-X-X-S/T-X-K/R, K/R-X-S/T, K/R-X-S/T-X-K/R (Pearson and Kemp, 1991). Recently, a new technique for elucidating the substrate specificity of protein kinases has been developed and applied to PKC (Nishikawa et al., 1997). This techniques allows one to test the potential of more than 2.5 billion peptide substrates by using a degenerate oriented peptide library. All PKC isozymes prefer sequences with a basic amino acid at -3 and a hydrophobic amino acid at +1 relative to the phosphorylated Ser. In addition to these amino acids, cPKCs selected substrates with basic amino acids at -6, -4, -2, +2, and +3. nPKCs and aPKCs were similar, however, with hydrophobic residues at +2 and +3. The difference in the substrate specificity between PKC isoforms was explained by the proposed amino acid contacts modelled from PKA. Interestingly, PKC does not have a stereospecific requirement for the phosphorylated Ser, as it can phosphorylate both the L and D stereoisomers (Kwon et al., 1994).

Apart from containing the proper phosphorylation site sequence, the best PKC substrates are phospholipid binding proteins (Bazzi and Nelsesteun, 1987c). There does exists a class of cofactor-independent PKC substrates (Bazzi and Nelsestuen, 1987b) which will be discussed later. The requirement of a substrate to be bound at the phospholipid bilayer seems obvious since PKC is activated at the membrane surface. However, a bound PKC substrate is not necessarily sufficient for phosphorylation. The binding of PKC's substrate seems to require to be bound to organized domains of PS in the membrane (Yang and Glasser, 1995). This would arrange PKC and the substrate in the same area of the membrane in a PS domain and thereby increase the activity of the enzyme. Native lysozyme is not phosphorylated by PKC, however, the addition of a myristoyl moiety targets the enzyme to the membrane where it undergoes PKC phosphorylation (Utsumi et al., 1994). However, the position of the myristoylation affected the extent of phosphorylation by PKC. Therefore, the topology of the membrane bound substrate also seems to be important.

1.4.3 Protein kinase C cofactor-independent phosphorylation

Protein kinase C is capable of phosphorylating a class of substrates in the absence of phospholipid and Ca^{2+} (Bazzi and Nelsestuen, 1987b). The mechanism by which these substrates activate PKC is not fully understood. It has been shown that a commonly used substrate from this class, protamine sulfate, induces a conformational change in the enzyme

that causes the pseudosubstrate domain to become susceptible to proteolytic cleavage (Orr and Newton, 1994). The characteristic feature of these substrates is the high positive charge density provided by the multiple Arg residues. The minimal requirement of a substrate to undergo cofactor independent phosphorylation by PKC was defined to be extended clusters of arginyl residues on both sides of the target Ser/Thr (Ferrari et al., 1987). The peptide Arg_4-Tyr-Gly-Ser-Arg_6-Tyr was found to be the smallest sequence capable of being phosphorylated by PKC in a cofactor independent manner. Peptides with arginyl clusters on either the N-terminus or C-terminus alone required cofactors for phosphorylation by PKC.

Protamine as well as poly-L-arginine can stimulate the autophosphorylation of PKC in the absence of cofactors (Leventhal and Bertics, 1993). Additionally, this stimulation displayed positive cooperativity with respect to protamine. The authors suggest that multiple protamine molecules bind and activate PKC at a specific "arginine-selective activator-independent site (s)". The site(s) was suggested to be apart from the active site due to the autophosphorylation that was observed. Also, the authors proposed that contacts between PKC molecules may be occurring since, the binding of protamine or poly-L-arginine to PKC induces the formation of aggregates which can be sedimented under nominal centrifugation (Bazzi and Nelsestuen, 1987b; Leventhal and Bertics, 1993). Models of the catalytic domain may suggest an alternative route for PKC activation by such substrates. A cluster of acidic residues appear to be masking part of the pseudosubstrate domain (Orr and Newton, 1994). A peptide analog of this acidic region activates PKC independently of cofactors and it was

suggested that this peptide competed with the active site for the binding of the pseudosubstrate (House et al., 1989). The binding of these Arg-rich substrate may be at this acidic region and thereby activating PKC by competing with the pseudosubstrate for these residues.

The role of the Arg residues in these substrates has been suggested to be a partial substitute for Ca^{2+} (Kimura et al., 1987). This is an interesting proposal since Ca^{2+} has been found to inhibit the phosphorylation of protamine sulfate by PKC (Sakai et al., 1987). Additionally, an Arg residue has been implied to be a possible mimic for Ca^{2+} in the nPKCs (Newton, 1995). This proposal still remain as speculation and requires experiment evidence to truly elucidate the role of the Arg residues.

1.4.4 Substrate analogs of Protein kinase C

Recently, a group has been developing peptide substrate analogs for PKC to use an active site inhibitor (Ward et al., 1995; Ward et al., 1996). The peptides, in addition to the essential residues present in the consensus sequence for PKC, have a Cys residue in place of a Ser/Thr (Table 1.1). These peptides were inhibitory towards the PKC catalyzed phosphorylation of histone. The inhibited PKC-peptide complex was stable upon dilution, however, the inhibition was sensitive to dithiothreitol. Additionally, peptides with an Ala in place of the Cys residue were ineffective as inhibitors of PKC. PKC could be partially

Name	Peptide
SAA	N-biotinyl-Arg-Arg-Arg-Cys-Leu-Arg-Arg-Leu
SAB	N-biotinyl-Arg-Lys-Arg-Cys-Leu-Arg-Arg-Leu
SAC	N-biotinyl-Lys-Lys-Lys-Cys-Leu-Lys-Lys-Leu
SAS	N-biotinyl-Arg-Arg-Arg-Thr-Leu-Arg-Arg-Leu
BAR	N-biotinyl-Arg-Arg-Arg-Arg-Tyr-Gly-Cys-Arg-Arg-Arg-Arg-Arg-Arg-Tyr
ARP	Arg-Arg-Arg-Arg-Tyr-Gly-Ser-Arg-Arg-Arg-Arg-Arg-Arg-Tyr
MARCKS-R	Ac-Phe-Arg-Arg-Ser-Phe-Arg-Leu-NH_2
MARCKS-K	Ac-Phe-Lys-Lys-Ser-Phe-Lys-Leu-NH_2

Table 1.1 Substrates and substrate analogs for protein kinase C.

protected from inactivation by a peptide substrate and by MgATP. These indicated that the peptide may be forming a disulfide bond in the active site of the enzyme. A further study utilized the same peptides, however, a biotin group was added onto the N-terminus. The addition of the biotin group made for easy detection of the PKC-peptide complex by Western blot and subsequent detection by avidin-conjugated horseradish peroxidase. The biotinylated peptides mirrored the findings from the earlier study. The labelling of PKC by the biotinylated peptides was sensitive to DTT, MgATP and substrates. The biotinylated peptides did not label PKC if the enzyme was denatured. This indicated that the enzyme provided a specific binding site for the peptides. Also, limited proteolysis of PKC demonstrated the peptides labelled the only the catalytic domain of PKC. When the Cys was replaced with a Ser, these peptides became substrate of PKC. The maximal rate of phosphorylation of these peptides was seen in the presence of Ca^{2+} and phospholipid. However, it was indicated that a significant rate of phosphorylation was observed in the absence of cofactors.

1.5 Objective

There is great interest in studying protein kinase C and elucidating its role in cellular signalling. Although, many studies have been done and are being performed, the structural information on this enzyme is limited and is slowly appearing one domain at a time (see

above for ref.). Apart from the crystallographic data elucidated and possible data to come, the conformational change the enzyme undergoes upon binding to membranes and the concomitant activation is difficult to study by the common physical methods due to the presence of membranes. However, if this conformational changes in the enzyme produced from the translocation to membranes could be mimicked apart from the membrane, different techniques could be used to study this conformational change. Knowing that Arg-rich substrates activated PKC in the absence of membranes and that the minimal sequence for this activation had been defined, the ability of this minimal sequence Arg-rich peptide to mirror the conformational change that normally occurred in PKC upon binding Ca^{2+} and phospholipid was investigated.

2. PROTEIN KINASE C AND PEPTIDE INTERACTION STUDIES

2.1 Introduction

Protein kinase C requires a conformational change to remove the pseudosubstrate domain from the enzymes active site before any substrate phosphorylation occurs (Orr et al., 1992; Orr and Newton, 1994). Typically, Ca^{2+}, PS and DG perform this task (Brumfeld and Lester, 1990; Lester et al., 1990), however, Arg-rich substrates are competent in achieving cofactor independent phosphorylation by PKC (Bazzi and Nelsestuen, 1987b). The mechanism by which the Arg-rich substrates induce the activation of PKC is unclear. Additionally, the extent of the conformational change in PKC produced by these substrate is also vague. In this chapter, interactions between PKC and Arg-rich peptides and the subsequent conformational change in the enzyme are investigated.

2.2 MATERIALS AND METHODS

2.2.1 Materials

All phospholipids were from Avanti Polar lipids, Inc. (Alabaster, AL). Bovine serum albumin fraction V, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), benzamidine, leupeptin, N-(5-dimethylaminonaphtalene-1-sulfonyl)-L-α-phosphatidylethanolamine (DNS-PE), and ATP were from Sigma Chemical Co. (St Louis, MO). Q-Sepharose fast flow and Phenyl Sepharose were obtained from Pharmacia (Quebec). Hybond ECL nitrocellulose membrane was purchased from Amersham International (Buckinghamshire, England). The peptide $[Arg]_4$-Tyr-Gly-Ser-$[Arg]_5$-Tyr was purchased from the University of Kentucky. The peptide acetyl-Phe-Lys-Lys-Ser-Phe-Lys-Leu-amide was purchased from the Institute for Biological Science, National Research Council (Ottawa, Ontario) while the peptide acetyl-Phe-Arg-Arg-Ser-Phe-Arg-Leu-amide was a generous gift of R.E. Williams from the Institute for Biological Science, National Research Council (Ottawa, Ontario). The peptides, N-biotinyl-Arg-Arg-Arg-Cys-Leu-Arg-Arg-Leu, N-biotinyl-Arg-Lys-Arg-Cys-Leu-Arg-Arg-Leu, N-biotinyl-Lys-Lys-Lys-Cys-Leu-Lys-Lys-Leu, N-biotinyl-Arg-Arg-Arg-Thr-Leu-Arg-Arg-Leu, and N-biotinyl-$[Arg]_4$-Tyr-Gly-Cys-$[Arg]_5$-Tyr were a generous gift of Catherine O' Brian (Houston, Texas). [γ-^{32}P]ATP was supplied by NEN (Montréal, Québec, Canada). The fluorophores PRODAN and 1,1' bis(4-anilino)naphthalene were purchased from Molecular Probes (Eugene, OR).

2.2.2 Methods

2.2.2.1 Purification of Protein Kinase C α

Protein Kinase C α was purified from both insect cells overexpressing PKC α in the baculovirus system and rat brains. The insect cells overexpressing PKC α in the baculovirus system were kindly provided by Robert Burns and Nancy Rankl of the Sphinx Pharmaceutical Co. The procedure for purification of PKC α from these two different sources was similar to a previously described procedure (Stabels et al., 1991) with the following modifications. The entire purification procedure was done on ice or at 4 °C. Brains from 24 Sprague-Dawley rats were removed as quickly as possible, washed and homogenized in 150 ml of a buffer containing 2 mM benzamidine, 1% (w/w) Triton X-100, 50 mM Tris/HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 40 μg/ml leupeptin, and 200 μM PMSF. Harvested insect cells were lysis in the same buffer. The cells were gently homogenized and then the lysate was centrifuged for 1 hr at 100 000 x g in a Beckman SW28 rotor. The supernatant from this spin was applied to a Q-Sepharose fast flow column (2.5 x 20 cm, 70 ml) previously equilibrated with in 1 mM EDTA, 1 mM EGTA, 20 mM Tris/HCl, pH 7.5, and 10% (v/v) glycerol (DEAE buffer). The column was then washed with 200 ml of DEAE buffer. The column was then washed with 150 ml of DEAE buffer plus 0.05 M KCl. Fractions were collected when the column was eluted with 150 ml of DEAE buffer plus

0.15 M KCl. The collected fractions were assayed for PKC activity in the mixed micelle assay as previously described (Hannun et al., 1985). The fractions displaying calcium and phospholipid dependent activity were pooled, concentrated with a YM30 Diaflo Ultrafiltration Membrane in an Amicon Ultrafiltration Cell to approximately one half of the original volume and adjusted to 1.5 M KCl. This was then loaded onto a phenyl Sepharose column (1.0 x 10 cm, 10 ml) equilibrated with 1.5 M KCl DEAE buffer. This column was then washed with 100 ml of 1.5 M KCl DEAE buffer. A 100 ml linear gradient from 1.5 to 0.0 M KCl was applied and fractions collected. The collected fractions were assayed for PKC activity in the mixed micelle assay as previously described (Hannun et al., 1985). The fractions displaying calcium and phospholipid dependent activity were pooled, concentrated with a YM30 Diaflo Ultrafiltration Membrane in an Amicon Ultrafiltration Cell to a 3 or 4 ml, brought to 50 % glycerol (v/v) and stored in working portions at -70 °C. Typically, this procedure produced a single band on a silver-stained electrophoresis gel with specific activities ranging from 0.1 to 2 μmoles of phosphate incorporated per milligram of protein per minute. Additionally, the phospholipid independent activity did not exceed 8% of the total kinase activity.

2.2.2.2 Mixed micellar assay for Protein kinase C

The mixed micellar assay was performed essentially as described previously (Hannun

et al., 1985). The 240 µl reaction consisted of 25 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 0.2 mg/ml histone, 3 mM EGTA or 300 µM $CaCl_2$, 0.3% (v/v) Triton X-100 or 0.3 mg/ml PS/DG (4:1, w/w) in 0.3 % (v/v) Triton X-100. To this mixture 5 µl of the fraction to be tested was added. The reaction was initiated by the addition of 20 µl of 200 µM [γ^{32}-P] ATP. The reaction mixture was then vortexed and allowed to proceed for 10 min at 30°C. Termination of the reaction was with 2 ml of ice cold 25 % (w/v) TCA. The stopped reactions were filtered on GF/C Whatman filters and washed 3 times with 2 ml of ice cold 25 % (w/v) TCA. The filters were dried at 37°C for 30 min and subsequently counted using efficiency corrected Cerenkov counting.

2.2.2.3 Preparation of large unilamellar vesicles

A mixture of 2 mM lipid in chloroform:methanol (2:1, v/v) was dried in a 16 x 100 cm test tube under a stream of N_2 followed by 2 hours under high vacuum. The lipid was resuspended in 100 mM KCl, 5 mM $MgCl_2$, and 20 mM Tris/HCl, pH 7.0 by subjecting this mixture through five freeze-thaw cycles. This mixture was then extruded through two stacked 0.1 µm polycarbonate filters in a Lipofast microextruder. This produced large unilamellar vesicles (LUVs) with an average diameter of 100 nm. LUVs consisted of 1,2 diolein (DAG) (0-1 mol%), 1-palmitoyl-2-oleoyl phosphatidylserine (POPS) (10-50 mol%), and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) (the remainder; 49-90 mol%). The

LUVs were dilute 1:1 (v/v) with 100 mM KCl, 5 mM $MgCl_2$, and 20 mM Tris/HCl, pH 7.0 to give a working concentration of 1 mM.

2.2.2.4 Intrinsic tryptophan fluorescence of Protein kinase C

The fluorescence spectra of PKC was measure as previously described (Lester and Brumfeld, 1990) with the following modifications. The excitation wavelength was 295 nm and the emmision spectra was recorded between 305 and 365 nm. Measurement were performed on a SLM-Aminco Series 2 luminescence spectrometer. The emmision spectra of PKC (130 nM) were measured in a 200 µl reaction consisting of 100 mM KCl, 5 mM $MgCl_2$, and 20 mM Tris/HCl, pH 7.0 with 500 µM EGTA or 200 µM $CaCl_2$. When present, the LUVs were at a concentration of 100 µM while the peptide, RRRRYGSRRRRRY, was at a concentration of 60 µM.

2.2.2.5 Resonance energy transfer binding assay for Protein kinase C

The binding of PKC to lipid membranes evaluated by resonance energy transfer was performed as previously described (Bazzi and Nelsestuen, 1987a). LUVs used for this assay contained DAG, POPS, POPC and 10 mol% N-(5-dimethylaminonaphtalene-1-sulfonyl)-L-α-phosphatidylethanolamine (DNS-PE). PKC was mixed with 5 µM LUVs in a ratio of 1:5

(w/w). All measurement were conducted in 100 mM KCl, 5 mM $MgCl_2$, and 20 mM Tris/HCl, pH 7.0 with 500 μM EGTA or 200 μM $CaCl_2$. When added, the peptides were at the indicated concentrations. Measurement were performed on a SLM-Aminco Series 2 luminescence spectrometer.

2.2.2.6 Protein kinase C activity assay

The activity of PKC towards peptide substrates was achieved by a previously established method (Chakaravarthy et al., 1991). The 250 μl reaction mixture contained 200 ng/ml PKC, 100 mM KCl, 0.3 mg/ml BSA, 5 mM $MgCl_2$, and 20 mM Tris/HCl, pH 7.0 with 500 μM EGTA or 200 μM $CaCl_2$. LUVs were at a concentration of 100 μM, while the peptide substrate was present at 5-70 μM. When added, histone was at 0.1-0.2 mg/ml. The reaction was initiated by the addition of 20 μM [γ-^{32}P] ATP (0.2 μCi/ml) and incubated at 25 °C. After 10 minutes the reaction was terminated with 75 μl of ice cold 5 % (v/v) acetic acid or with 5X SDS-PAGE sample buffer and boiled for 5 min.. The samples stopped with acetic acid were then spotted onto Whatman ion exchange P81 paper and washed four times with 0.4 % phosphoric acid. The filters were then dried and counted using efficiency corrected Cerenkov counting. The samples stopped with sample buffer were loaded and run on a 18 % SDS-PAGE. The gel was dried and exposed to Kodak X-AR film overnight at -70°C.

2.2.2.7 Triton X-114 assay

The partitioning of hydrophobic proteins into Triton X-114 has been previously described (Bordier, 1981; Alcaraz et al., 1984; Brusca and Radolf, 1994; Sanchez-Ferrer et al., 1994). PKC-α (250 ng) was incubated with 1% (w/v) Triton X-114 in a 300 µl mixture consisting of 100 mM KCl, 5 mM $MgCl_2$, 20mM Tris/HCl, pH 7.0, 500 µM EGTA, 0.3mg/ml BSA, 40 µM bromophenol blue, and 0 µM or 60 µM RRRRYGSRRRRRY. This was overlaid on a 300 µl sucrose pad consisting of 0.25 M sucrose, 0.06% Triton X-114, 100 mM KCl, 5 mM $MgCl_2$, and 20mM Tris/HCl, pH 7.0 on ice. This suspension was then incubated at 37 °C for 5 min., afterwhich it was centrifuged at 8000 rpm for 5 min at room temperature. The aqueous and detergent phases were then analyzed for enzyme activity towards the ARP as previously described in the PKC activity assay.

2.2.2.8 Probing hydrophobic sites on protein kinase C

Fluorescence measurements were accomplished on an SLM-Aminco Series 2 luminescence spectrometer. The analyzed solution contained 100 mM KCl, 5 mM $MgCl_2$, and 20mM Tris/HCl, pH 7.0, 500 µM EGTA, 0 µM or 60 µM RRRRYGSRRRRRY, PKC-α (0.25 µM), 1.5 µM bis-ANS or 60 µM PRODAN (in methanol). The excitation wavelength for bis-ANS was 395 nm with the emission wavelength was 495 nm. For PRODAN, an

emission spectrum was taken from 400 nm to 600 nm with an excitation wavelength of 350 nm.

2.2.2.9 Trypsin cleavage of Protein kinase C

The susceptibility of PKC-α to trypsin cleavage was performed essential as the published procedure (Newton and Koshland, 1989; Orr et al., 1992). A 50 µl reaction mixture containing 100 mM KCl, 5 mM $MgCl_2$, 20mM Tris/HCl, pH 7.0, 0.3 mg/ml BSA, 500 µM EGTA (or 200 µM $CaCl_2$), 0 µM or 60 µM of the peptide RRRRYGSRRRRRY, 0 µM or 100 µM lipid in the form of LUVs, 80 nM PKC and trypsin (1.7 or 17 units/ml). Upon addition of trypsin, the reaction was allowed to proceed for 25 min at 30°C after which cleavage was stopped by the addition of SDS-PAGE sample buffer and boiling for 5 min. The samples were then run on a 8% SDS-PAGE and subsequently silver stained.

2.2.2.10 Detection of complexes of protein kinase C and the biotinylated peptides

The crosslinking and detection of complexes of protein kinase C and the biotinylated peptides was performed essentially as previously described by Ward et al., 1996, with the following modifications. Protein kinase C (≈ 150 ng) was incubated with 1-10 µM of a biotinylated peptide in 100 mM KCl, 0.3 mg/ml BSA, 5 mM $MgCl_2$, and 20 mM Tris/HCl,

pH 7.0 with 500 μM EGTA or 200 μM $CaCl_2$. When present, LUVs were at a concentration of 100 μM. The reaction was allowed to proceed for 15 min at 30°C after which it was stopped by the addition of SDS-PAGE sample buffer, without β-mercaptoethanol, and boiled for 15 sec. The samples were then immediately loaded and run on a 10 % SDS-PAGE.

The protein from the gel was then transfered to nitrocellulose by the following procedure. A piece of prewetted Hybond ECL nitrocellulose membrane was placed on the gel and subsequently positioned between 6 pieces of 3 MM Whatman paper (3 on each side). This sandwich, with the nitrocellulose paper towards the positive electrode, was placed in an electroblotting cassette (Bio-Rad Trans Blot) in 25 mM Tris, 192 mM glycine and 20 % methanol. A current of 20 V was applied overnight.

The biotinylated peptide and PKC complexes were detected using the Western-light Plus chemiluminescent detection system (TROPIX, Inc., Bedford, MA). Briefly, the nitrocellulose was washed for 5 min. in 25 ml of 137 mM NaCl and 20 mM Tris/HCl, pH 7.6. The nitrocellulose was then incubated in 25 ml of blocking buffer (0.2 % I-Block (casein), 0.1 % Tween-20, 137 mM NaCl and 20 mM Tris/HCl, pH 7.6) for 30 min and then for 1 h in 20 ml of blocking buffer plus 1μl of Avidx-AP conjugate. The nitrocellulose was subsequently washed 3 times with 50 ml of wash buffer (0.1 % Tween-20, 137 mM NaCl and 20 mM Tris/HCl, pH 7.6) for 5 min per wash. Two, 2 min washes with 20 ml of assay buffer (10 mM $MgCl_2$ and 20 mM Tris/HCl, pH 9.8) followed. The excess assay buffer was drained from the nitrocellulose by touching one corner on paper towels, without letting the

blot dry. The nitrocellulose was then laid on a piece of Saran Wrap on a flat surface and a thin layer (≈ 1-3 ml) of CSPD Ready-to-Use substrate solution containing 1:20 Nitro-Block was applied for 5 min.. The excess substrate solution was drained from the nitrocellulose by touching one corner on paper towels, without letting the blot dry. The nitrocellulose was then placed between two pieces of Saran Wrap and air bubbles and wrinkles were removed. This was then exposed to film (Kodak X-AR) for 30 secs to 30 minutes.

A digital image of the film was aquired with a Kodak digital science DC-40 camera. This image was then examined with 1D image analysis software (Eastman Kodak Comp., Rochester, N.Y.).

2.3 RESULTS

2.3.1 Protein kinase C catalyzed phosphorylation of peptide substrates

2.3.1.1 Cofactor requirements

The cofactors requirements for the protein kinase C catalyzed the phosphorylation of substrates differs amongst the classes of substrates (Bazzi and Nelsestuen, 1987b). Phosphorylation of two of these substrate classes by PKC is displayed in Figure 2.1. Both MARCKS-K (Ac-Phe-Lys-Lys-Ser-Phe-Lys-Leu-NH_2) and MARCKS-R (Ac-Phe-Arg-Arg-Ser-Phe-Arg-Leu-NH_2) peptides are phosphorylated by PKC in a Ca^{2+} and phospholipid dependent manor. The replacement of the Lys residues with Arg does increase the rate of PKC catalyzed phosphorylation in the MARCKS peptide sequence. ARP (Arg-Arg-Arg-Arg-Tyr-Gly-Ser-Arg-Arg-Arg-Arg-Arg-Tyr) is phosphorylated by PKC both in the presence and absence of Ca^{2+} and phospholipid. This is in agreement with previous results which suggested that ARP is the minimal sequence requirement to undergo cofactor independent PKC catalyzed phosphorylation (Ferrari et al., 1987).

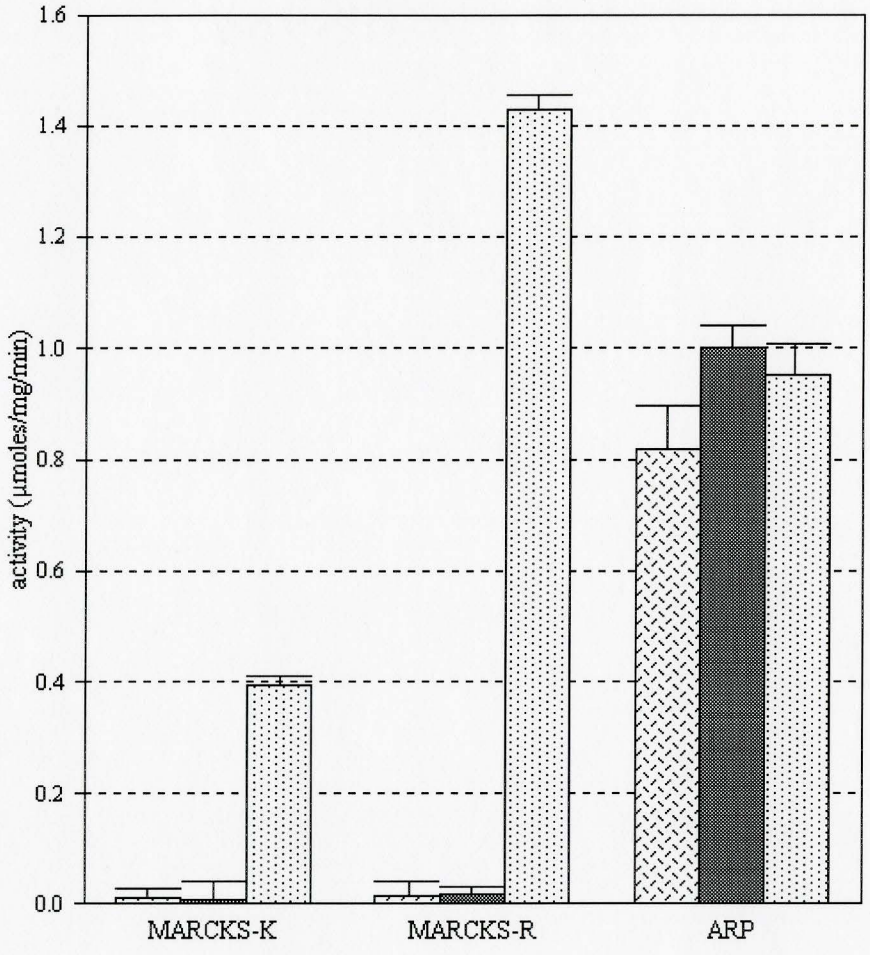


Figure 2.1 Protein kinase C catalyzed phosphorylation of peptide substrates in the presence and absence of cofactors. The first lane of each is in the absence of cofactors, the second is with phospholipid alone, while the last is with Ca^{2+} and phospholipid. The phospholipid was in the form of LUVs which were composed of 20 mol% POPS, 79 mol% POPC, and 1 mol% DG. The peptide substrate was at a concentration of 60 μM. Reproduced from Bruins and Epand, 1995.

2.3.1.2 PKC catalyzed phosphorylation of ARP and inhibition by histone

The PKC catalyzed phosphorylation rates of ARP with various substrate concentrations is displayed in Figure 2.2. The rate of phosphorylation of ARP by PKC does increase in the presence of phospholipid alone. The addition of 20 μM Ca^{2+} to this mixture causing only a slight further increase. Interestingly, the addition of 0.2 mg/ml histone inhibits the PKC catalyzed phosphorylation of ARP in the presence of phospholipid alone. The phosphorylation rate of ARP in the absence of cofactors as well as in the presence of 20 μM Ca^{2+} and phospholipid is not affected by the addition of 0.2 mg/ml of histone. When a lower concentration of histone (0.1 mg/ml) is added to the phosphorylation reaction of ARP with phospholipid alone, no inhibition is seen (Figure 2.3). The PKC catalyzed phosphorylation of ARP in the presence of 0.2 mg/ml histone with phospholipid alone resembles the phosphorylation of ARP in the absence of cofactors.

The inhibition of the PKC catalyzed phosphorylation of ARP by histone does not appear to be due to the phosphorylation of histone. The autoradiograph presented in Figure 2.4 displays the phosphorylation of different substrates by PKC. Histone (0.2 mg/ml) and MARCKS-K (90 μM) are phosphorylated to a minor extent with 20 μM Ca^{2+} in the presence of phospholipid, while 200 μM Ca^{2+} greatly increases the phosphorylation in this system. With phospholipid in the absence of Ca^{2+}, histone inhibits the phosphorylation of ARP,

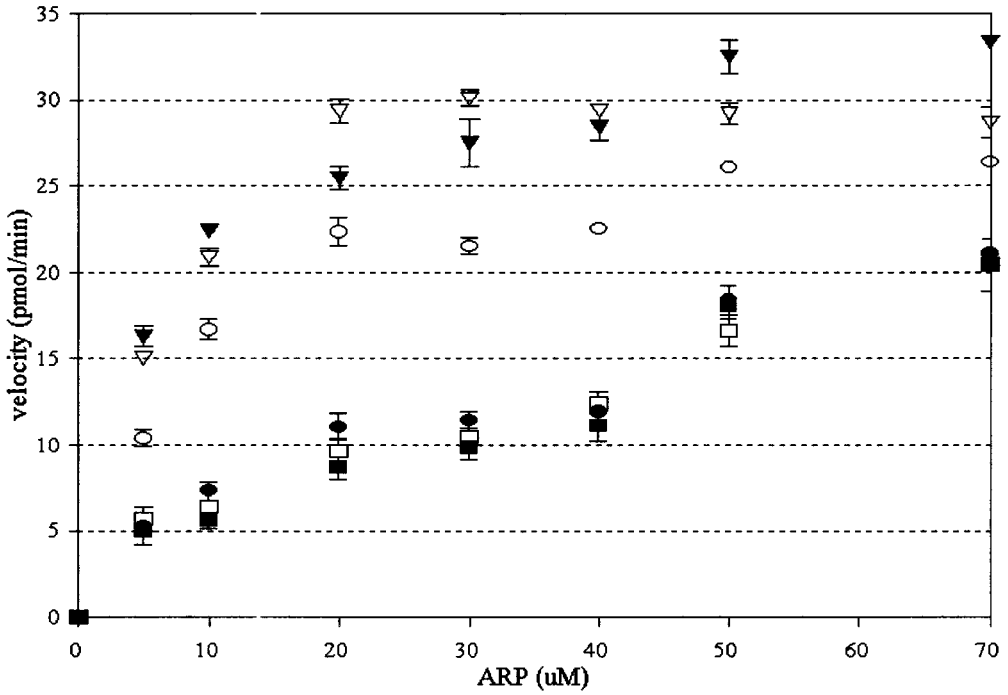


Figure 2.2 The rate of phosphorylation of ARP by PKC with various cofactors and the inhibition by histone. The PKC catalyzed phosphorylation of ARP was measured in the absence of cofactors (squares), in the presence of phospholipid alone (circles) and with calcium (20 uM) and phospholipid (triangles). The rates were measure in the absence (empty symbols) and presence of 0.2 mg/ml histone (filled symbols). The phospholipid was in the form of LUVs composed of 50 mol% POPS and 50 mol% POPC.

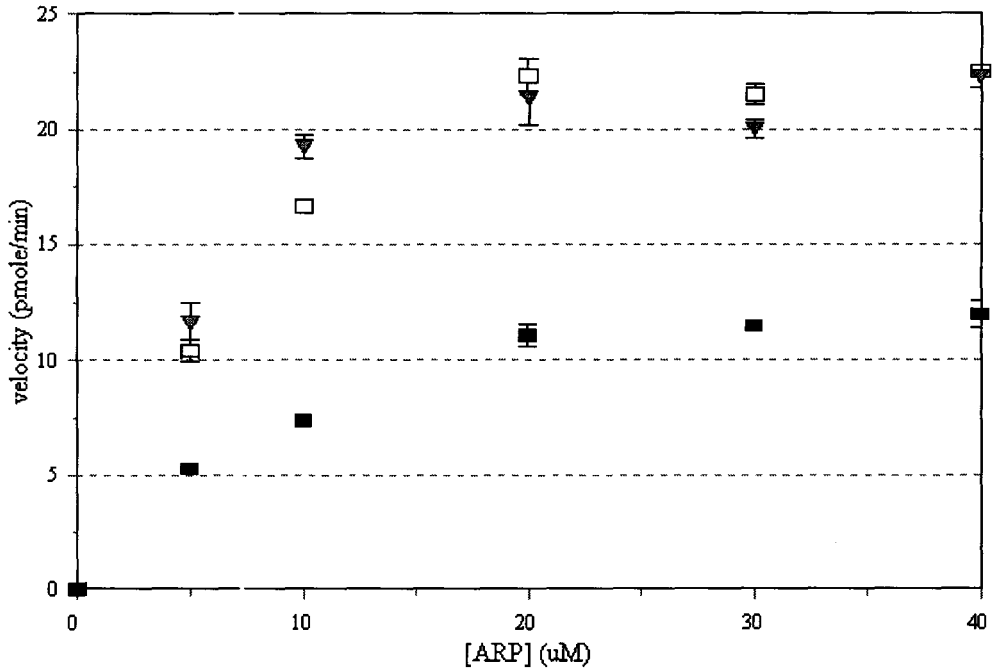
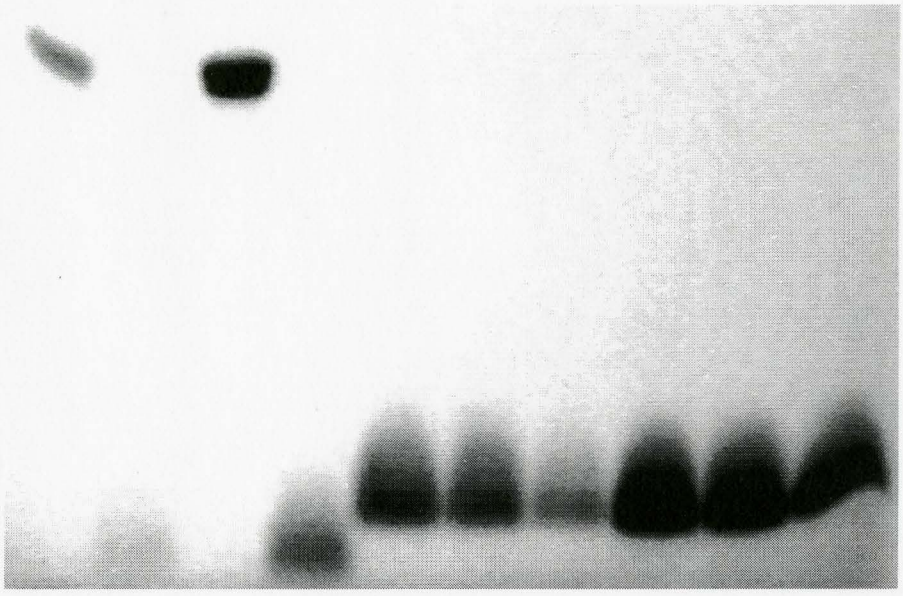


Figure 2.3 The rate of phosphorylation of ARP by PKC and the inhibition by histone. The PKC catalyzed phosphorylation of ARP was measured in the presence of phospholipid alone. The rates were measure in the absence (empty squares), in the presence of 0.1 mg/ml histone (triangles) or with 0.2 mg/ml histone (filled squares). The phospholipid was in the form of LUVs composed of 50 mol% POPS and 50 mol% POPC.



+	-	+	-	-	-	+	-	-	+	histone
-	-	-	-	+	+	+	+	+	+	ARP
-	+	-	+	-	+	-	-	+	-	MARCKS-K
20	20	200	200	-	-	-	20	20	20	Ca^{2+} (μM)

Figure 2.4 The PKC catalyzed phosphorylation of substrates as detected by an autoradiograph. All lanes contained phospholipid in the form of LUVs composed of 50 mol% POPS and 50 mol% POPC. The concentration of histone, ARP, and MARCKS-K were 0.2 mg/ml, 7 μM, and 90 μM respectively.

without being phosphorylated itself. The addition of 20 μM Ca^{2+} increases the phosphorylation of the ARP and overcomes the inhibition by histone. The MARCKS-K peptide does not inhibit the phosphorylation of ARP to any great extent.

The fitting of the data in Figure 2.2 and Figure 2.3 allows the estimation of the K_m and the k_{cat} of the PKC catalyzed phosphorylation of ARP in the presence of various cofactors. The calculated values for the K_m and k_{cat} are tabulated in Table 2.1. The K_m values for ARP are lower than previously reported values while the k_{cat} values are lower and higher (Ferrari et al., 1987). However, the differences may be explained by the enzyme preparation used. The protein kinase C used in the previous study was purified from bovine brains and did contain at least one major contaminant while, the protein kinase used for this study was from a baculovirus expression system and appeared homogeneous on a silver stained SDS-PAGE. Additionally, the state of the phospholipid (PS alone) is not stated in the previous study and may be another discrepancy between the values.

The K_m for ARP with the assorted cofactors does not vary significantly from around 10 μM. The k_{cat} for ARP does increase from approximately 0.38 s^{-1} in the absence of cofactors to approximately 0.67 s^{-1} and 0.87 s^{-1}in the presence of phospholipid without and with Ca^{2+}, respectively. The inhibition of 0.2 mg/ml histone reduces the k_{cat} of the ARP by 50 % in the presence of phospholipid alone without significantly altering the K_M. A lower amount of histone (0.1 mg/ml) has no significant affect on the K_M or k_{cat} of ARP in the presence of phospholipid alone.

Cofactors	histone (mg/ml)	K_m (μM)	k_{cat} s^{-1}
none	0	10.7 ± 3.4	0.38± 0.04
	0.2	12.2 ± 3.6	0.36 ± 0.04
phospholipid	0	6.8 ± 1.9	0.70 ± 0.05
	0.1	4.3 ± 1.1	0.64 ± 0.05
	0.2	9.5 ± 1.8	0.39 ± 0.01
phospholipid, Ca^{2+}	0	6.7 ± 1.5	0.93 ± 0.06
	0.2	4.6 ± 0.2	0.81 ± 0.01

Table 2.1 The kinetic constants determined for protein kinase C towards ARP. The phospholipid was in the form of LUVs composed of 50 mol% POPS and 50 mol% POPC. Calcium was at a concentration of 20 μM.

2.3.2 The translocation of PKC in the absence of Ca^{2+}

The translocation of protein kinase C to the membrane can be monitored by a variety of techniques (Epand, 1994). Resonance energy transfer is one of these techniques and was first performed by Bazzi and Nelsestuen (1987a). This technique monitors the translocation of PKC by using liposomes containing N-dansyl-phosphatidylethanolamine. When PKC translocates to the membrane, an increase in dansyl fluorescence occurs as a result of energy transfer from the excited Trp of PKC. The translocation of PKC is easily observed when Ca^{2+} is present with LUVs containing PS and DG (Figure 2.5). Minimal increase in fluorescence is observed in the absence of Ca^{2+}. However, when ARP is added to PKC with these vesicles in the presence of 500 μM EGTA, a comparable increase to the Ca^{2+} induced signal in dansyl fluorescence is noticed. ARP did increase the dansyl fluorescence in the absence of PKC, however, this value was subtracted from the signal with PKC present and the resultant value is displayed in Figure 2.5. Two other peptide substrate, MARCKS-K and SAA, do not cause any notable change in the dansyl fluorescence. These results indicate that ARP is causing PKC to associate with the phospholipid membrane in the absence of Ca^{2+}.

To confirm the results obtained using resonance energy transfer, the intrinsic tryptophan spectra of PKC was monitored. The fluorescence maximum of PKC's Trp occurs at 338 nm and the magnitude at this wavelength is significantly reduced upon binding to membranes (Lester and Brumfeld, 1990). In the absence of phospholipid the tryptophan

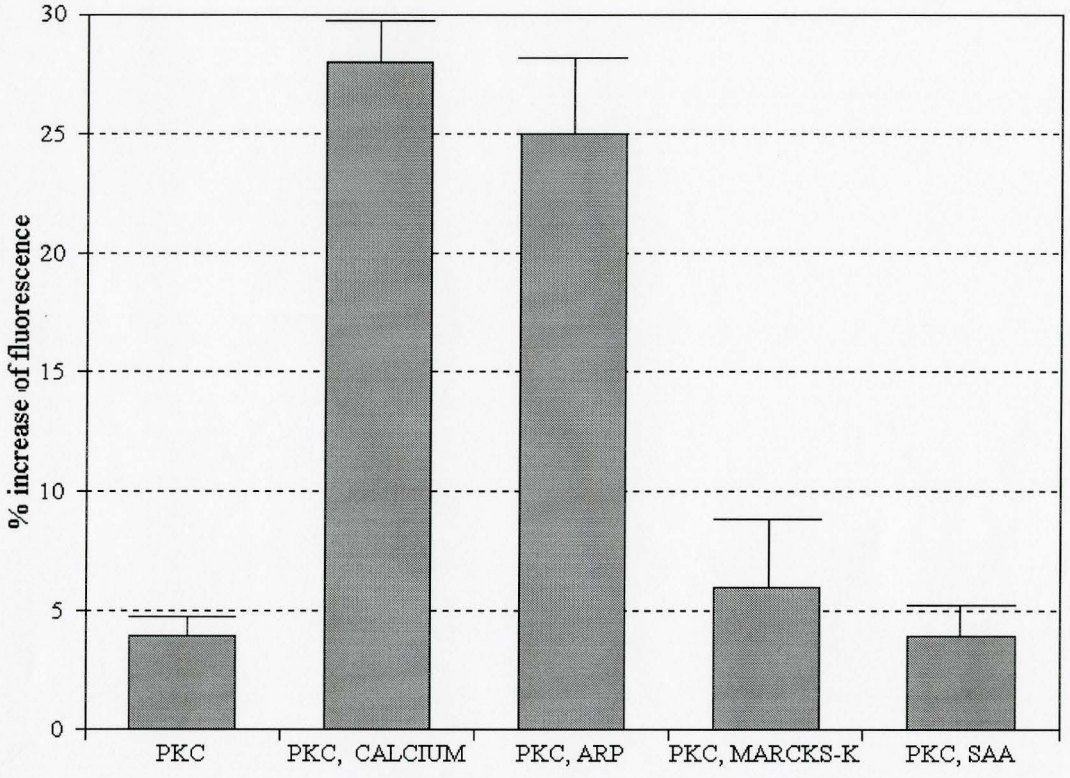


Figure 2.5 The translocation of protein kinase C as monitored by resonance energy transfer. All measurements contained LUVs composed of 20 mol% POPS, 69 mol% POPC, 1 mol% DG and 10 mol% N-dansyl-phosphatidylethanolamine and 500 μM EGTA (except when 200 μM calcium was present as indicated). The concentration of the ARP, MARCKS-K, and SAA were 60 μM, 60 μM and 25 μM respectively. The increase in fluorescence is relative to an identical measurement without PKC present. Reproduced from Bruins and Epand, 1995.

spectra of PKC displays a maxima around 338 nm which does not change in magnitude upon the addition of ARP (Figure 2.6). The same maxima of PKC's Trp fluorescence is seen in the presence of phospholipid vesicles containing 20 mol % POPS, 79 mol % POPC, and 1 mol % DG, however, upon the addition of Ca^{2+} the amplitude of the signal decreases approximately 50 % (Figure 2.7). ARP mimics the decrease seen with Ca^{2+} and phospholipid producing an approximate 60 % decrease in the extent of PKC's fluorescence at 338 nm. The results obtained from both intrinsic tryptophan fluorescence and resonance energy transfer experiments are analogous, demonstrating that ARP can function in a similar manner to Ca^{2+} and induce the translocation of PKC to a phospholipid bilayer.

2.3.3 The exposure of hydrophobic sites on PKC by ARP

The association of protein kinase C with the membrane produces a conformational change in the enzyme which results in the insertion of a portion of the enzyme into the membrane (Lester et al., 1990). It was of interest to examine if ARP could generate the exposure of hydrophobic site(s) on PKC which may promote its association with a phospholipid bilayer. This hypothesis was tested with the use of polarity sensitive fluorescent probes and with a partitioning experiment.

The first fluorophore used was the water soluble bis-ANS, which is sensitive to the to the polarity of its environment. In polar surroundings, the fluorescence of this probe is

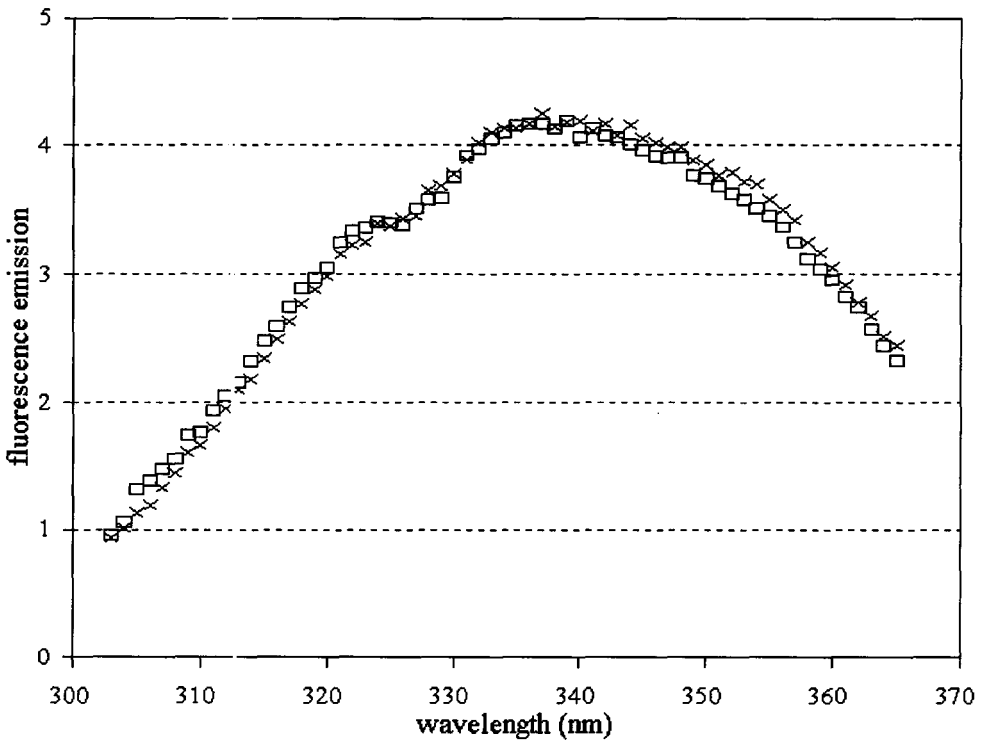


Figure 2.6 Intrinsic tryptophan spectra of protein kinase C in the presence (squares) and absence (crosses) of ARP. When present, ARP was at a concentration of 60 μM. Reproduced from Bruins and Epand, 1995.

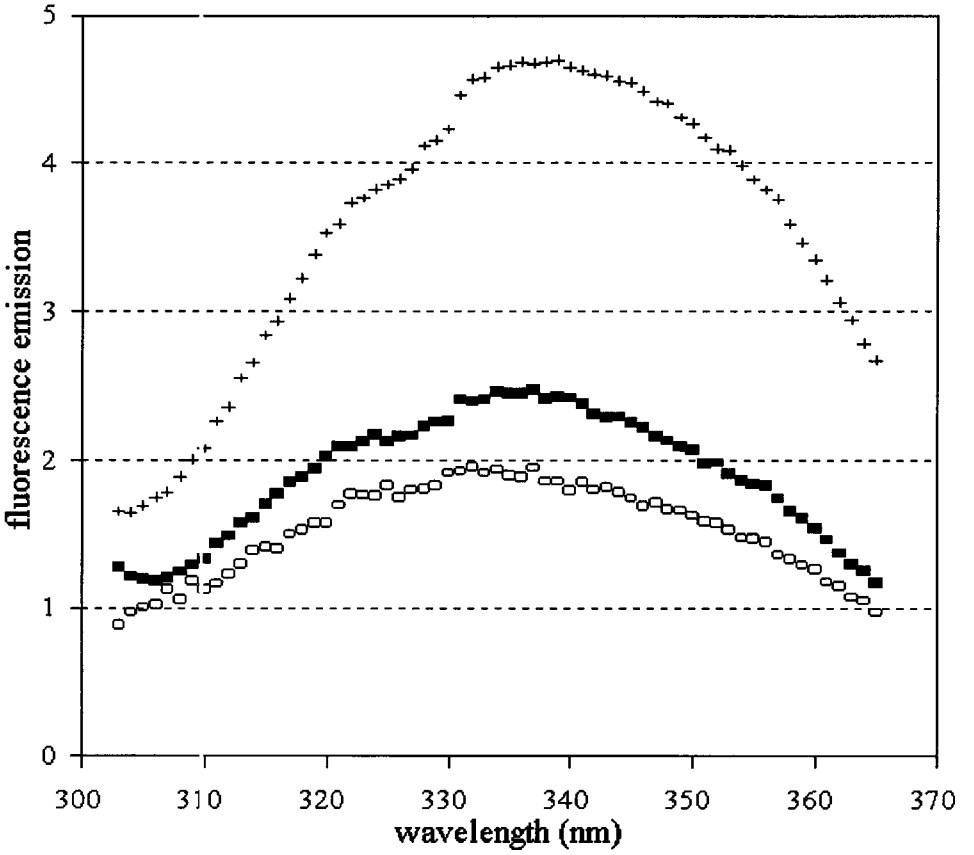


Figure 2.7 Intrinsic tryptophan spectra of protein kinase C in the presence of phospholipids and the modulation by calcium or ARP. The spectra of PKC with phospholipids was taken with EGTA alone (plus), EGTA with 60 μM ARP (circles) or with 200 μM calcium (squares).The phospholipid was in the form of LUVs composed of 20 mol% POPS, 79 mol% POPC, and 1 mol% DG. Reproduced from Bruins and Epand, 1995.

minimal, while in a hydrophobic environment, a notable increase in the quantum yield is observed (Cardamone and Puri, 1992). When protein kinase C is mixed with bis-ANS, no increase in fluorescence is noticed. The addition of ARP to this mixture does cause an almost 4 fold increase in bis-ANS fluorescence (Figure 2.8). This indicates that ARP is causing the exposure of hydrophobic site(s) on PKC.

Further evidence supporting the exposure of hydrophobic site(s) on PKC induced by ARP came from another fluorophore, PRODAN. This fluorophore displays a fluorescence emission maximum around 520 nm in a polar environment. However, in a hydrophobic environment, PRODAN shifts its fluorescence emission maximum to 420 nm (Weber and Faris, 1979). PRODAN displays a lower quantum yield than bis-ANS and higher concentration of the fluorophore is required. PRODAN is not highly water soluble and therefore was dissolved in methanol and then added to our aqueous system (no precipitation was observed). The spectrum of PRODAN in our experimental system with PKC present is displayed in Figure 2 9. Three peaks are evident at 420 nm, 490 nm and at 520 nm. If PKC and PRODAN are mixed prior to the addition of ARP, the addition of ARP induces an increase in the peak at 420 nm while the other peaks are minimally changed. This would indicate that PRODAN was in a more hydrophobic environment when ARP interacts with PKC. Interestingly, if PKC and ARP are mixed prior to the addition of PRODAN, a significantly different spectrum is observed. A notable decrease in the 420 nm peak with a striking increase in the peaks at 490 nm and 520 nm signify that PRODAN is in a more polar

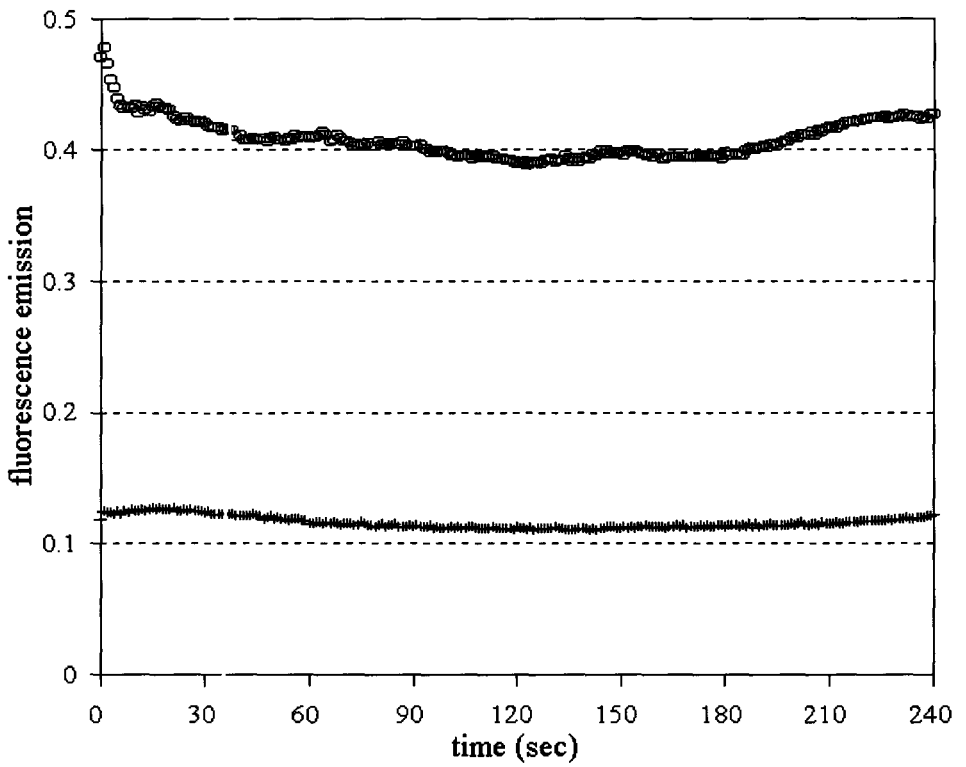


Figure 2.8 Exposure of hydrophobic domains of protein kinase C by ARP detected by bis-ANS. The fluorscence of bis-ANS was measured with PKC (squares) as well as with PKC and 60 μM ARP (X) Reproduced from Bruins and Epand, 1995.

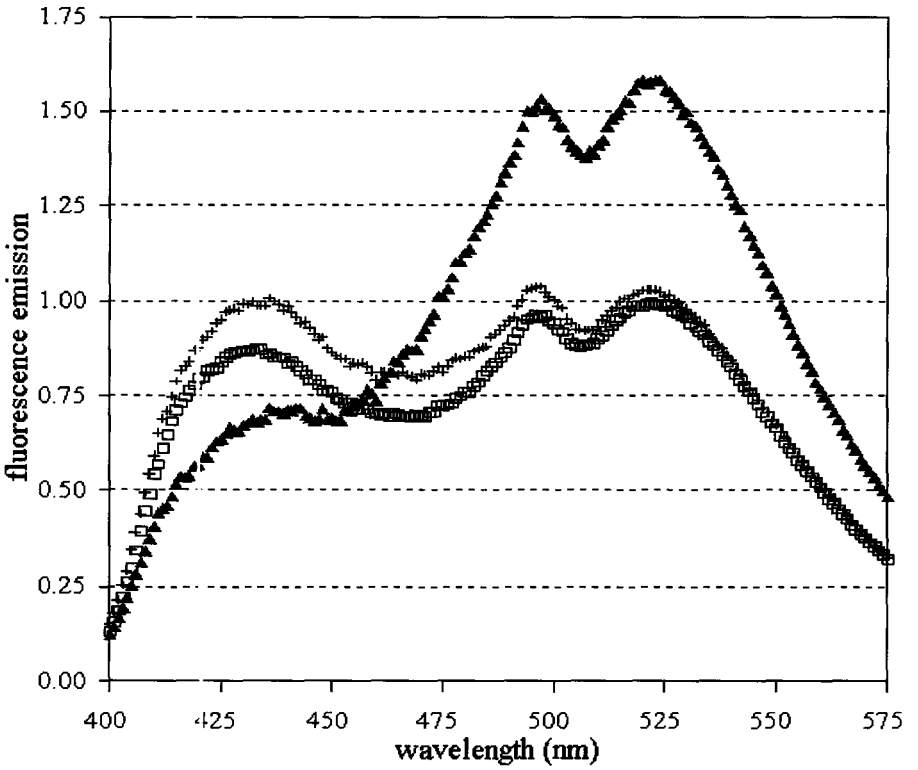


Figure 2.9 Exposure of hydrophobic domains of protein kinase C by ARP detected by PRODAN. The spectra of PRODAN was measured with PKC (empty squares). The specta of PRODAN with PKC was also assessed when 60 μM of ARP was added before (filled squares) or after (triangles) the addition of PRODAN. Reproduced from Bruins and Epand, 1995.

environment in this circumstance than with PKC alone. Taken together, these results suggest that ARP causes the exposure of hydrophobic sites on PKC which in turn may initiate the aggregation of ARP and PKC complexes. Such aggregation complexes have been observed with protamine sulfate and PKC (Bazzi and Nelsestuen, 1987b) and lend support to these findings.

The partitioning of PKC into Triton X-114 micelles was used as a third independent test for the exposure of hydrophobic sites on PKC induced by ARP. This techniques is commonly used in the purification of integral membrane proteins (Brusca and Radolf, 1994; Sanchez-Ferrer, 1994). Triton X-114 has a cloud point of 22°C. Below this temperature Triton X-114 can solubilize and/or associate with proteins displaying hydrophobic surfaces. Then by raising the temperature above this point, Triton X-114 forms aggregated micelles with these proteins which can be pelleted by mild centrifugation. When PKC is mixed with Triton X-114 and subsequently separated into an aqueous and detergent phase, about 5 % of the total PKC activity is found in the detergent pellet (Figure 2.10). The presence of ARP with PKC increases the activity in the detergent phase about 4 fold. This constitutes about 15 % of the total PKC activity. ARP is a substrate for PKC and the amount of peptide that copartitions with PKC is difficult to evaluate. This may lead to an underestimation of the amount of PKC in the detergent phase. Regardless, ARP does increase the amount of PKC that partition into the Triton X-114 micelles.

The results from the fluorophore and the partitioning experiments are all in agreement.

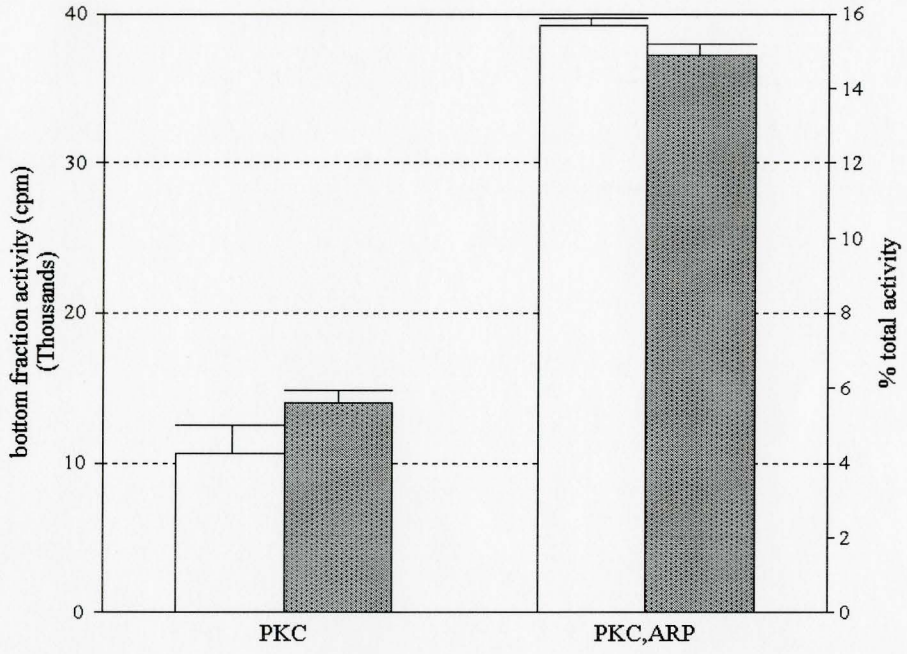


Figure 2.10 Protein kinase C partitioning into Triton X-114 micelles induced by ARP. The partitioning of PKC into Triton X-114 micelles in the presence and absence of 60 μM ARP was assessed by activity measurements of sedimented micelles. The bottom fraction activity is respresented by the empty bars while, the % total activity is represented by the filled bars. Reproduced from Bruins and Epand, 1995.

The binding of ARP to PKC induces the exposure of hydrophobic(s) on the enzyme. These site(s) are suspected to influence the membrane association of PKC as well as induce aggregation of the PKC and ARP complex in the absence of a phospholipid bilayer.

2.3.4 Conformational changes in PKC induced by ARP

The conformational change in protein kinase C that occurs upon membrane binding and subsequent activation can be monitored by the sensitivity to mild proteolytic cleavage. The removal of the pseudosubstrate domain from the active site of PKC exposes a proteolytic sensitive site which cleavages off the pseudosubstrate domain and produces a nicked form of PKC (Orr et al, 1992; Orr and Newton, 1994). Additionally, the binding of PKC to the membrane and concomitant activation discloses a second site of proteolytic cleavage at a hinge region between the catalytic domain and the regulatory domain and thereby separates these two domains.

With 1.7 units/ml of trypsin, both the catalytic and regulatory domains are evident when PKC is combined with Ca^{2+} and LUVs composed of 20 mol % POPS, 79 mol % POPC and 1 mol % DG (Figure 2.11a). PKC alone is not susceptible to trypsin cleavage under these conditions. ARP with either PKC alone or PKC with phospholipid appears to only render the pseudosubstrate domain of the enzyme susceptible to trypsin cleavage. However, ARP with its multiple Arg residues has the requirements of a substrate of trypsin and may inhibit the

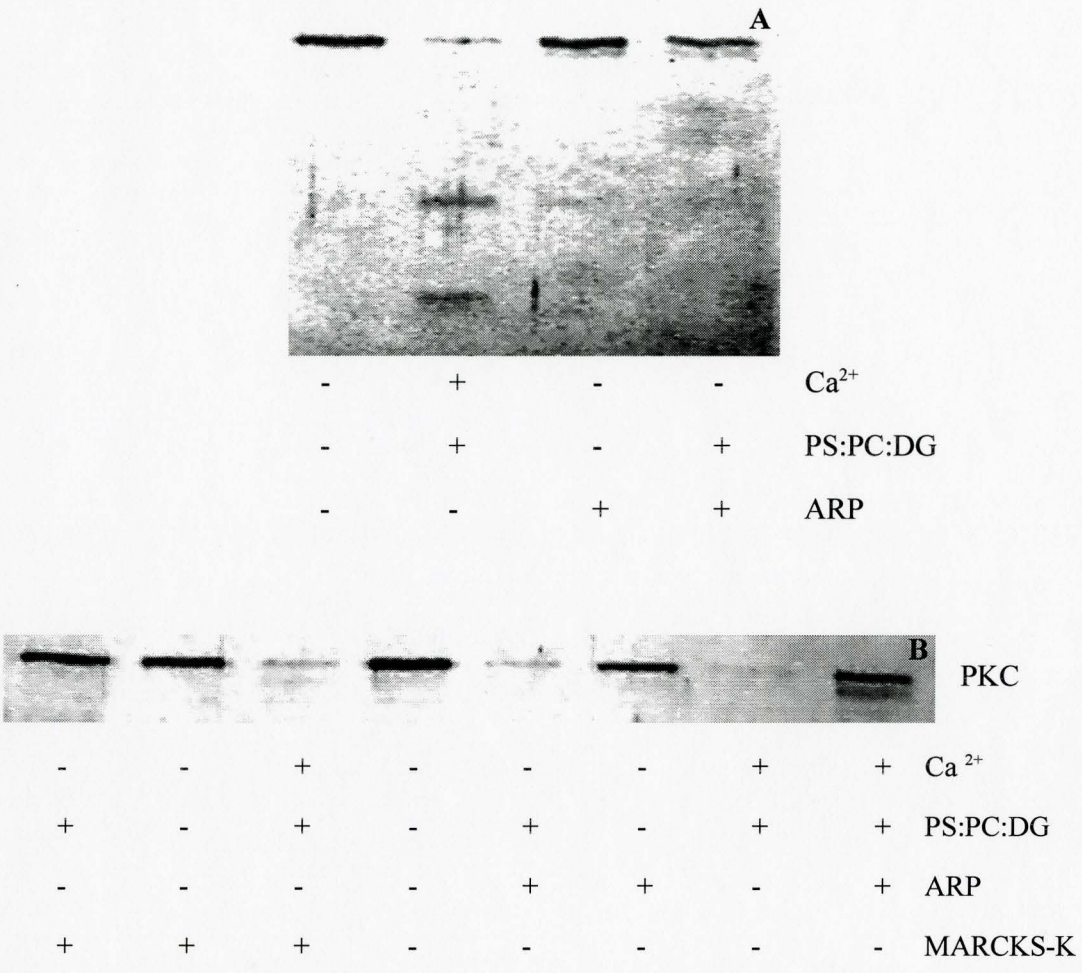


Figure 2.11 The trypsin susceptibiltiy of PKC. The phospholipid was in the form of LUVs composed of 20 mol% POPS, 79 mol% POPC, and 1 mol% DG. The reaction was run on a 7.5 % SDS-PAGE and subsequently silver stained. **A** was performed with 1.7 units/ml of trypsin, while **B** was performed with 17 units/ml of trypsin. Reproduced from Bruins and Epand, 1995.

enzyme. This was addressed by increasing the trypsin concentration 10 fold to 17 units/ml (Figure 2.11b). At this level of trypsin, the catalytic and regulatory domains are no longer clearly visible on a silver stained SDS-PAGE and therefore only the PKC band is shown. PKC in the absence of Ca^{2+} and phospholipid is still not susceptible to trypsin cleavage, while the addition of these cofactors almost eliminated the PKC band. With 17 units/ml of trypsin, the addition of ARP in the presence of phospholipid also diminishes the amount of PKC. In the absence of phospholipid, ARP minimally changes the susceptibility of PKC to trypsin. The MARCKS-K peptide does not seem to affect the susceptibility of PKC to trypsin either in the presence or the absence of phospholipid. Both ARP and MARCKS-K inhibit trypsin as seen when Ca^{2+} and phospholipid are present with PKC.

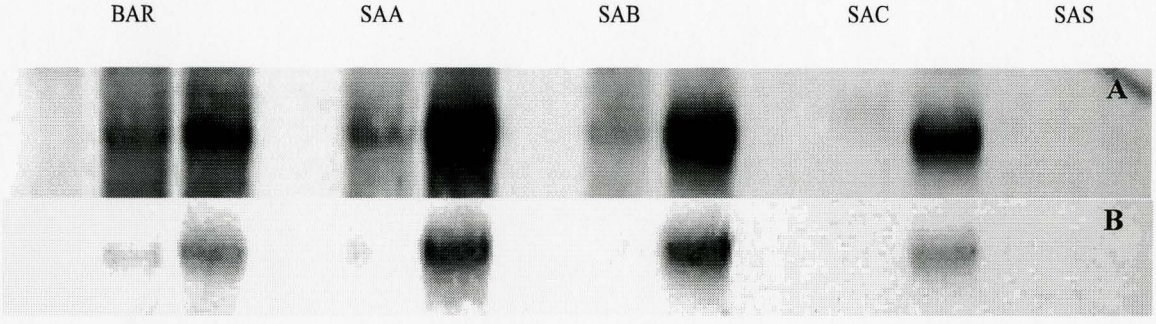
These results demonstrate that ARP binds to PKC in the absence of phospholipid and causes the removal of the pseudosubstrate domain. When a phospholipid bilayer is present with the ARP and PKC complex, a further conformational change occurs which renders the susceptibility of PKC to trypsin cleavage to the level seen with Ca^{2+} and phospholipid.

2.3.5 The crosslinking of PKC with substrate analogs

2.3.5.1 Crosslinking specificity of the substrate analogs and PKC

The crosslinking of substrate analogs of PKC by disulfide linkages has been

previously shown to be Ca^{2+} and phospholipid dependent (Ward et al., 1995; Ward et al., 1996). However, the substrates from which these analogs were developed displayed some cofactor independence for their phosphorylation. These biotinylated peptide analogs were a generous gift to us along with a biotinylated analog of ARP (Table 1.1). The biotin group on these peptides allows for easy detection after SDS-PAGE and western blotting by using enhanced chemiluminescence (ECL) with streptavidin conjugated alkaline phosphate. The crosslinking of PKC in the presence and absence of Ca^{2+} and LUVs composed of 50 mol% POPS, 49 mol% POPC and 1 mol% DG is displayed in Figure 2.12. In the absence of PKC there is no signal detected. Also, the biotinylated substrate, SAS (N-biotinyl-Arg-Arg-Arg-Thr-Leu-Arg-Arg-Leu) when incubated with PKC in the presence or absence of cofactors does not produce a signal due to the lack of a Cys residue. The other peptides, BAR (N-biotinyl-Arg-Arg-Arg-Arg-Tyr-Gly-Cys-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Tyr), SAA (N-biotinyl-Arg-Arg-Arg-Cys-Leu-Arg-Arg-Leu),SAB(N-biotinyl-Arg-Lys-Arg-Cys-Leu-Arg-Arg-Leu) and SAC (N-biotinyl-Lys-Lys-Lys-Cys-Leu-Lys-Lys-Leu) all crosslink with PKC in the presence of cofactors to varying degrees. In agreement with the previous studies, both SAA and SAB are much more potent than SAC, while BAR is a moderate crosslinker. In the absence of Ca^{2+} and phospholipid, BAR displays the greatest potency, both SAA and SAB show reactivity, while SAA displays no signal in the crosslinking of PKC. The amount of labelling of PKC by these substrate analogs in the absence of cofactors is substantially less than in the presence of cofactors. This is inspite of the fact that ARP is readily



-	+	+	-	+	+	-	+	+	-	+	+	+	+	PKC
+	-	+	+	-	+	+	-	+	+	-	+	-	+	PS:PC:DG

Figure 2.12 Crosslinking specificity of the substrate analogs with protein kinase C as detected by ECL. All lanes contain 200 μM Ca^{2+} and 5 μM of the biotinylated peptide. The lanes are in groups of 3 with the indicated peptide added except with SAS which was only in 2 lanes. The phospholipid was in the form of LUVs and were composed of 50 mol% POPS, 49 mol% POPC, and 1 mol% DG. **A** was exposed 5 times longer to film than **B**.

phosphorylated in the absence of cofactors.

2.3.5.2 The modulation by Ca^{2+}, DG and ARP of the labelling of PKC

Since the rate of phosphorylation of ARP by PKC was enhanced in the presence of phospholipid alone, the affect on the labelling of PKC by the substrate analogs was tested in the presence and absence of Ca^{2+} and/or DG. The crosslinking of PKC with SAA, SAB, and SAC all displayed maximal effectiveness with Ca^{2+} and LUVs composed of 50 mol% POPS, 49 mol% POPC and 1 mol% DG (Figure 2.13a,c). Almost equivalent labelling of PKC was also observed with SAB and SAC with Ca^{2+} and LUVs composed of 50 mol% POPS and 50 mol% POPC. SAA did not label PKC as well in the absence of DG. This suggests that membrane bound PKC in the presence of Ca^{2+} is in the same activation state in the presence and absence of DG. An interesting difference in the crosslinking potential of PKC can be noticed between LUVs with or without DG in the absence of Ca^{2+}. In the absence of Ca^{2+}, all the substrate analogs crosslink with PKC significantly more with LUVs containing 1 mol% DG than without DG. Interestingly, SAA did not induce the translocation of PKC to the membrane in the absence of Ca^{2+} as monitored by resonance energy transfer (Figure 2.5). However, the pseudosubstrate domain must be removed by a conformational change in the enzyme. These results suggest that PKC associates with a DG containing membrane in the absence of Ca^{2+} and undergoes a conformational change that removes the

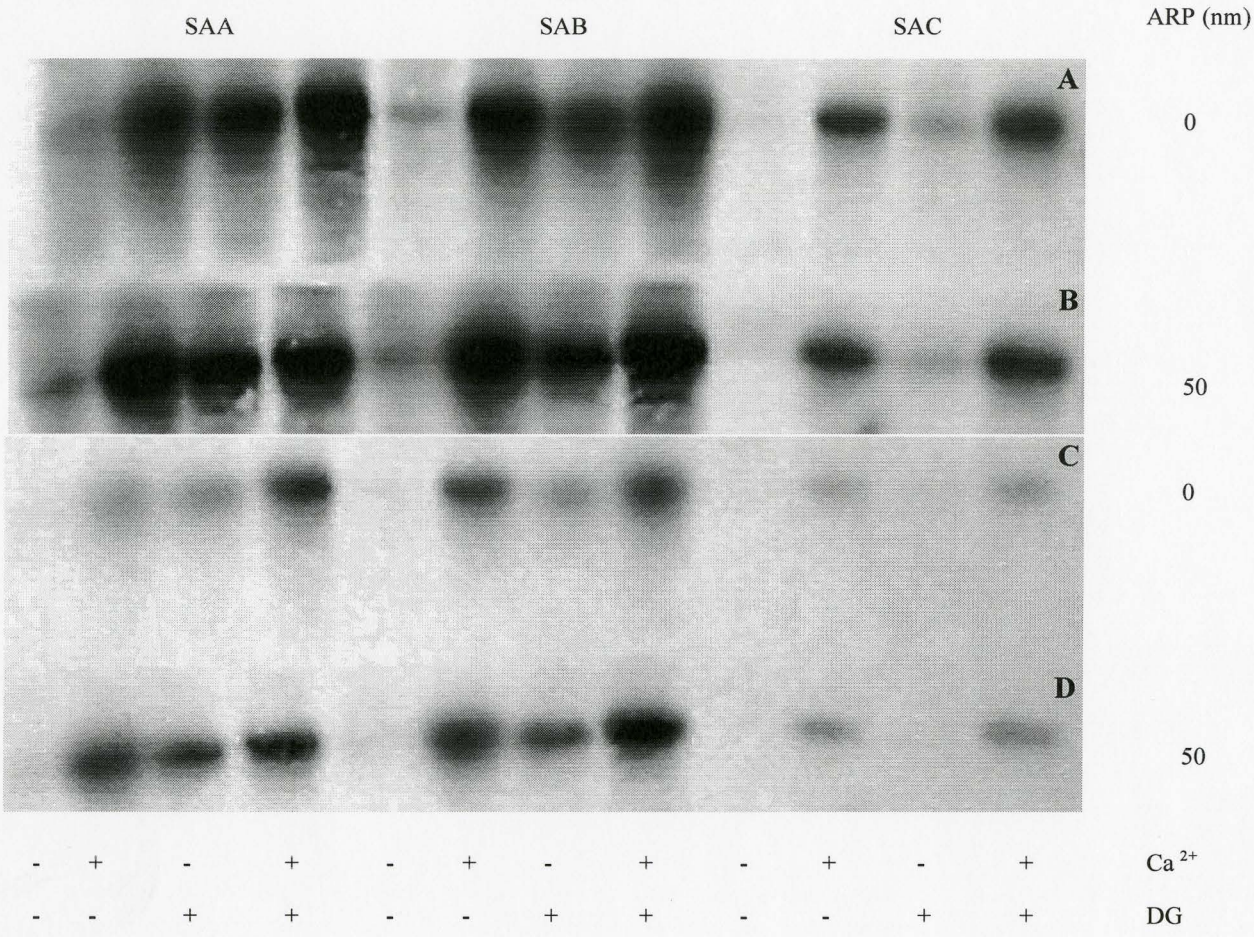


Figure 2.13 Labelling of protein kinase C in the presence and absence of cofactors and the modulation by the ARP. All lanes contain LUVs with 50 mol % POPS and 49-50 mol% POPC with the labelled lanes also containing 1 mol % DG and/or 200 μM Ca^{2+}. The lanes are in groups of 4 with the indicated biotinylated peptide added. Images **B** and **D** also contain 50 nm ARP. Images **A** and **B** were exposed to film for 5 times longer than **C** and **D**.

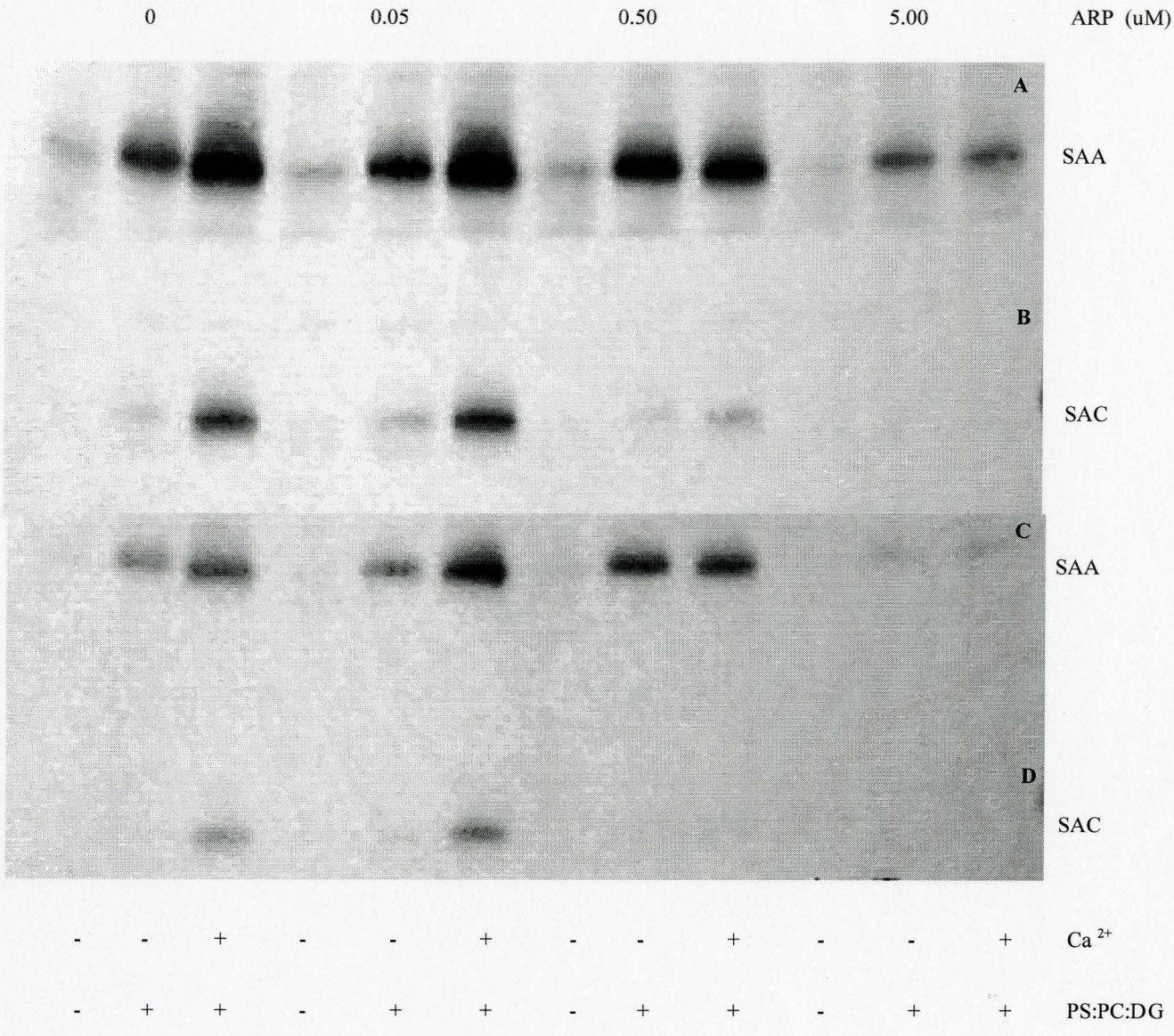


Figure 2.14 Labelling of protein kinase C in the presence and absence of cofactors and the modulation by increasing amounts of ARP. The LUVs were composed of 50 mol% POPS, 49 mol% POPC, 1 mol% DG. Calcium was at a concentration of 200 μM. The lanes are in groups of 4 with the indicated amount of the ARP added. Images **A** and **C** contain 5 μM of SAA while **B** and **D** contain 5 μM of SAC. Images **A** and **B** were exposed to film for 5 times longer than **C** and **D**.

pseudosubstrate domain but is not in a long lived membrane binding state.

ARP increases the labelling of PKC by the substrate analogs (Figure 2.13b,d). ARP noticeably elevates the crosslinking of PKC with SAA and SAB and slightly increase the labelling of PKC with SAC. The enhancement by of the crosslinking of PKC with SAA and SAB is most notable in the absence of Ca^{2+} with LUVs containing 1 mol% DG. Interestingly, this effect occurs with 50 nm of ARP which is approximately 2 magnitudes of order lower than the evaluated K_m. As the concentration of ARP increases in the reaction, the labelling by SAA increases with a maximum around 0.5 μM (Figure 2.14). At this concentration of ARP, the labelling of PKC by SAA in the presence of Ca^{2+} and phospholipid is slightly diminished, while in the absence of Ca^{2+} the labelling is maximum. The increase in PKC labelling by ARP is even apparent in the absence of cofactors. At 5 μM ARP inhibition of the crosslinking of PKC with either SAA or SAC is significant. This inhibition occurs at a concentration of ARP which is 40 times lower than the concentration of a pseudosubstrate peptide (Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln) needed for a similar extent of inhibition (Ward et al., 1996).

2.3.5.3 The modulation by various protein factors of the labelling of PKC

The labelling of PKC by SAA is not only affected by ARP but also by other factors (Figure 2.15). With LUVs comprised of 50 mol% POPS and 50 mol% POPC, the labelling

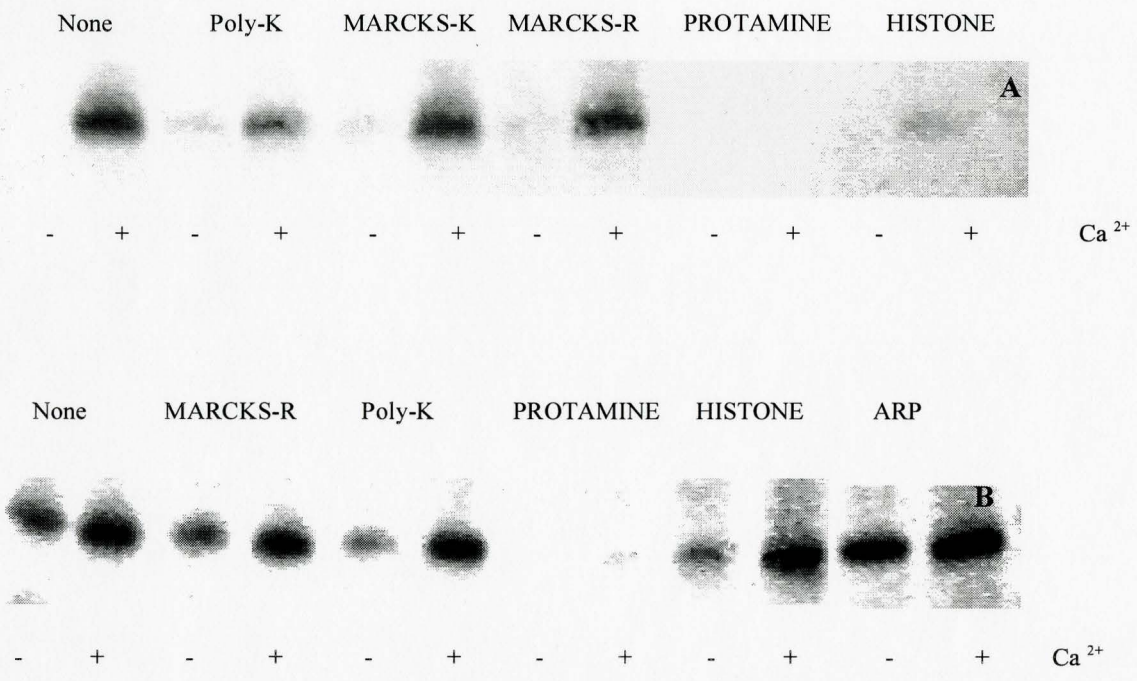


Figure 2.15 Modulation of the crosslinking with 5 μM SAA of protein kinase C. **A.** All lanes contain LUVs composed of 50 mol% POPS and 50 mol % POPC with 200 μM Ca^{2+} when indicated. The lanes are in groups of 2 with the added modulator indicated above. The concentrations of the modulators was as follows: poly-K, MARCKS-K and MARCKS-R were at 5 μM; protamine sulfate and histone were at 0.2 mg/ml. **B.** All lanes contain LUVs composed of 50 mol% POPS, 49 mol % POPC and 1 mol% DG with 200 μM Ca^{2+} when indicated. The lanes are in groups of 2 with the added modulator indicated above. The concentrations of the modulators was as follows: poly-K, MARCKS-R and ARP were at 0.5 μM; protamine sulfate and histone were at 0.02 mg/ml.

of PKC by SAA is not significantly affected by 5 μM of MARCKS-K or MARCKS-R. The presence of DG in the LUVs along with 0.5 μM MARCKS-R does not produce a difference in the labelling of PKC by SAA. Both 5 μM poly-K and to a lesser extent 0.5 μM poly-K inhibit the labelling of PKC by SAA in the presence and absence of cofactors. Interestingly, 0.2 mg/ml of histone or protamine sulfate with LUVs comprised of 50 mol% POPS and 50 mol% POPC in the presence or absence of Ca^{2+} almost completely abolishes the labelling of PKC by SAA. With DG in the LUVs and with a 10 times lower concentration of modulator, the inhibition by histone is not as compelling while protamine sulfate is still highly effective in the inhibition of PKC labelling.

The labelling of PKC with BAR produces substantially different results with these modulators (Figure 2.16). MARCKS-K, and MARCKS-R at 5 μM concentrations enhance the crosslinking of PKC with 5 μM BAR in the absence of Ca^{2+}. Poly-K (0.5 μM) inhibits the labelling of PKC with BAR. Interestingly, neither protamine sulfate (0.02 mg/ml and 0.2 mg/ml) or histone (0.02 mg/ml) inhibit the labelling of PKC with BAR as seen with SAA. Rather, these PKC substrates seem to strengthen the labelling of PKC with BAR in the absence of Ca^{2+}. This effect is strongest in the absence of cofactors similar to what is observed with MARCKS-K and MARCKS-R. With Ca^{2+} and phospholipid, the PKC substrates suppress the labelling of PKC with BAR to varying degrees. Protamine sulfate and MARCKS-R are the most potent while the influence of histone and MARCKS-K are modest.

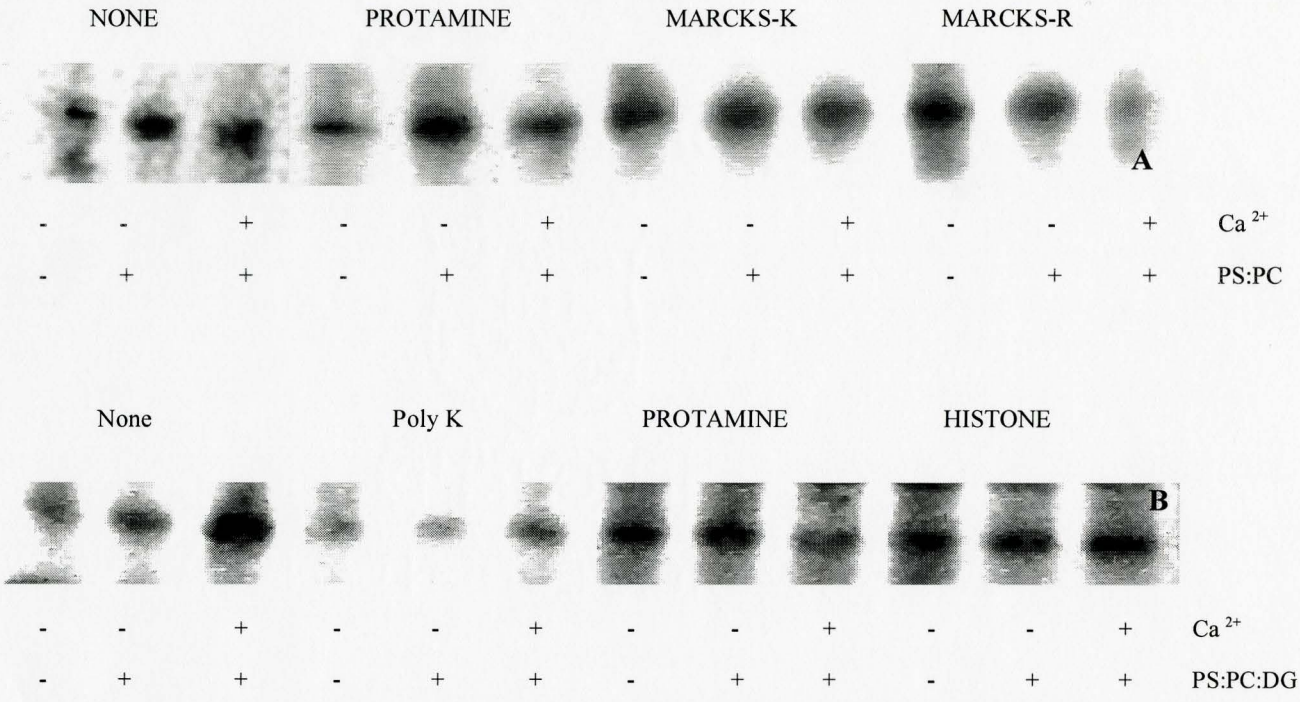


Figure 2.17 Modulation of the crosslinking with 5 μM BAR of protein kinase C. **A**. LUVs, composed of 50 mol% POPS and 50 mol % POPC, and/or 200 μM Ca^{2+} was added when indicated. The lanes are in groups of 3 with the added modulator indicated above. The concentrations of the modulators was as follows: MARCKS-K and MARCKS-R were at 5 μM; protamine sulfate were at 0.2 mg/ml. **B**. LUVs, composed of 50 mol% POPS, 49 mol % POPC and 1 mol% DG and/or 200 μM Ca^{2+} were added when indicated. The lanes are in groups of 3 with the added modulator indicated above. The concentrations of the modulators was as follows: poly-K, was at 0.5 μM; protamine sulfate and histone were at 0.02 mg/ml.

2.4 DISCUSSION

2.4.1 Protein kinase C cofactor independent phosphorylation

Protein kinase C catalyzes the phosphorylation of protamine sulfate in the absence of Ca^{2+} and phospholipid (Bazzi and Nelsestuen, 1987b). It was subsequently elucidated that the minimal sequence of protamine that is able to undergo cofactor independent phosphorylation by PKC was Arg-Arg-Arg-Arg-Tyr-Gly-Ser-Arg-Arg-Arg-Arg-Arg-Arg-Tyr (ARP) (Ferrari et al., 1987). This peptide was obtained and readily underwent phosphorylation by PKC in the absence of cofactors (Figure 2.1). Both MARCKS-K (Ac-Phe-Lys-Lys-Ser-Phe-Lys-Leu-NH_2) and MARCKS-R (Ac-Phe-Arg-Arg-Ser-Phe-Arg-Leu-NH_2) which lacked the clusters of Arg residues on both sides of the phosphorylated residue required cofactors for phosphorylation. The replacement of the Lys residues in the MARCKS peptide sequence with Arg residues does significantly increase the rate of phosphorylation by PKC in the presence of Ca^{2+} and phospholipid.

Further investigation of the cofactor independent phosphorylation of ARP by PKC was pursued through kinetic analysis. The K_m and k_{cat} for ARP were determined with the classical Michaelis-Menten kinetics. The values obtained from such an analysis are displayed in Table 2.1. The values obtained in the presence of phospholipid are an estimation since Michaelis-Menten kinetics does not regard the on and off rates for the binding of PKC, the

substrate and the phosphorylated product to the phospholipid bilayer. The K_m for ARP was around 10 μM and was not significantly different in the presence or absence of cofactors. This figure is lower than the previously reported values of 33 μM and 20 μM for ARP in the absence and presence of cofactors, respectively (Ferrari et al., 1987). However, as mentioned earlier, the PKC used in this prior study was purified from bovine brain and did contain at least one major contaminant. The k_{cat} for ARP was approximately 0.38 s^{-1} in the absence of cofactors but increase to approximately 0.67 s^{-1} and 0.87 s^{-1} in the presence of phospholipid without and with Ca^{2+}, respectively. The increase in k_{cat} in the presence of phospholipid seems to be most easily explained by both PKC and the substrate binding to these bilayers and thereby reducing the dimensionality of the interaction. These values were obtained with membranes containing 50 mol% POPS and 50 mol% POPC but lacking DG. PKC binds and are activated by these membrane in the presence of Ca^{2+} (Mosior and Epand, 1993) but requires DG in the membrane for Ca^{2+}-independent binding (Epand et al., 1992; Mosior and Epand, 1994). Therefore, the kinetic data suggests that the ARP is promoting the association of PKC with these membranes in the absence Ca^{2+}.

Histone (0.2 mg/ml) inhibits the PKC catalyzed phosphorylation of 7 μM ARP in the presence of phospholipid alone while itself is not phosphorylated (Figure 2.4). The kinetic data would suggest that the type of inhibition (at this amount of histone) seems to be non-competitive. However, histone does not seem to compete for the active site of PKC since it is not phosphorylated under these conditions and the binding of histone to another site on the

enzyme has never been shown and seems unlikely (given its cofactor requirements). Additionally, histone did not affect the phosphorylation of ARP in the absence of phospholipid or with phospholipid and 20 μM Ca^{2+}. A lower concentration of histone did not affect the phosphorylation at all. These elements suggests that the inhibition displayed by histone may not be directly related to the binding of histone to the enzyme but by rather altering the membrane bound ARP and PKC complex in the absence of Ca^{2+}. Domains of PS have been shown to be important in PKC's activity at the membrane surface (Yang and Glasser, 1995). A high enough concentration of histone may act by sequestering PS in domains that the ARP and PKC complex could not bind. Alternatively, the binding of histone to the membrane in the absence of Ca^{2+} may produce repulsive forces for the association of the ARP and PKC complex to the bilayer. However, it can not be ruled out that histone at higher concentrations inhibit the interactions of ARP with PKC.

When the Cys in SAA is replaced with a Thr, this peptide can be moderately phosphorylated by PKC in the absence of cofactors. However, the crosslinking of PKC in the absence of cofactors is nominal. A similar observation is made with BAR, the biotinylated analog of ARP with a Cys in place of the Ser. The reason for this discrepancy is not easily identified. PKC's reaction mechanism with Ca^{2+} and phospholipid follows that of a sequential bi bi mechanism with MgATP binding into the active site first followed by histone (Leventhal and Bertics, 1991). Cofactor independent phosphorylation should follow a similar mechanism. The binding of the substrate is suspected to occur on the catalytic

domain at a location outside the active site at a cluster of acidic residues. This would subsequently result in the removal of the pseudosubstrate domain. (Orr and Newton, 1994). Additionally, the binding pocket for ATP in PKC, from homology modelling, is buried in the active site (Orr and Newton, 1994). Therefore, substrate binding at these acidic residues on PKC removes the pseudosubstrate domain and then, presumably, MgATP would bind first in the active site. The ensuing binding of a substrate molecule in the active site might occur through a variety of different ways. Positive cooperativity has been noticed with the protamine phosphorylation by PKC (Leventhal and Bertics, 1993). This suggests that a second substrate molecule would bind in the active site after MgATP while the first substrate molecule remains bound. Also, these authors proposed that the binding site for the first substrate molecule was at a distinct site from the catalytic domain. Alternatively, the binding of MgATP may alter the active site conformation causing the pseudosubstrate domain to bind with lower affinity such that the equilibrium between an open active site and closed active site is shifted towards the open state. This would then allow the originally bound substrate molecule or another substrate molecule to bind in the active site without the requirement of an additionally bound substrate molecule.

The inequality between the cofactor independent phosphorylation and labelling may be due to inaccessibility of the Cys residue in the active site. It has been observed that the conformation change that PKC undergoes in the absence of Ca^{2+} and phospholipid is not to the same extent as in the presence of cofactors. Apart from increasing the membrane affinity

of PKC, DG elevates the maximal rate of phosphorylation by the enzyme (Mosior and Epand, 1993). This may be due to the active site conformation. Therefore, the active site conformation of PKC with distinct cofactors may be different. The Cys residue that forms the disulfide bridge with the substrate analogs may not be as available in the absence of cofactors. However, in the presence of phospholipid and EGTA, the ARP and PKC complex seems to be in a similar conformation as the Ca^{2+} membrane bound form of the enzyme as detected by proteolytic susceptibility. Yet, the degree of labelling of PKC by BAR does not appear to be equivalent to the amount of phosphorylation observed. Therefore, the active site conformation and the availability of the Cys may not be the explanation for the deviation between the level of labelling and the phosphorylation.

Since MgATP was not added in the labelling of PKC by the substrate analogs in this study, it is difficult to elucidate the precise mechanism by which cofactor independent phosphorylation transpires. However, the addition of MgATP in the previous study with these peptides, in the presence of Ca^{2+} and phospholipid, reduced the labelling of PKC by 70 % (Ward et al., 1996). Therefore, it was surmised that the binding of MgATP was blocking the Cys in the active site that was forming a disulfide bond with the substrate analogs. Consequently, the presence of MgATP in this study would presumably block the labelling and provide negligible information as to the mechanism of substrate binding.

The addition of ARP to the labelling of PKC with SAA and SAB does provide a little insight into the possible mechanism. ARP enhances the labelling of PKC by SAA and SAB

in the presence and slightly in the absence of cofactors. This implies that ARP binds apart from the active site and possibly stabilizes the enzymes open conformation while the substrate analogs reacts in the active site. In the absence of cofactors, the degree of PKC labelling promoted by ARP still appears weaker than expected for the amount of cofactor independent phosphorylation that occurs. Therefore, the binding of MgATP in the active site might be an important complementary stabilization along with the initial bound substrate molecule that allows the second substrate molecule to bind into the active site.

ARP enhances the labelling of PKC by the substrate analogs at concentrations 2 orders of magnitude below the evaluated K_M. Additionally, significant competition (inhibition of labelling) with SAA for the binding at the active site of PKC does not occur until the concentration of ARP is around its K_M. The kinetic data presented does not indicate any positive cooperativity. However, the concentrations of protamine sulfate where positive cooperativity has been observed are at least 1 order of magnitude below the evaluated K_M. Consequently, it appears that PKC may contain a high affinity binding site for Arg rich substrates separate from the active site. Since, BAR, in the labelling of PKC, displays more cofactor dependence than ARP, the replacement of Ser with Cys would seem to hamper the interaction of a substrate in this site.

2.4.2 Substrate induced translocation of PKC

The induction of the binding of PKC to the membrane by ARP in the absence of Ca^{2+} is demonstrated by the resonance energy transfer and intrinsic tryptophan fluorescence experiments. Both these techniques require the physical association of the Trp residue(s) on PKC to interact with the membrane. The Trp on PKC are excited and then either, the emission energy is transferred to a dansyl moiety in the membrane or the magnitude of the Trp spectra is decreased upon binding to membranes. ARP in both techniques clearly causes the association of PKC with a membrane in the absence of Ca^{2+} (Figure 2.5, Figure 2.7). Not surprisingly, the MARCKS-K peptide did not influence the Ca^{2+}-independent binding of PKC to membranes. However, an analog of a PKC substrate, SAA, which displays moderate cofactor independent phosphorylation by PKC (Ward et al., 1996) also did not induce the translocation of PKC to the membrane in the absence of Ca^{2+}. This indicates that ARP possesses additional elements apart from those required for PKC cofactor independent phosphorylation which can induce the Ca^{2+}-independent translocation of PKC to a phospholipid bilayer. The main difference between ARP and SAA is the length of the Arg clusters on either side of the phosphorylated residue. Therefore, the removal of the pseudosubstrate domain by the substrate may require the binding of a certain amount of positive charge while the promotion and stabilization of a membrane bound form of the enzyme by a substrate would necessitate additional positive charge.

Membranes with DG and high PS can support the binding of PKC in the absence of Ca^{2+} (Epand et al., 1992; Mosior and Epand, 1994). Under these conditions it is evident that the substrate analogs can weakly interact and label PKC. In the absence of DG and Ca^{2+}, the labelling is minimal. ARP does, however, strengthen the labelling of PKC in the presence and absence of DG and Ca^{2+}. This is most notable with membranes with DG and high PS in the absence of Ca^{2+}. ARP may be acting by translocating more PKC to the membrane or by stabilizing the open conformation of the already membrane associated enzyme. It has been suggested that the Arg residues in protamine sulfate may be a partial substitute for Ca^{2+} in the activation of PKC (Kimura et al., 1987; Sakai et al., 1987). Therefore, the interaction of nanomolar amounts ARP with PKC plausibly stabilizes the membrane bound state of the enzyme as well as increasing the amount of membrane bound enzyme.

2.4.3 Conformational changes in PKC upon interacting with ARP and phospholipid

The binding of ARP to PKC produces a conformational change in the enzyme. In the absence of phospholipid, the binding of ARP to PKC removes the pseudosubstrate from the enzymes active site as detected by proteolytic cleavage (Figure 2.11). This is in accord with previous findings (Orr et al., 1992; Orr and Newton, 1994) and is expected since the substrate is phosphorylated independent of cofactors. Additionally, the association of ARP with PKC produces the exposure of hydrophobic site(s) on the enzyme. In the absence of a

phospholipid bilayer, these sites may interact to form larger aggregates of PKC and ARP. Such aggregates have been observed to form with protamine sulfate and PKC (Bazzi and Nelsestuen, 1987b). The presentation of the hydrophobic site(s) on PKC by ARP seems sufficient to promote the association of the enzyme substrate complex with the membrane. Upon association with the membrane, the enzyme undergoes a subsequent change in conformation such that the hinge region becomes exposed to proteolytic cleavage. PKC at the membrane in complex with ARP appears to be in a similar membrane associated conformation as the enzyme bound to Ca^{2+} and phospholipid.

2.4.4 PKC, substrates and substrate analogs

The labelling of PKC with SAA and BAR is affected in different ways by PKC substrates. Most notably is the influence of the addition of protamine sulfate. The labelling of PKC by SAA is greatly inhibited by protamine sulfate in the presence and absence of Ca^{2+}. The binding of protamine sulfate to PKC blocks any site to which SAA may bind and indicates a much higher affinity of binding to PKC. Conversely, 0.2 mg/ml histone blocks the labelling of PKC by SAA in the presence and absence of cofactor, while lower concentrations of histone mainly inhibit the Ca^{2+} independent labelling of PKC. The MARCKS peptides do display minimal inhibition of labelling by SAA but this is presumably due to competition for the active site. The labelling of BAR is enhanced by protamine

sulfate. This supports the notion for positive cooperativity between Arg-rich substrates. In the presence of Ca^{2+} and phospholipid, protamine slightly inhibits the labelling of PKC by BAR which is suggestive that the protamine sulfate is now competing for the active site with BAR. Interestingly, both MARCKS-R and lower histone concentrations also enhance the labelling of PKC in the absence of Ca^{2+}. This seems odd since neither of these substrates can undergo cofactor independent phosphorylation. Once bound to the membrane, PKC may associate with eight Ca^{2+} (Bazzi and Nelsestuen, 1990; Bazzi and Nelsestuen, 1993) which may reflect the appearance of a number of lower affinity sites on PKC produced by the conformational change in the enzyme. Therefore, the enhancement of labelling by histone and MARCKS-R may be a result of binding to such low affinity sites after an Arg-rich substrate binds and induces a conformational change. Histone may bind to these low affinity sites due to its positive charge and at higher concentration may competing for the binding of PKC with ARP through electrostatics. However, histone would not be able to induce a conformational change in the enzyme due to the lack of Arg. Poly-K inhibits the labelling of PKC by both SAA and BAR. This may be due to disruption of membrane domains of PS as noted earlier or due to pure competition for binding sites through electrostatic interactions. Nevertheless, the binding of BAR and SAA to PKC is different as can be observed by their responses to other PKC substrates.

3. SUMMARY AND FUTURE STUDIES

In the absence of phospholipid, Arg-rich substrates induce the exposure of hydrophobic sites on protein kinase C. Arg-rich substrates can subsequently promote the translocation of protein kinase C to the membrane in the absence of Ca^{2+}. The conformational change that occurs upon membrane binding of the enzyme and substrate complex is similar to the Ca^{2+} translocated enzyme. The binding of Arg-rich substrates to PKC appears to transpire through a high affinity site apart from the active site. Additionally, cofactor independent phosphorylation and the promotion of the translocation of PKC to membrane by Arg-rich peptides have different requirements for the number of Arg residues flanking the target Ser/Thr residue. This study has demonstrated new insights into cofactor independent phosphorylation by PKC as well as illustrated a novel mechanism by which a substrate can promote the translocation of PKC in the absence of calcium. However, other investigations are needed to further elucidated the precise mechanism of by which cofactor independent phosphorylation and substrate induced translocation transpire. Additional kinetic studies with PKC and protamine sulfate and/or ARP would be most fruitful. Since both these substrates do not require a membrane, the kinetic analysis would be relatively straight forward. Both product and dead-end inhibition studies would help clarify the reaction mechanism and define the order of binding of the substrates and release of the products. These studies could

be done with the substrate analogs, nonhydrolyzable ATP analogues, ADP and poly-K. Furthermore, the kinetic analysis between the intact enzyme and its catalytic domain under these conditions would also help determine the interaction of PKC with Arg-rich substrates. Also, to examine if BAR crosslinks to PKC apart from the active site, one could crosslink PKC's active site with non-biotinylated substrate analogs in the presence of Ca^{2+} and phospholipid. Then, EGTA would be added with BAR and subsequently detected by ECL. A negative result, however, would not rule out the binding of BAR at another site(s) since a Cys may not be available at the site to form a disulfide bridge with. Unfortunately, physical studies of PKC and ARP apart from the membrane do not see feasible since it appears that the ARP and PKC complex aggregates in solution.

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