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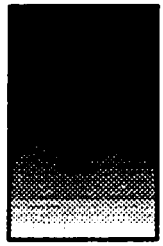

CHARACTERIZATION AND REGULATION OF CYTOSOLIC PHOSPHOLIPASE A_2 ACTIVITY FOLLOWING BRADYKININ STIMULATION OF RABBIT CORTICAL COLLECTING DUCT CELLS

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ABSTRACT

The mammalian kidney, in particular the collecting duct segment of the nephron, is a major site of renal sodium and water reabsorption. One of the many agonists known to regulate collecting duct function is bradykinin (BK), a nonapeptide which exerts natriuretic and diuretic effects through its ability to increase arachidonic acid (AA) availability and prostaglandin production. Through the use of an established cell line of rabbit cortical collecting duct (RCCD) epithelial cells representing a mixed population of principal and intercalated cell types, a study was established in order to reveal which enzyme is responsible for BK-induced AA release. Evidence presented herein indicates that 85 kDa cytosolic phospholipase A_2 ($cPLA_2$) is the enzyme accountable for BK-mediated AA. This is based on the following observations: RCCD cells express $cPLA_2$ protein, an arachidonyl trifluoromethyl ketone inhibitor of $cPLA_2$ completely blunted BK-stimulated AA release, both basal and BK-stimulated PLA_2 activities were significantly diminished following treatment with an antibody to this enzyme, and finally, PLA_2 *in vitro* activity could be stimulated by submillimolar calcium concentrations and was dithiothreitol (DTT)-insensitive. Strategies were also employed to reveal the involvement of protein kinase C (PKC) in BK-mediated AA release, $cPLA_2$ activation and $cPLA_2$ phosphorylation. Chemical inhibition of PKC reduced both BK-stimulated AA release and $cPLA_2$ activity; however, BK-stimulated AA release appeared limited by calcium mobilization rather than by PKC. Cytosolic PLA_2 activity could be stimulated by phorbol ester (PMA) and the magnitude of this activation was similar to that caused by BK. A direct correlation between the level of $cPLA_2$ phosphorylation and

activation was confirmed by the phosphatase-mediated reversal of both BK- and PMA-stimulated responses. Furthermore, the increase in $cPLA_2$ phosphorylation was found to occur on serine residues. The mitogen-activated protein kinase (MAPK) cascade was also implicated in the events leading to BK-induced AA release but simultaneous inhibition of PKC and MAPK did not result in an additive reduction. Protein kinase C also appears involved in the sequential activation of MAPK. Additional examination of PKC isozyme expression revealed the presence of at least four types, PKCα, γ, ϵ, and ζ. Upon BK-receptor stimulation, only PKCϵ translocated to the particulate fraction, and as a result, this isozyme may represent the predominant PKC species activated by this agonist. Taken together, the results presented herein provide an examination of the signalling mechanisms responsible for $cPLA_2$ activation in response to BK.

DEDICATION

To my family and fiancée Camilla, who provide me with the support and encouragement to achieve my goals and aspire to reach new ones.

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DECLARATION

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LIST OF ABBREVIATIONS

A23187	calcium ionophore
$AACOCF_3$	arachidonyl trifluoromethyl ketone
AA	arachidonic acid
AVP	arginine-vasopressin
BCS	biodegradable counting scintillant
BK	bradykinin
BSA	bovine serum albumin
Ca^{2+}	calcium
CaLB	calcium-dependent lipid-binding
cAMP	cyclic-adenosine-3',5'-monophosphate
CHO	Chinese hamster ovary
$cPLA_2$	cytosolic phospholipase A_2
DAG	diacylglycerol
DMEM/F12	Dulbecco's Modified Eagle Medium:Nutrient mixture F-12
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EGTA	ethylenebis(oxyethylenenitrilo)tetraacetic acid
EP	E-type prostanoid receptor
ERK	extracelluar signal-regulated kinase
FCS	fetal calf serum
Gö6976	12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole
G-protein	guanine nucleotide-binding protein
HBSS	Hank's balanced saline solution
HELSS	haloenol lactone suicide substrate
IL	interleukin
$iPLA_2$	calcium-independent PLA_2
IP_3	inositol trisphosphate
ITS	insulin/transferin/selenium
kDa	kilodalton
MAPK	mitogen-activated protein kinase
MAPKK or MEK	mitogen-activated protein kinase kinase
MEKK	mitogen-activated protein kinase kinase kinase
MDCK	Madin-Darby canine kidney cell
MW	molecular weight
PAP	phosphatidic phosphohydrolase
PBS	phosphate-buffered saline

PC	phosphatidylcholine
PD98059	2'-amino-3'-methoxyflavone
PDD	4α-phorbol-12,13-didecanoate
PE	phosphatidylethanolamine
PGE_2	prostaglandin E_2
PGHS	prostaglandin endoperoxide synthase
PGI_2	prostaglandin I_2 or prostacyclin
PI	phosphatidylinositol
PI-PLC	phosphatidylinositol-specific phospholipase C
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol-12-myristate-13-acetate
PMSF	phenylmethylsulfonylfluoride
RCCD	rabbit cortical collecting duct
RHC-80267	DAG lipase inhibitor
Ro31-8220	{3-[1-[3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide methane sulfonate}
RT-PCR	reverse trancription-polymerase chain reaction
S.D.	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S.E.M.	standard error of the mean
$sPLA_2$	secretory phospholipase A_2
TBS	Tris-buffered saline
TTBS	tween Tris-buffered saline
T_3	3,5,3'-triodothyronine
$[Ca^{2+}]_i$	intracellular calcium concentration

SECTION 1: INTRODUCTION

1.1 The kidneys

The kidneys are paired organs situated on the posterior wall of the abdomen. In humans, although the kidneys constitute less that 0.5% of the total body weight of an adult, they receive a blood flow equal to about 25% of total cardiac output. The kidneys are critical to survival through their ability to filter the blood, reabsorb water and solutes, secrete selected solutes into the tubular lumen, and their ability to modulate the volume and composition of the body fluids. Indeed, the kidneys can be viewed more appropriately as regulatory, rather than as excretory organs.

1.1.1 The nephron

The basic functional structure of the kidney that permits this homeostatic regulation is that of the renal tubule or nephron. The human kidney contains approximately 1.2 million nephron units, each of which is organized into a series of defined and continuous segments (renal corpuscle, proximal tubule, loop of Henle, distal tubule, and collecting duct system) that perform very specialized functions. Following glomerular sieving, an ultrafiltrate of plasma is produced and presented to Bowman's capsule and the distal tubule segments. Each of the different tubule segments are comprised of unique epithelial cells that possess specific and distinct properties which consequently allow the kidney as a whole to maintain body fluid homeostasis. This homeostasis is achieved through the individual properties of each segment

to either secrete into the urine, or reabsorb into the circulation, those substances that need to be closely regulated. Accordingly, the tubules are able to control precisely the volume, osmolality, composition, and pH of the intracellular and extracellular compartments.

Quantitatively, the primary function of the nephron is to reabsorb sodium and water. The major segment involved in this phenomenon is the proximal tubule and it accounts for the reabsorption of approximately 70% of the filtered water, whereas the descending thin limb and collecting duct system are responsible for the remainder. The collecting duct itself is the predominant nephron region under the control of the hormone, arginine-vasopressin (AVP), which subsequently modulates water permeability. Furthermore, since this is the last segment of the nephron, it represents a critical site for the final determination of urine composition.

1.1.2 The cortical collecting duct

The collecting duct system of the nephron can be further divided into the initial collecing tubule, cortical collecting duct (CCD), outer medullary collecting duct, inner medullary collecting duct, and papillary collecting duct. The CCD begins at the initial collecting tubule and extends to the junction of the cortex and outer medulla. It is composed of two primary cell types, the principal or light cells, and the intercalated (α and β) or dark cells. The ratio of principal to intercalated cells is approximately 2:1 at the cortical end of the CCD and progressively increases to 4:1 as it reaches the cortico-medullary junction. The principal cells are responsible for sodium and water reabsorption, and potassium secretion. The α-intercalated cells mediate net acid secretion, the β-intercalated cells mediate net bicarbonate secretion, and together these two cell types are important in maintaining acid-

base balance.

For the purpose of examining the structure-function relationships of the various nephron segments, one can employ strategies utilizing either isolated perfused tubules, or cultured cells. Early experimental evidence relied largely on the use of the former technique, but the use of cultured renal tubule epithelia is now widely accepted as an effective tool with which the involvement and function of individual factors can be studied. Indeed this tool features a more readily accessible and homogeneous population of cells with which to more definitively reveal the unique operation of specific intracellular mechanisms.

1.1.3 The rabbit cortical collecting duct

While studies of primary cultures of CCD cells have been used for determining those hormone interactions and intracellular signalling pathways which exist in this segment, there was the necessity for the creation of an immortalized CCD cell line which would facilitate in depth studies of these events. Until recently, this has however been difficult to achieve due to problems associated with the isolation procedure and contamination from other nephron segments (Smith and Garcia-Perez, 1985).

One particular cell line that has been extensively used for the examination of collecting duct function and cellular physiology is the Madin-Darby canine kidney (MDCK) epithelial cell line (Madin and Darby, 1958). Although these cells express many characteristics associated with the principal cells of the distal nephron (distal tubule and early collecting duct), the precise origin of this cell type has never been unequivocally determined. Recently, a permanent rabbit cortical collecting duct (RCCD) cell line has been developed by Burns et

al., (1996). Using an isolation technique based on the use of successive Percoll and Ficoll gradient centrifugations of collagenase-digested renal cortices (Lajeunesse et al., 1995), an enriched population of RCCD cells was prepared and subsequently immortalized by transfection with a plasmid encoding the large T antigen of SV40.

Following the isolation and generation of a new cell line, it must be critically proved that neither immortalization of the cells nor subsequent cell culture techniques significantly alter the properities displayed by these tubular cells *in vivo*. In order to be recognized as a proper model of CCD cells, the immortalized RCCD cells were subject to rigorous examination. Antibodies to epithelial cell markers (cytokeratins) stained all cells positively while no immunofluorescence was observed with antibodies to smooth muscle myosin, Tamm Horsfall protein, or von Willebrand factor, thus indicating the lack of contaminating cell types within this homogeneous CCD population (Burns et al., 1996). Based on scanning electron microscopic evidence, cells had either apical microplicae, characteristic of α-intercalated cells, or few blunt microvilli, common to either principal or β-intercalated cells. The prevalence of individual cell types within this population was further assessed by the use of cell type-specific antibodies. Peanut lectin agglutinin, a marker for β-intercalated cells immunolabelled 27% of the cells while antibodies mAb 703 and mAb 503, to principal cells and α-intercalated cells respectively, stained 26% of the cells. The remaining epithelial cells remained unlabeled, a possible result of the loss of surface antigens usually expressed by the CCD.

In addition to expression of the appropriate cellular morphology associated with CCD tubules, immortalized RCCD cells also reveal hormonal responses similar to those previously characterized in freshly isolated CCD segments (Burns et al., 1996). As expected, receptor

responses to either prostaglandin E_2, AVP, or isoproterenol were each associated with significant increases in cAMP production. On the other hand, treatment of cells with parathyroid hormone or calcitonin did not result in detectable cAMP elevation (Burns et al., 1996). These two agonists stimulate cAMP production in distal and connecting tubule cells, and the absence of a stimulatory effect on cAMP accumulation in RCCD cells indicates the purity and homogeneity of the cell population. Since this cell line retained morphological and hormonal properties characteristic of this nephron segment *in vivo*, it represents an excellent system in which to further and more completely examine CCD function and regulation.

1.1.4 Arginine-vasopressin effect on salt and water absorption

While many agonists demonstrate an ability to regulate collecting duct function, the physiological role of AVP is most clearly established as being the only hormone that significantly stimulates either salt and/or water absorption. When picomolar concentrations of circulating AVP bind toV_2 receptors located on the basolateral membrane of the CCD principal cell, a signalling cascade ensues that results in the elevation of cAMP and subsequent activation of protein kinase A (Jard, 1988). Following this, sodium entry increases via apically-located amiloride-sensitive sodium channels and the water-tight epithelium is transformed to a water-permeable epithelium (Frindt and Burg, 1972). Increased water permeability associated with AVP stimulation is a result of the exocytic insertion of aquaporin-2 water channels into the apical membrane (Deen et al., 1994). In the unstimulated cell, water channels reside in subapical vesicles, but following stimulation these channels are shuttled to and inserted into the luminal membrane where they facilitate water entry into the

cell. The main effect is that solute-free water reabsorption by the kidney is increased. One particular hormone that plays a key role in the modulation of AVP's ability to stimulate water transport in the CCD is bradykinin, BK.

1.2 Bradykinin

The existence of BK has been known since the late 1940s when Rocha E. Silva discovered the presence of a substance that possessed potent vasodilator and smooth muscle-stimulating properties (Silva, 1949). Today, BK is known to be involved in many biological processes including vasodilation, neurotransmission, pain production, inflammation, and cell proliferation (Bhoola et al., 1992).

Bradykinin is a nonapeptide derived from the precursor protein, kininogen, through the action of tissue kallikreins. Kallikreins are serine proteases that cleave low molecular weight kininogen substrate to the decapeptide, lysyl-bradykinin, which is then selectively cleaved of one amino acid by an aminopeptidase, thereby yielding the final product, BK (Carretero et al., 1980).

The kallikrein-kinin system is best considered as a local autocrine/paracrine system rather than as a hormonal system because the plasma concentration of BK is seldom sufficient to cause an acute decrease of arterial blood pressure (except under experimental conditions) (Carretero et al., 1991) and because bradykinin has a short half-life of 15 sec in plasma (McCarthy et al., 1965). In fact, Nasjletti and colleagues (1975) found that when BK was infused into the renal artery, greater than 90% of the BK infused was inactivated in one

passage through the kidney and that less than 0.2% appeared in the urine. This is largely the result of the significant amount of kininase expressed by the proximal tubule, which thereby prevents filtered BK from reaching the more distal segments of the nephron (Hall et al., 1976).

All the components of the kallikrein-kinin system (low molecular weight kininogen, tissue kallikrein, kininases II, and the BK-B_2 receptor) are located locally within the kidney, and as such it appears to regulate renal glomerular function, renin release, and sodium and water metabolism in conjunction with the renin-angiotensin vasopressor system (Lortie et al., 1992). While new sites of kininogen and B_2 receptor colocalization have been recently noted in examined nephron segments, it appears by immunohistochemistry (Figueroa et al., 1988) and in situ hybridization histochemistry (Song et al, 1996) that the distal tubules and collecting ducts are the nephron regions that reveal the most intense signals for those components of the kallikrein-kinin system. In fact, there is considerable evidence to suggest that the diuretic effects of endogenous BK take place largely at the CCD where the peptide inhibits AVP-stimulated salt and water transport (Schuster, 1985; Schuster et al, 1984). For BK to exert its action, as with any signalling peptide molecule, it must interact with a specific receptor located on the target cell. As previously mentioned, the CCD possesses a high density of BK receptors of the B_2 subtype (Song et al., 1996).

1.2.1 The bradykinin-B_2 receptor subtype

The cellular actions of BK are mediated through interaction with two different receptors, B_1 and B_2 (Regoli and Barabe, 1980). The B_1 receptor has a weak affinity to intact BK, but has a strong affinity to kinin's metabolites [des-Arg^9]bradykinin and [des-Arg^{10}]kallidin. This receptor does not appear to be expressed in normal mammalian tissues but is expressed in pathological states such as inflammation and trauma (Bhoola et al., 1992). The B_2 receptor, in contrast, has a high affinity to BK, and is the receptor that mediates most of the biological processes of this peptide. The rat and human B_2 receptors have been cloned and they share 81% homology at the gene level (Hess et al., 1992; McEachern et al., 1991).

1.2.2 Bradykinin-receptor signalling

The B_2 receptor is a member of the seven-transmembrane receptor family and is linked to the guanine nucleotide-dependent regulatory proteins G_i and G_q, depending on the cell type examined. Coupling to these G-proteins results in the activation of a polyphosphoinositide-specific phospholipase C (PI-PLC) and a transient increase in the production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). This is followed by the subsequent activation of protein kinase C (PKC) and the biphasic elevation of free intracellular calcium ($[Ca^{2+}]_i$) characterized by a rapid initial peak (due to Ca^{2+} release from intracellular stores) and a secondary plateau phase (due to prolonged transmembranous Ca^{2+} influx) (Aboolian et al., 1989; Dixon et al., 1989; Shayman et al., 1987). In addition to this established pathway of receptor-mediated signalling, BK receptor occupation has been shown to induce tyrosine phosphorylation of cellular substrate proteins (Jong et al., 1993) and to result in the

production of cGMP (Boulanger et al., 1990). Therefore, the signalling mechanisms activated by BK display distinct variability.

1.2.3 Bradykinin's effect on the collecting duct

As mentioned earlier, it appears that the diuretic effects of BK are mediated largely through its ability to inhibit AVP-stimulated water and salt transport in the CCD (Schuster et al., 1984). Since AVP is critical to CCD urine concentration and overall conservation of body water, the importance of BK's ability to antagonize this response can not be dismissed. Indeed, there has been considerable investigation into the biochemical mechanisms by which BK mediates its effects upon CCD water transport. While the propensity for BK to inhibit AVP-stimulated cAMP production (and therefore water transport) is well established, the intracellular mechanisms through which these processes occur have not been fully elucidated. Among those factors involved, it appears that PKC, Ca^{2+}, and PGE_2 each play important roles.

Studies performed on isolated perfused rabbit CCD tubules (Ando et al., 1988) and on cultured rabbit CCD cells (Dixon et al., 1988: Dixon et al., 1989) have revealed that AVP-stimulated hydraulic conductivity and adenylate cyclase activity can be partially suppressed by the activation of PKC. Although PKC was clearly implicated in these studies, the precise mechanism by which PKC abrogated the response to AVP remained uncertain. The role of PKC was attributed to either a post-cAMP event in perfused CCDs, or to inhibitory events at the hormone/receptor level or receptor/G_s level in cultured CCDs. The apparent differences between the mechanism of PKC inhibition have been suggested to result from

cellular changes induced upon the growth of CCD cells in culture (Dixon et al., 1989). This is further supported by the demonstration that PGE_2-mediated inhibition of hormone-stimulated cAMP accumulation is rapidly lost in cultured collecting tubular cells, a result linked to the loss of the G_i-linked receptor response (Sonnenburg and Smith, 1988). Regardless of the mechanism, PKC inhibits vasopressin-stimulated cAMP production, and since BK stimulation of CCD cells activates this kinase in both perfused CCD tubules and cultured CCD cells, the importance of PKC in signalling by this nonapeptide is confirmed.

Activation of PI-PLC upon BK-receptor coupling results in elevated $[Ca^{2+}]_i$. Kusano and colleagues (1985) have shown that increased $[Ca^{2+}]_i$ can inhibit AVP-stimulated adenylate cyclase activity and cAMP accumulation. While these investigators demonstrated that this response is mediated by cAMP phosphodiesterase activation, it may also be a result of possible effects on prostaglandin production. Support for this conclusion is provided by Ando and colleagues (1988) who observed that elevated $[Ca^{2+}]_i$ levels inhibit AVP action primarily through the effect of a cyclooxygenase product, a result independent of phosphodiesterase effects upon cAMP levels.

Prostaglandins represent another important mechanism through which BK exerts its effects upon CCD function (Schuster, 1985; Hébert et al., 1995). Biochemical studies confirm that BK can stimulate prostaglandin E_2 (PGE_2) synthesis in collecting duct cells (Shayman et al., 1987; Slivka and Insel, 1988), and that its production can be inhibited by the cyclooxygenase inhibitor, indomethacin. However, whereas PGE_2 is the major prostaglandin produced by the CCD, numerous other prostanoid metabolites, such as prostacyclin (PGI_2), are synthesized by the CCD and regulate its function (Hébert et al., 1995).

The actions of all naturally occurring prostanoids are mediated by the activation of distinct receptor subtypes. With respect to PGE_2 action, at least four EP receptor subtypes have been identified. Receptors of the EP_1 subtype activate PLC, EP_2 and EP_4 receptor subtypes couple to a G_s protein and activate adenylate cyclase, and lastly EP_3 receptor subtypes couple to a G_i protein and inhibit adenylate cyclase. Interestingly, the effects of PGE_2 on both water and salt transport are mediated by the action of all four receptor species. In the absence of AVP, PGE_2 stimulates water reabsorption via EP_2 receptor subtype activation, and inhibits sodium reabsorption via EP_1 receptor subtype activation (Hébert et al, 1991; Hébert et al., 1993). In contrast, while under the influence of AVP, stimulation of EP_1 and EP_3 receptor subtypes each contribute to inhibition of AVP-induced water permeabililty, either via activation of PKC (Hébert et al., 1990) or via stimulation of the pertussis-toxin sensitive G_i protein (Sonnenburg and Smith, 1988).

In summary, BK is an important regulator of CCD function, and many of the physiological responses to this agonist can be attributed to the production of various prostanoid metabolites. Although prostaglandin endoperoxide synthase (PGHS) is responsible for the conversion of AA to prostaglandin endoperoxide H_2 (which is susbsequently isomerized to the various prostanoids), it is the availability of AA that limits the production of these mediators. Therefore, examination of the enzymes and intracellular signalling pathways activated by BK-receptor stimulation and resulting in the release of AA, are of paramount importance.

1.3 Arachidonic acid mobilization

1.3.1 Eicosanoids and pathways of arachidonic acid mobilization

Eicosanoids are synthesized from polyunsaturated fatty acids, predominantly AA, and represent a class of autocrine/paracrine factors that act as important physiological regulators of cellular function. They include those products that result from the action of cyclooxygenase (prostaglandins, thromboxanes), lipoxygenase (leukotrienes, lipoxins), and cytochrome P450 enzymes. Since these products are not stored in cells in appreciable amounts, their synthesis is dependent upon and limited by the availability of the precursor molecule, AA (van den Bosch, 1980). The major cellular store for AA is the intracellular diacyl phospholipids, within which this fatty acid is located predominantly at the *sn*-2 position.

Three major enzymatic pathways are thought to account for the liberation of AA and the consequent increase in its availablity. One involves the hydrolysis of phosphatidylinositol (PI), phosphatidylcholine (PC), or phosphatidylethanolamine (PE) via the sequential actions of phospholipase C (PLC), diglyceride lipase and monoglyceride lipase. The second pathway involves the hydrolytic action of phospholipase D (PLD) which accounts for the generation of phosphatidic acid which can then be hydrolyzed by the action of phosphatidic acid phosphohydrolase (PAP) to yield diacylglycerol (DAG). This latter product is the substrate of lipase activity which liberates AA. Finally, the third pathway for AA mobilization relies on the direct action of a PLA_2, which selectively releases fatty acids from the *sn*-2 position of membrane phospholipids. While the PLC/DAG lipase (Konrad et al., 1994) and PLD/phosphatase pathways (Lassegue et al., 1991) represent the predominant mode of AA

release in some experimental models, it would seem probable that, since PLA_2 specifically cleaves fatty acids from the phospholipid ester position where AA is enriched, this acylhydrolase would be the predominant enzyme accounting for AA release in a variety of other cells.

1.3.2 Phospholipases A_2

Phospholipases A_2 are a group of lipolytic esterases that catalyze the hydrolysis of the *sn*-2 acyl ester bond of glycerophospholipids resulting in the liberation of fatty acids and the production of lysophospholipids. They are a diverse class of enzymes that play a central role in diverse cellular processes including membrane phospholipid digestion and metabolism, immunologic responses, and signal transduction. Related to these numerous and diverse functions, PLA_2 can be divided into several groups.

1.3.3 Secreted phospholipase A_2

The best characterized of the PLA_2 species are the secretory PLA_2 enzymes ($sPLA_2$) which themselves can be categorized into the traditional groups I, II, and III. The members of these groups have all been isolated as extracellular enzymes, have high disulfide bond content, low molecular mass (14-18 kDa), and require relatively high concentrations (mM) of Ca^{2+} for catalysis. While these enzymes have been characterized in snake venoms where they occur in abundance, a growing number of cases involving group I and II enzymes have been reported for mammalian tissues (Mukherjee et al., 1994).

Group I PLA_2 enzymes are released as zymogens from the pancreas, but are also

found in other organs such as the lung, spleen, kidney, and gastric mucosa (Hanasaki et al., 1992). Pancreatic PLA_2 was initially thought to be a predominantly digestive enzyme, but surprisingly, it has now been demonstrated to exert several biological activities that are also receptor-mediated and unrelated to the catalytic activity of these enzymes (Mukherjee et al., 1994). Among these activities are a proliferative effect upon Swiss 3T3 cells (Arita et al, 1991), and a contractile effect upon guinea pig lung parenchyma (Kanemasa et al, 1992) and isolated porcine cerebral arteries (Nakajima et al., 1992).

Group II PLA_2 was first identified in synovial fluid and platelets, but has now been described in several cells and tissues. Due to its increased levels in inflammatory exudates, tissue fluids, and serum during diseases such as septic shock and rheumatoid arthritis, this group appears implicated in the processes of inflammation and defense against microorganisms (Kudo et al., 1993). Evidence exists that upon inflammatory insult and cytokine induction during host defense, group II PLA_2 is released as an acute phase protein from the liver (Crowl et al, 1991).

Despite the fact that members of the $sPLA_2$ class are able to cleave AA from the *sn*-2 position of phospholipids, they do not exhibit a preference towards this fatty acid. These low molecular weight PLA_2 enzymes are largely extracellular and require millimolar calcium concentrations (present only outside the cell) for their catalytic activation. Since AA is the precursor to the physiologically active prostanoids, this class of enzymes does not likely play an important role in agonist-mediated signal transduction.

1.3.4 Calcium-independent phospholipase A_2

While virtually all major organs (predominantly brain, lung, heart, and liver) possess calcium-independent forms of PLA_2 ($iPLA_2$), their activity remains largely uncharacterized and may represent that of one or more isozymes. The two best characterized intracellular $iPLA_2$s are represented by the 80 kDa and 40 kDa enzymes. The 80 kDa $iPLA_2$ was first purified from $P388D_1$ macrophages (Ackermann et al., 1994) and has since also been cloned and sequenced from CHO cells (Tang et al., 1997). Evidence suggests that this enzyme is responsible for phospholipid fatty acid remodelling under resting conditions (Balsinde et al., 1995; Balsinde and Dennis, 1997). The 40 kDa $iPLA_2$ has been purified from myocardial tissue as a calcium-independent enzyme activated by ATP. It exhibits specificity towards arachidonyl-containing plasmalogen substrates and associates with calmodulin in a calcium-dependent manner (Wolf and Gross, 1985; Hazen et al, 1990; Wolf and Gross, 1996). According to Wolf and Gross (1996), calmodulin associates with myocardial $iPLA_2$ to maintain the enzyme in an inactive state, but upon dissociation of this complex, the 40 kDa enzyme is rendered catalytically active. This lipase may have a physiological role in the events leading to the development of metabolic alterations that precede cell death during myocardial ischemia (Hazen et al., 1991a).

Recently, a novel $iPLA_2$ has been purified and characterized from rabbit kidney cortex (Portilla and Dai, 1996). These investigators reported the presence of a 28 kDa protein with calcium-independent PLA_2 activity selective against arachidonylated-plasmalogen substrates. This activity was localized within the proximal tubule. Furthermore, when this segment was challenged by hypoxic conditions, there was an accelerated release of AA and catabolism of

phospholipids in a similar manner to that seen during ischemia-hypoxic cell injury to the kidney (Portilla et al., 1994; Portilla and Creer, 1995). Although the mechanism of activation and regulation of this iPLA$_2$ remains unknown, similar to the 40 kDa type PLA$_2$, it does not appear that this isoform is likely involved in agonist-mediated enzyme stimulation and subsequent AA mobilization.

1.3.5 Cytosolic phospholipase A$_2$

Group IV PLA$_2$ (cPLA$_2$) is a cytosolic enzyme present in a variety of species and cell types, but was first purified and characterized in RAW 264.7 mouse macrophage cells by Leslie and collaborators (1988). The cDNA of cPLA$_2$ has been cloned in many species and comprises 2880 nucleotides that encode for a 749 amino acid protein with a predicted molecular weight of 85.2 kDa (Clark et al., 1991; Sharp et al., 1991). When the inferred sequence of murine cPLA$_2$ was compared with that from the human, a homology of greater than 95% was revealed between the two species (Clark et al., 1991). Cytosolic PLA$_2$ displays no homology with the sPLA$_2$ groups and preferentially cleaves AA-containing phospholipids including PC, PE, and PI (Leslie et al., 1988). The deduced cPLA$_2$ protein sequence reveals several intriguing features that may be important to its regulation and mechanism of action. Among them, a 68 amino acid stretch in the N-terminal portion of the enzyme that shares significant sequence homology with the C2 Ca^{2+}-dependent phospholipid-binding (CaLB) domain of PKC, GTPase activating protein, PLCγ_1, and the synaptic vesicle protein p65 (Clark et al., 1991); a segment in the middle of the sequence deficient in hydrophobic amino acids that may serve as a flexible "hinge" region; a proline enriched domain towards the C-

terminus; nine free cysteine residues; and the presence of numerous phosphorylation sites for both tyrosine and threonine/serine kinases.

1.3.6 Receptor activation of cytosolic phospholipase A_2

The pathway leading from receptor occupation to enzyme activation often appears dependent upon the cell type examined and the nature of receptor-G protein coupling. Evidence has implicated both pertussis toxin-sensitive G_i (Winitz et al., 1994; Kruger et al., 1995) and Gq protein families (Burch et al., 1986) in this event, but it appears in these cases that $cPLA_2$ is activated secondary to that of an initial effector, possibly through the lipid products of PLC or PLD, as demonstrated in MDCK cells stimulated with BK (Kennedy et al., 1996). However, support for the direct coupling of $cPLA_2$ to a pertussis toxin-sensitive G protein is provided by Axelrod (1990) and Xing and Mattera (1992).

Numerous studies recognize the 85 kDa PLA_2 as the most likely intracellular PLA_2 enzyme responsible for the liberation of AA upon stimulation by extracellular ligands. Lin and co-workers (1992b) created a stably transfected Chinese hamster ovary (CHO) cell line overexpressing $cPLA_2$ and were able to demonstrate that treatment with either ATP or thrombin resulted in increased AA release when compared to wild-type CHO cells. In contrast, when CHO cells were manipulated to overexpress $sPLA_2$, AA release was not enhanced upon receptor activation. Based upon its likely role in cell signalling events, the regulatory mechanisms responsible for $cPLA_2$ activation has been extensively studied in recent years.

The importance of calcium in stimulating the release of AA and the generation of

prostaglandins has been recognized for quite some time (Hassid, 1981). Purified $cPLA_2$ demonstrates an ability to translocate to natural membrane fractions in response to submicromolar, physiological concentrations of calcium, i.e., those Ca^{2+} transients likely to occur in the cytosol following extracellular ligand-receptor occupation. It has been shown that the CaLB domain likely facilitates this association (Clark et al., 1991; Nalefski et al., 1994). Through the use of immunofluorescence microscopy and immuno-gold electron microscopy techniques, it was revealed that the nuclear envelope and endoplasmic reticulum are the primary sites for the liberation of AA and binding of $cPLA_2$ in CHO cells overexpressing $cPLA_2$ (Schievella et al., 1995) and in rat basophilic leukemia cells (Glover et al., 1995). Thus, in contrast to $sPLA_2$ group enzymes that require micromolar Ca^{2+} as a catalytic cofactor, it appears that the calcium requirement of $cPLA_2$ is mainly for its translocation to substrate phospholipids.

Cytosolic PLA_2 undergoes a post-translational phosphorylation following appropriate cell stimulation. This was first demonstrated in macrophages stimulated with phorbol ester (an activator of PKC) (Wijkander and Sundler, 1992) and $cPLA_2$-transfected CHO cells stimulated with ATP or thrombin (Lin et al., 1992b). Phosphorylation was shown to be at least partially responsible for the activation of $cPLA_2$ and mobilization of AA in these cell types. In a series of experiments, using $cPLA_2$-overexpressed CHO cells, Lin and colleagues (1993) were able to establish that phosphorylation of $cPLA_2$ at Ser-505 was specifically responsible for the enhanced catalytic activity of this enzyme following stimulation by phorbol ester. Furthermore, these investigators revealed that this critical catalytic site was located within a mitogen-activated protein kinase (MAPK) phosphorylation consensus sequence and

that replacement of Ser-505 by Ala at this position, completely prevented activation of $cPLA_2$. Therefore, taken together, the evidence supports a role for both PKC and MAPK in the regulation of $cPLA_2$ activation.

1.4 Protein kinase C and the mitogen-activated protein kinase signalling cascade

1.4.1 Protein kinase C

In 1977, PKC was isolated from bovine cerebellum as a phospholipid- and calcium-dependent enzyme (Takai et al., 1977). The potential importance of this enzyme later came to light upon the discovery that unsaturated diacylglycerols selectively bind and activate PKC (Kishimoto et al, 1980). Since signals that stimulate members of the large families of G protein-coupled receptors, tyrosine kinase receptors, or non-tyrosine kinase receptors activate various phospholipases and cause DAG and IP_3 release (and subsequent Ca^{2+} mobilization), PKC has now become recognized as an important mediator of signal transduction processes.

Protein kinase C actually exists as a family of distinct, single subunit polypeptides that can be divided into conserved (C1-C4) and variable (V1-V5) regions. The N-terminal region is regulatory in function and the C-terminal region possesses catalytic activities. The C1 domain contains a Cys-rich motif present in most isozymes and forms the DAG/phorbol ester binding site. The C2 domain represents the site for recognition of acidic phospholipids (ex. phosphatidylserine) and in some isozymes, the Ca^{2+}-binding site . The C3 and C4 domains form the ATP- and substrate-binding lobes of the kinase core.

Currently, the PKC family is classified into three groups based on their structure and

cofactor regulation. The first discovered and best characterized are the Ca^{2+}/diacylglycerol-sensitive conventional PKCs (cPKC; PKCα, PKCβ_1, PKCβ_2, and PKCγ). The next well characterized are the Ca^{2+}-insensitive/DAG-sensitive novel PKCs (nPKC; PKCδ, PKCε, PKCη, PKCθ, and PKCμ) which are structurally similar to the cPKCs, except that the C2 domain does not mediate Ca^{2+} binding. The least well understood isozymes are the atypical PKCs (aPKC; PKCλ and PKCζ). These isozymes differ significantly in structure from the other two classes and do not respond to Ca^{2+} or DAG.

Protein kinase C typically catalyzes serine or threonine phosphorylation reactions, but it also displays ATPase and phosphatase activity (O'Brian and Ward, 1991). Given the plethora of substrates for PKC action, a multiplicity of functions have been attributed to this enzyme. Among the many functions, PKC appears involved in receptor densensitization, modulating membrane structure events, regulating transcription, controlling cell growth and differentiation, and learning and memory (reviewed by Hug and Sarre, 1993).

Recent interest into the role of PKC in cell signalling events has been highlighted by the existence of the many PKC isozymes, and the possibility that the maintenance of such heterogeneity is related to specific functions. Indeed, there is abundant evidence supporting the existence of PKC isozyme-specific differences in terms of localization, cofactor dependence, and substrate range that thereby may allow the cell to finely and precisely modulate its response to numerous signals (reviewed by Hug and Sarre, 1993). In fact, with respect to $cPLA_2$ regulation by PKC, Godson et al., (1993) have demonstrated that inhibition of PKCα expression by antisense cDNA, but not that of PKCβ, is uniquely associated with the loss of phorbol ester-mediated arachidonic acid release in MDCK cells.

1.4.2 The mitogen-activated protein kinase cascade

The transmission of many extracellular signals to intracellular targets is determined by complex networks of proteins that are organized into signalling cascades. One of the best characterized is the MAPK signalling cascade and it represents the critical intracellular machinery associated with cellular responses to growth factors and ligands for numerous seven transmembrane receptors. Up to five or possibly six tiers of amplification may exist within this cascade (Saito et al., 1994) and the MAPK protein itself represents a point of convergence for signals originating from the different classes of receptors.

The MAP kinase enzymes are a family of serine-threonine kinases whose activation by growth factors was first shown to involve the receptor-mediated sequential activation of the low-molecular weight G-protein Ras and the protooncogene serine kinase c-Raf-1 (Zhang et al., 1993). This appears to be followed by the Raf-mediated phosphorylation of MAPK kinase (MEK), which in turn selectively phosphorylates and activates MAPK (Kyriakis et al., 1992). Alternatively, and in addition to the above classical cascade, agonists of seven transmembrane receptors coupled to G proteins can activate MAPK in a PKC-dependent manner that does not involve activation of ras (reviewed by Seger and Krebs, 1995). These ras-independent mechanisms of MAPK activation may occur via direct PKC stimulation (Nemenoff et al., 1993) of MAPK, or also via PKC activation of raf (Kolch et al., 1993) and MEKK (Lange-Carter et al., 1993) upstream of MAPK. It therefore appears that the pathways involved in MAPK activation are likely to be both cell-specific as well as agonist-specific.

SECTION 2: RATIONALE

Given the above discussion regarding the importance of BK-mediated AA metabolism in the CCD, it can be readily appreciated that gaining an understanding into the intracellular signalling mechanisms involved in AA liberation, will allow a more complete description of CCD function. Whereas the critical importance of $cPLA_2$ activation in liberating AA from the collecting duct-type MDCK cell line has been thoroughly examined in recent years, the physiological basis for much of the current understanding of water and salt homeostatic regulation has been achieved through studies of microperfused rabbit cortical collecting tubules. For the purpose of achieving an appropriate cell culture model to allow the extrapolation of data from those tubule-perfusion studies, Burns et al. (1996) generated an immortalized cell line of RCCD origin. With this new model at hand, a series of biochemical experiments were designed to establish the mechanism by which BK controls AA metabolism in RCCD cells.

SECTION 3: HYPOTHESIS

It is hypothesized that rabbit CCD cells release AA upon treatment with BK, and that this response is mediated by $cPLA_2$ activation.

SECTION 4: OBJECTIVES AND APPROACH

4.1 Objectives

Through the use of cultured RCCD cells:

① to establish which enzyme mediatesAA release in response to BK treatment

② to elucidate the mechanism of BK-induced $cPLA_2$ activation and AA release

4.2 Approach

The following strategies were implemented:

① To reveal which enzyme mediates BK-stimulated AA release, the response to a $cPLA_2$ inhibitor, to a disulfide-bond reducing agent (DTT), and to immuno-inhibition (with a polyclonal $cPLA_2$ antibody) was assessed by measuring the consequent effect upon AA release and $cPLA_2$ activity.

② To display the correlation between $cPLA_2$ activation and its enhanced serine phosphorylation following agonist stimulation, the response to phosphatase treatment and anti-phosphoserine antibody were assessed.

③To determine the role of PKC and MAPK in the signalling cascade following BK stimulation, phorbol ester, enzyme inhibitors, and kinase-specific antibodies were utilized.

SECTION 5: MATERIALS AND METHODS

5.1 Materials

Biodegradable counting scintillant (BCS), [5,6,8,9,11,12,14,15-^{3}H]AA, adenosine 5'-[α-^{32}P]triphoshate, horseradish peroxidase-conjugated donkey anti-rabbit Ig and sheep anti-mouse Ig, ECL hyperfilm, Hybond nitrocellulose, ECL reagents, and PKC activity assay kit were products from Amersham Canada (Oakville, ON, Canada). Protein A-agarose for immunoprecipitation was purchased from Boehringer Mannheim (Laval, QB, Canada). Phorbol-12-myristate-13-acetate (PMA), RHC-80267, and $AACOCF_3$ were obtained from Biomol Research Laboratories (Plymouth, PA, USA). Ro31-8220, Gö6976, PD98059, and 4α-phorbol-12,13-didecanoate (PDD) were supplied by Calbiochem-Novabiochem Int. (La Jolla, CA, USA). The rabbit polyclonal antibody to phosphoserine (purified from rabbit antiserum by phosphoserine-specific affinity chromatography and shows no significant cross-reactivity to either phosphothreonine or phosphotyrosine) and mouse anti-p42-MAP kinase antibody was bought from Dimension Laboratories Inc. (Mississauga, ON, Canada). Phosphatidylcholine, (L-α-1-stearoyl-2-[5,6,8,9,11,12,14,15-^{3}H]arachidonyl) was obtained from Dupont NEN (Mississauga, ON, Canada). The Genetics Institute (Boston, MA, USA) kindly provided the purified 85 kDa $cPLA_2$ standard protein and the rabbit polyclonal antibody to $cPLA_2$ which is directed against amino acids 42-58 located within the CaLB domain. Anti-protein kinase C isozyme (α, β, γ ,δ ,ϵ, ζ) sampler set complete with competing peptides, 5000 units penicillin/5000 μg/mL streptomycin, and fetal calf serum (FCS) were

bought from GIBCO BRL (Burlington, ON, Canada). Bradykinin, bovine serum albumin (BSA), A23187, potato acid phosphatase, insulin/transferin/selenium (ITS) and 1-stearoyl-2-arachidonyl-phosphatidylcholine were purchased from Sigma Chemical (Mississauga, ON, Canada).

5.2 Methods

5.2.1 Cell Culture

Immortalized RCCD cells (Burns et al., 1996), passages 3-25, were cultured in 75 cm^2 flasks containing DMEM/F12 defined media supplemented with 10% FCS, 0.4% penicillin/streptomycin solution (GIBCO), 15 mM *N*-2-hydroxyethyl piperazine-*N*'2-ethanesulfonic (HEPES), 50 nM hydrocortisone, 2.5 nM 3,5,3'-triiodothyronine, 44 mM sodium bicarbonate, and ITS. Cells maintained in an atmosphere of 5% CO_2 at 37°C were passsaged at confluence. Experiments were performed with RCCD cells cultured in 12-well cluster dishes (for measurement of AA release), 100 x 20 mm dishes (for PLA_2 assays, PKC assays, and Western blotting), and 20 mm glass coverslips for Ca^{2+} signal measurements prior to forming a complete monolayer of cells.

5.2.2 Measurement of arachidonic acid release

Cells grown in 12-well dishes were serum-starved overnight in DMEM/F12 supplemented media containing 0.05% (w/v) bovine serum albumin (BSA) and 0.3 µCi [^{3}H]AA. After an 18-24 hour incubation period, labelled medium was aspirated and cultures

were freed of unincorporated [^{3}H]AA by two successive washes with Hanks' Balanced Saline Solution (HBSS) containing 0.05% (w/v) BSA. This was followed by a 30 min preincubation period during which cells were treated with appropriate concentrations of the various inhibitors and agonists prepared in HBSS. The medium was subsequently aspirated and replaced with fresh HBSS containing the inhibitors and stimulatory agents to be tested. Controls were prepared without the agent(s) under study. Following a 15 min incubation at 37°C, the medium was immediately removed and centrifuged at 5000*g* for 5 min in order to pellet any cellular debris. An aliquot of the supernatant was measured for [^{3}H]AA release by scintillation counting in 10 mL BCS. Results were normalized for total label incorporated into the cells by dividing the dpm [^{3}H]AA released by the total dpm [^{3}H]AA incorporated into the cells (as determined by solubilizing the cells in 5% SDS).

5.2.3 Protein kinase C assay

Cells grown on 100 x 20 mm culture dishes were washed twice with DMEM/F12 defined media containing 0.05% BSA and incubated over night for 18-24 hrs. The following day, cells were rinsed twice with HBSS+0.05% BSA and preincubated for 30 min prior to stimulation at 37°C with HBSS, 100 nM BK, or 100 nM PMA. Stimulation was terminated after 2 min by aspirating the medium and washing the cells two times with ice-cold phosphate buffered saline (PBS) (pH 7.5). Cells were then scraped off the plates with a rubber policeman, and following centrifugation at 1000*g*, were resuspended in homogenization buffer containing 50 mM Tris(hydroxymethyl)aminomethane (Tris)·HCl (pH 7.5), 20 µg/mL leupeptin, 10 µg/mL aprotinin, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM ethylene

glycol-bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT) and sonicated (4 pulses x 5 sec each) on ice using the small probe of an Ultrasonics cells disruptor set at 5. In order to separate cytosolic and particulate fractions, the supernatant was subjected to a centrifugation at 100,000*g* for 60 min. The high speed pellet was subsequently resuspended in homogenization buffer and the protein was determined using the Bio Rad microassay procedure with BSA as standard. Protein kinase C activity was measured according to the protocol described in the Amersham assay kit, which is based on the phosphorylation of a PKC-specific peptide. The activity is expressed as the amount of phosphate transferred to a PKC specific-substrate (pmol of phosphate transferred per mg of protein per min).

5.2.4 Determination of Phospholipase A_2 activity

Cells grown in 100 x 20 mm dishes were serum-starved over night in DMEM/F12 defined media containing 0.05% BSA. The next day, cultures were washed twice with HBSS+0.05% BSA and preincubated for 30 min at 37°C with or without PKC (Ro31-8220 and Gö6976) and MAPK (PD98059) inhibitors. Cells were then treated for 2 min with HBSS, BK, or PMA in the absence or presence of inhibitors. Stimulation was stopped by immediately placing the dishes on ice, aspirating the medium, and washing the cells twice with ice-cold wash buffer containing 50 mM Tris·HCl (pH 7.5), 250 mM sucrose, and 1 mM EGTA. Cells were then scraped off the plates into wash buffer, centrifuged at 1000*g* for 5 min, and subsequently resuspended in cell lysis buffer composed of 50 mM Tris·HCl (pH 7.5), 250 mM sucrose, 1 mM EGTA, 5 mM DTT, protease inhibitors in μg/mL [100 benzamidine,

20 leupeptin, 2 PMSF, 100 aprotinin], and phophatase inhibitors in mM (10 sodium vanadate, 10 sodium pyrophosphate, 1 levamisole). The resuspended cells were then sonicated on ice after which protein concentrations were determined by the Bio Rad microassay.

Total cell lysates were subsequently assayed for PLA_2 activity according to the protocol described by Leslie (1990). Briefly, lysates were incubated in assay buffer [50 mM Tris·HCl (pH 7.5), 250 mM sucrose, 0.05% BSA, 5 mM DTT, and 1 mM Ca^{2+}] containing 30 µM 1-stearoyl-2-arachidonyl phosphatidylcholine substrate with 55000 dpm 1-stearoyl-2-[^{3}H]arachidonyl phosphatidyl choline tracer. The substrate was prepared by evaporating the solvent under nitrogen followed by its sonication on ice (4 pulses of 45 sec each with 15 sec pauses) in assay buffer. Incubations were carried out at 37°C and were terminated after one hour by the addition of 2.5 mL Dole reagent consisting of 2-propanol/heptane/0.5 M H_2SO_4, 20:5:1, v/v/v) (Dole and Meinertz, 1960). This was followed by the addition of 1.5 mL heptane containing 20 µg unlabelled AA as cold carrier. In order to visualize separate phases, 1 mL of H_2O was added, and an aliquot of the top layer was subsequently purified by silicic acid column chromatography. Columns were then washed with diethyl ether and the final collected eluent was dried under nitrogen and analyzed by liquid scintillation spectrometry in 10 mL BCS.

5.2.5 Measurement of intracellular calcium concentration

Cultures of RCCD cells grown on 20 mM glass coverslips, serum-starved over night in DMEM/F12 defined media containing 0.05% BSA, were subject to calcium measurements, largely as previously described by Hébert et al. (1990). Briefly, cells were loaded with 5 µM

acetoxymethyl ester of fura-2 (fura-2/AM) (Molecular Probes, Eugene, OR) for 45 min at 37°C. The intensity of fura-2 fluorescence was measured with the rapidly alternating excitation provided by dual monochromators set at 340 nM and 380 nM. The monochromator output was coupled to an inverted microscope and the corrected emission intensity ratio (340:380, R) was monitored continuously. Once cells had equilibrated, a baseline of 50-100 sec was taken prior to stimulation with 100 nM BK. Following the experiment, an *in situ* calibration was performed. In order to obtain a maximum ratio, R_{max}, the HBSS bath was changed to one that contained 5 µM ionomycin. This was followed by changing the bath contents with a calcium/magnesium free solution containing 2 mM EGTA and 5 µM ionomycin thereby permitting the determination of the minimum ratio, R_{min}. Intracellular calcium was calculated from the following equation: $[Ca^{2+}]=K_d[(R-R_{min})/(R_{max})](380_{min}/380_{max})$, where K_d for the fura-2-Ca^{2+} complex is assumed to equal 224 nM at 37°C.

5.2.6 Preparation of samples for Western blotting of cytosolic phospholipase A_2, protein kinase C isozymes, and mitogen-activated protein kinase

Cells grown in 100 x 20 mm dishes were washed twice with serum-free DMEM/F12 defined media containing 0.05% BSA and subsequently incubated overnight for 18-24 hrs. Immediately following, medium was rapidly aspirated and cells were rinsed twice with ice-cold 50 mM Tris·HCl (pH 7.5) buffer containing 150 mM NaCl and 1 mM EGTA. Kept on ice, they were then scraped off the dishes and centrifuged at 1000*g* for 5 min before being resuspended in Western lysis buffer composed of 50 mM Tris·HCl (pH 7.5), 150 mM NaCl,

1 mM EGTA, 20 µg leupeptin, 2 µg PMSF, 100 µg aprotinin, and 1 mM levamisole. The resuspended cells were then sonicated on ice by four pulses of 5 sec each and the resultant cell lysates were analyzed by Western blotting.

5.2.7 Isolation of cytosolic and particulate fractions used for Western blotting of protein kinase C isozymes

Cells grown in 100 x 20 mm dishes were washed twice with DMEM/F12 defined media containing 0.05% BSA and incubated overnight for approximately 18-24 hrs. They were subsequently washed again and preincubated for 30 min with HBSS + 0.05% BSA followed by further treatment with 100 nM BK or 100 nM PMA for 2 min at 37°C. Immediately following, the medium was aspirated, dishes were placed on ice, and cells were washed twice with ice-cold 50 mM Tris·HCl (pH 7.5) buffer containing 150 mM NaCl and 1 mM EGTA. Cells were then scraped off the dishes and centrifuged at 1000*g* for 5 min before being resuspended in Western lysis buffer. The resuspended cells were then sonicated on ice as described above and the resultant cell lysates were centrifuged at 100,000*g* for 60 min in order to separate cytosolic and particulate fractions. Following this, the high speed pellet was resuspended in Western lysis buffer, and both this fraction and the cytosolic portion were adjusted to the desired protein concentration for Western blotting.

5.2.8 Immunoprecipitation of phosphorylated cytosolic phospholipase A_2

Cells were serum-starved and stimulated as described previously in section 4.2.4. Following stimulation, cells were washed and scraped off the plates with ice-cold buffer solution containing 50 mM Tris·HCl (pH 7.5), and 1 mM EGTA. They were subsequently centrifuged at 1000*g*, resuspended, and then lysed in immunoprecipitation buffer [50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1% Nonidet P40 (NP40), 0.5% sodium desoxycholate, 20 µg/mL leupeptin, 10 µg/mL aprotinin, 1 mM PMSF, 30 µg/mL bacitracin, 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 1 mM levamisole]. Protein concentrations were determined and diluted to 50 µg/500 µL of immunoprecipitation buffer in a fresh centrifuge tube. To this aliquot, 25 µL protein A-agarose suspension was added, and the solution was subsequently rocked on a platform for 3 h at 4°C in order to reduce background that may be caused by non-specific adsorption of cellular debris. Following a low speed centrifugation, the resultant supernatant was transferred to a new centrifuge tube, incubated with 4 µL of $cPLA_2$ antibody for 1 h, and left overnight upon the addition of a further 25 µL protein A-agarose. The beads were then pelleted and washed twice with 500 µL immunoprecipitation buffer, twice with 500 µL wash buffer 1 (50 mM Tris·HCl (pH 7.5), 500 mM NaCl, 0.1% NP40, 0.05% sodium desoxycholate) and once with 500 µL wash buffer 2 (50 mM Tris·HCl (pH 7.5), 0.1% NP40, 0.05% sodium desoxycholate). Following the last wash, the immunoprecipitates were dried with strips of filter paper and then used for Western blotting.

5.2.9 Preparation of Western blots

Cell samples prepared in buffer were adjusted to the desired protein concentrations and subsequently extracted in Laemmli sample buffer, boiled for five min in a water bath and subject to SDS-PAGE using a Bio Rad Mini Protean II apparatus set at 200V. Determination of $cPLA_2$ and PKC isozyme protein content was performed on 7.5% SDS-PAGE gels run for approximately 50 min, while examination of MAPK content was performed on 12% SDS-PAGE gels run for an additional hour following the run-off of the Coomassie blue tracking dye. Proteins from the gels were then transferred to Hybond nitrocellulose membranes according to the manufacturer's instructions and were used immediately or stored at -20°C until needed.

Nitrocellulose membranes were blocked between 3 and 20 hours with 5% non-fat skim milk in Tris-buffered saline (TBS) followed by repeated washings with 0.1% Tween-20/TBS (TTBS). Individual blots were then incubated for one hour with either: rabbit polyclonal $cPLA_2$ antiserum (1:2000 dilution), mouse polyclonal anti-p42-MAP kinase antibody (1:1000 dilution), the various rabbit polyclonal anti-PKC isozyme antibodies (1:500 dilution), or with rabbit polyclonal antibody to phosphoserine dissolved in TTBS containing 2% skim milk. After additional washing with TTBS, the blots were incubated with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (1:2000) as required for 1 h. Following further washes with TTBS, the blots were visualized with the use of Amersham's Enhanced Chemiluminescence (ECL) reagents according to the manufacturer's specifications.

5.3 Statistics

Data are expressed as averages of duplicate determinations from individual experiments and are presented as the mean ±SEM where $n \geq 4$, or the mean ±SD where $n=3$. Differences were evaluated by analysis of variance (ANOVA) with Student-Newman-Keuls (SNK) multiple comparisons procedure. Statistical significance was accepted at $P<0.05$.

SECTION 6: RESULTS

6.1 Examination of the enzyme implicated in bradykin-induced arachidonic acid release

6.1.1 Determination of cytosolic phospholipase A_2 involvement in bradykinin-stimulated arachidonic acid release

Since BK is an important mediator of CCD function, reportedly exerting its effects through enhanced AA hydrolysis and prostanoid production, experiments were set up to determine whether the recently characterized RCCD cell line (Burns et al., 1996) could be used as an appropriate model to gain a greater understanding of the intracellular mechanisms that mediate BK signalling in this kidney segment. Elevation of $[Ca^{2+}]_i$ is a well established response to BK stimulation in many cell types, including MDCK cells (Aboolian et al., 1989) and primary cultures of rabbit cortical collecting tubular cells (Dixon et al., 1989). As shown in **Figure 6.1**, BK treatment of RCCD cells caused a significant increase in $[Ca^{2+}]_i$ that was characterized by a rapid initial peak, followed by a slow and steady sustained elevation, a result similar to that described by Dixon et al. (1989). Next, to determine whether BK treatment could also induce AA release, cells were labeled with radioactive AA and stimulated with BK for 15 min. Results shown in **Figure 6.2** reveal that RCCD cells release AA in a dose dependent manner and that cells were responsive to BK at concentrations as low as 10 pM with peak release at approximately 10 nM. Although $cPLA_2$ was shown to be the enzyme responsible for BK-mediated AA release in MDCK-D1 cells (Kennedy et al., 1996; Xing et

Figure 6.1
Effect of bradykinin on intracellular calcium concentration

RCCD cells grown on 20 mm glass cover slips were serum-starved overnight in DMEM/F12 defined media containing 0.05% (w/v) BSA and subsequently loaded with fura-2 for 45 min. After achieving a steady baseline value, cells were stimulated with 100 nM BK and the $[Ca^{2+}]_i$ measured at 37°C. The result shown is representative of three independent experiments.

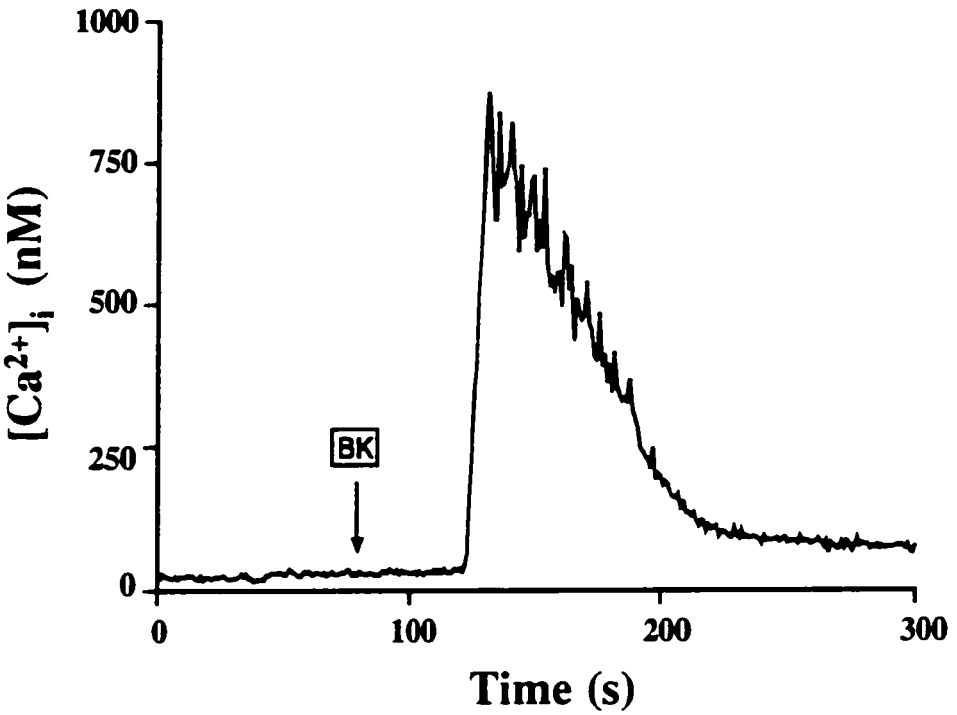
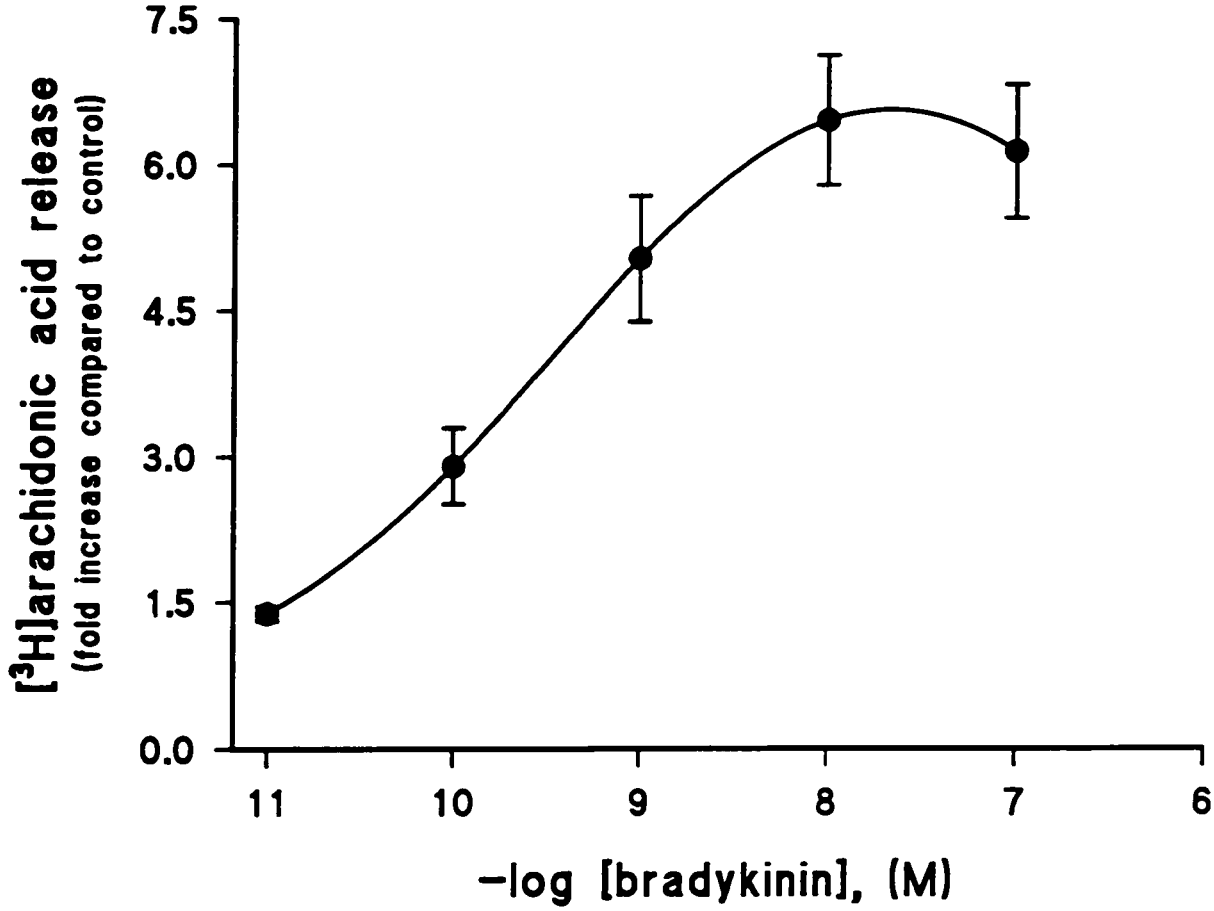

1000
750
500
250
0
$[Ca^{2+}]_i$ (nM)
BK
0
100
200
300
Time (s)

Figure 6.2
Dose dependent arachidonic acid release in response to bradykinin

RCCD cells were labelled overnight with 0.3μCi [^{3}H]AA in DMEM/F12 defined media containing 0.05% (w/v) BSA. Cells were subsequently washed twice with HBSS+0.05% BSA and then preincubated for 30 min prior to a 15 min stimulation with the indicated concentrations of BK. [^{3}H]AA released into the media and total cell label incorporated into cells were counted. The amount of label released was divided by the total incorporation and was expressed as the fold increase in [^{3}H]AA release compared control. (n=3)


[3H]arachidonic acid release
(fold increase compared to control)
7.5
6.0
4.5
3.0
1.5
0.0
11
10
9
8
7
6
−log [bradykinin], (M)

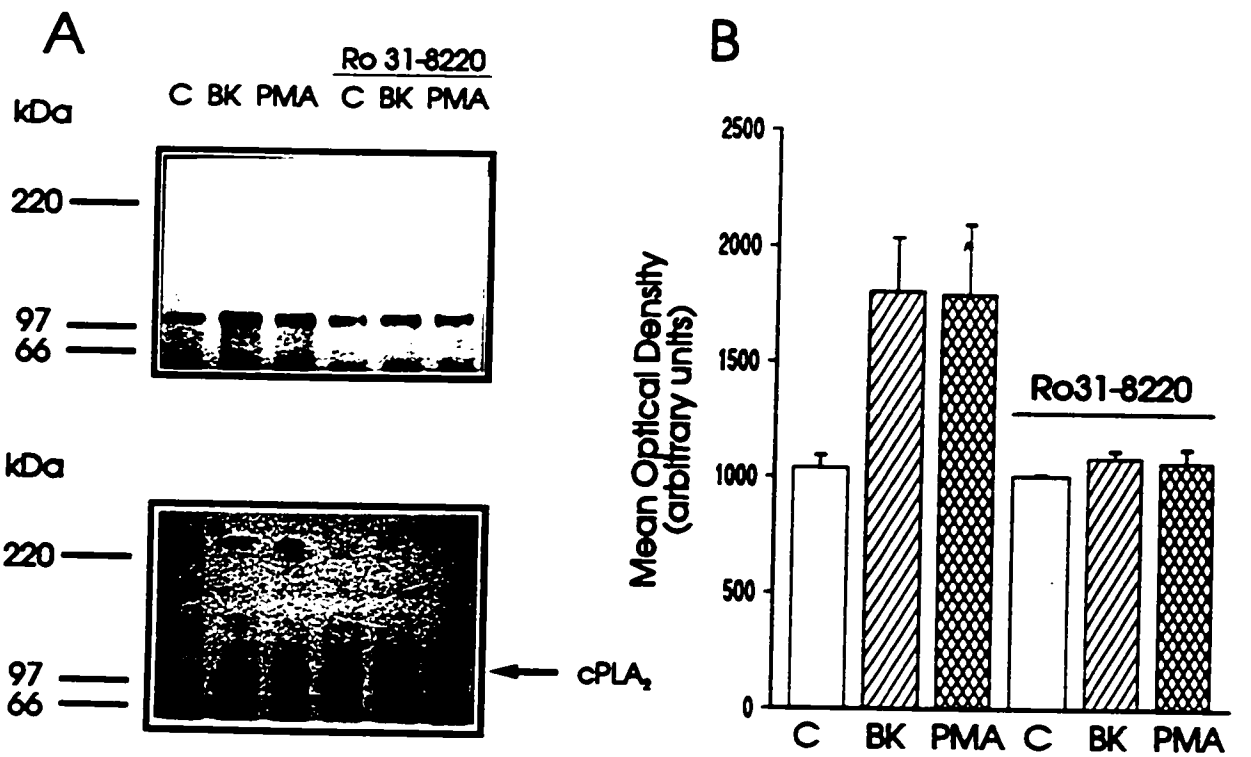
al., 1996), such information was not yet available for RCCD cells.

Western blotting with a rabbit polyclonal antibody to the 85 kDa PLA_2 protein revealed the presence of $cPLA_2$ in RCCD cells (**Figure 6.3**). Samples derived from whole cell lysates possessed a component that migrated to the same position as that of a pure $cPLA_2$ standard. The observation that $cPLA_2$ migrated with a molecular weight of about 100 kDa, rather than 85 kDa, as would be expected based upon its amino acid sequence (Clark et al., 1991), has been previously reported and may be the result of hindered electrophoretic mobility on SDS-PAGE related to the presence of a proline-rich (>12% of total residues) domain towards the C-terminus (Leslie et al., 1988; Clark et al., 1990).

To establish whether this phospholipase was responsible for mediating BK-induced AA release, several approaches were considered. The agent, arachidonyl trifluoromethyl ketone ($AACOCF_3$), an analogue of AA that preferentially inhibits $cPLA_2$ over the $sPLA_2$ isoforms (Riendeau et al., 1994), was first tested. As shown in **Figure 6.4**, BK-stimulated AA release was 92% inhibited by 50 μM $AACOCF_3$. In contrast, an inhibitor of DAG lipase (RHC-80267) was without significant effect on the response to BK. This latter result precluded the combined action of PLC and DAG lipase as a major pathway for releasing AA, and is similar to what was found for the MDCK cell line (Slivka and Insel, 1988; Kennedy et al., 1996). Recently, it was shown that $AACOCF_3$ also demonstrates inhibitory activities towards Ca^{2+}-independent PLA_2 (Ackermann et al., 1995). However, a role for this enzyme was unlikely since treatment with haloenol lactone suicide substrate (HELSS), a potent and specific inhibitor of the 40 kDa $iPLA_2$ (Hazen et al., 1991b), was unable to significantly attenuate BK-stimulated AA release.

Figure 6.3
RCCD cells express cytosolic phospholipase A_2

RCCD cells were lysed in buffer [50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 20 µg/mL leupeptin, 10 µg/mL aprotinin, and 1mM PMSF], boiled for 5 min in Laemmli sample buffer and subsequently separated by 7.5% SDS-PAGE. Gels were then transferred to nitrocellulose membranes, blocked overnight with 5% non-fat skim milk in Tris-buffered saline and probed with a $cPLA_2$ antibody (1:2000) for 1 h. Lane 1, 10 ng $cPLA_2$ standard; lanes 2-4, 2 µg, 5µg, and 10 µg of cell lysate protein. Values to left of blot are in kDa.


A
Ro 31-8220
C BK PMA C BK PMA
kDa
220
97
66
kDa
220
97
66
cPLA2
B
Mean Optical Density (arbitrary units)
2500
2000
1500
1000
500
0
Ro31-8220
C BK PMA C BK PMA

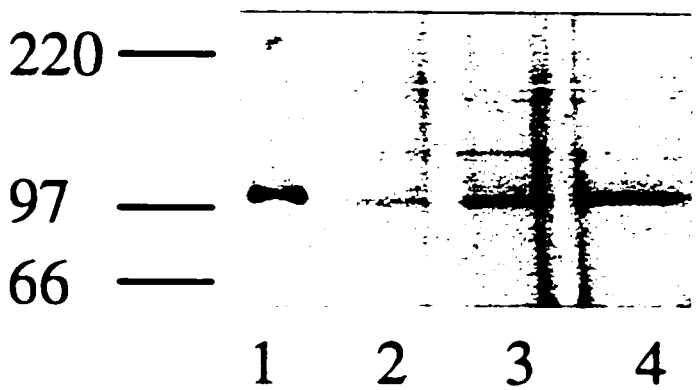
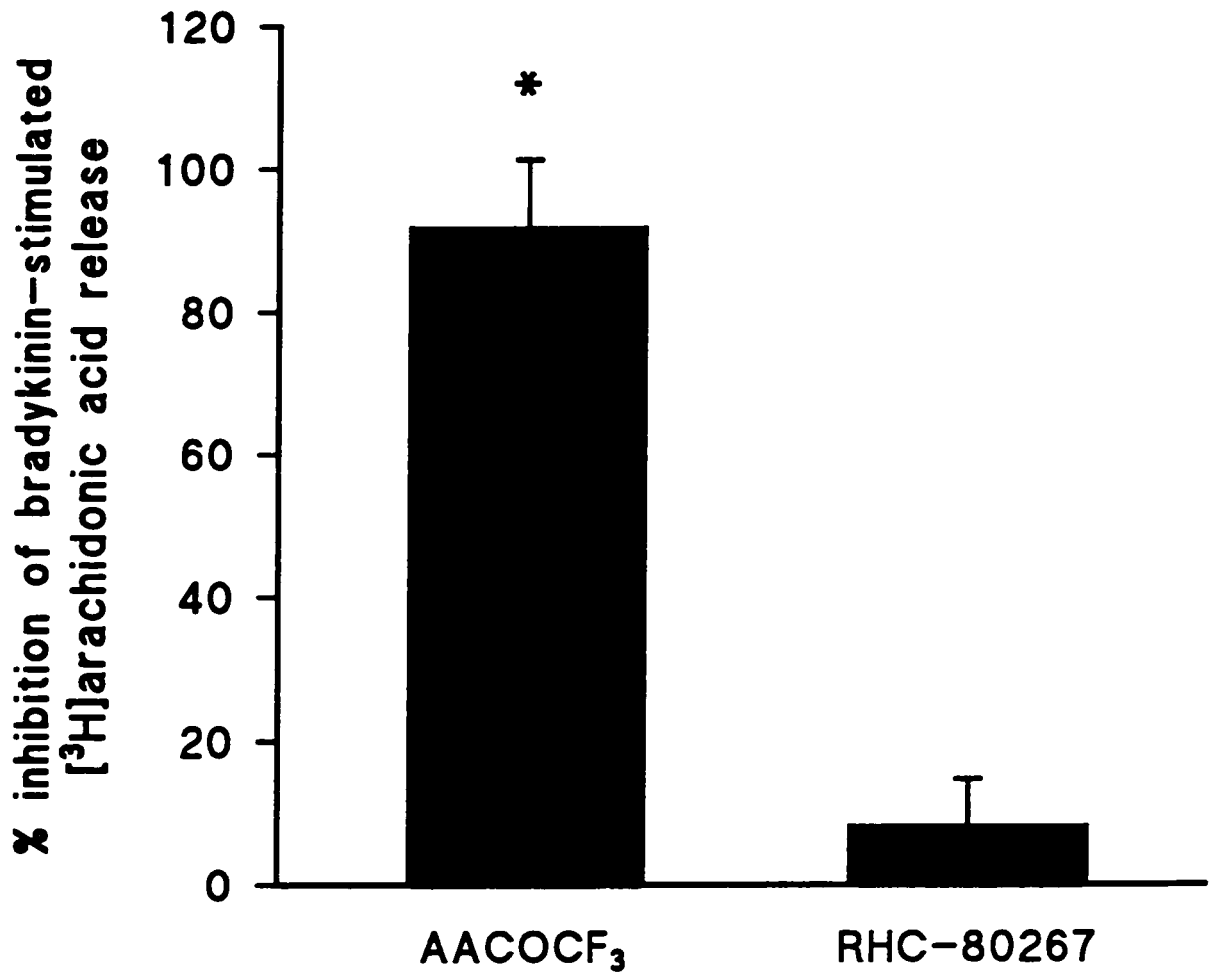

220
97
66
1
2
3
4

Figure 6.4
Effect of AACOCF$_3$ (an inhibitor of cytoplasmic phospholipase A$_2$) and RHC-80267 (an inhibitor of diacylglycerol lipase) on bradykinin-stimulated arachidonic acid release

RCCD cells were labelled overnight with 0.3 μCi [^{3}H]AA in DMEM/F12 defined media containing 0.05% (w/v) BSA, then washed twice with HBSS followed by a 30 min pretreatment with 50 μM AACOCF$_3$ or 10 μM RHC-80267. Cells were subsequently stimulated with 1 nM BK in the presence of inhibitors. Media was removed and counted for [^{3}H]AA release by the cells. The amount of label released was normalized for the total label incorporated into the cells and is presented as a percent inhibition of BK-stimulated AA release. The data are expressed as means ±SEM of 4 independent experiments performed in duplicate. *P<0.005 vs. BK alone.


% inhibition of bradykinin-stimulated [3H]arachidonic acid release
120
100
80
60
40
20
0
*
AACOCF3
RHC-80267

In order to demonstrate more definitively that the 85 kDa PLA_2 is directly responsible for BK-stimulated AA release, measurements of *in vitro* PLA_2 activity were performed. Determination of PLA_2 activity from whole cell lysates incubated either in the presence or absence of the reducing agent, DTT, were found to reveal no differences in the final values obtained, thus precluding a role for the 14 kDa PLA_2 in mediating the response. Further support favouring the presence of $cPLA_2$ was provided by the observation that the PLA_2 activity of RCCD cells could be maximally stimulated by submicromolar [Ca^{2+}]. In addition to these experiments, results summarized in **Table 6.1** provide more conclusive evidence implicating $cPLA_2$ in the signalling process triggered by BK stimulation of RCCD cells. Following maximal BK stimulation (100 nM) of the cells, PLA_2 activity measured *in vitro* was found to increase 2.0 fold, i.e., from 13.9±1.5 pmol·min^{-1}·mg^{-1} to 27.9±2.5 pmol·min^{-1}·mg^{-1}. In order to confirm that this activity was indeed attributable to a $cPLA_2$-mediated process, cell lysates were preincubated with a polyclonal antibody directed against amino acids 42-58 located within the CaLB domain of $cPLA_2$ (Clark et al., 1991). As suggested by Kennedy and colleagues (1996), formation of the enzyme/antibody complex may prevent the Ca^{2+}-dependent association of $cPLA_2$ with its substrate, or it may interfere with the enzyme's catalytic function. Irrespective of the actual mechanism of inhibition, the resultant activity from both control- and BK-stimulated responses were similarly blunted (compare 3.9±0.29 pmol·min^{-1}·mg^{-1} for control/anti-$cPLA_2$ and 4.14±1.1 pmol·min^{-1}·mg^{-1} for BK/anti-$cPLA_2$), thus indicating that $cPLA_2$ is accountable for BK-stimulated AA release. Based upon these data, the evidence as a whole indicates that the 85 kDa PLA_2 protein is the predominant enzyme mediating BK-stimulated AA release in RCCD cells.

Table 6.1
Effect of bradykinin and a cytosolic phospholipase A_2 antibody on in vitro phospholipase A_2 activity

RCCD cells were serum starved overnight in DMEM/F12 defined media supplemented with 0.05% (w/v) BSA. Cells were preincubated with HBSS +0.05% BSA followed by a 2 min stimulation with or without 100 nM BK. PLA_2 activity was determined and results expressed as means ±SEM of 4 individual experiments assayed in duplicate. [a]$P<0.05$ vs control, [b]$P<0.001$ vs BK.

Treatment	PLA_2 activity ($pmol \cdot min^{-1} \cdot mg^{-1}$)
control	13.9±1.5
control + anti-$cPLA_2$	3.90±0.29[a]
bradykinin	27.9±2.5[a]
bradykinin + anti-$cPLA_2$	4.14±1.1[b]

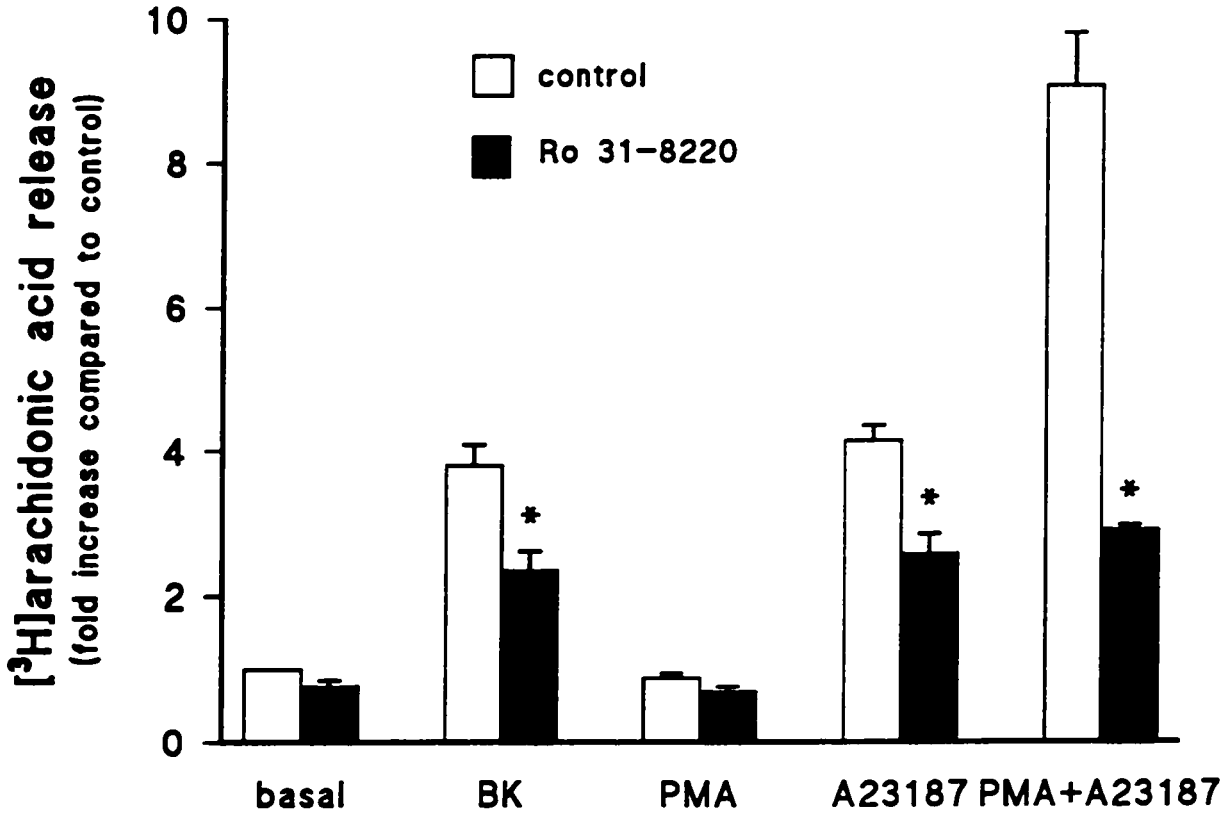
6.2 Determination of signalling events involved in bradykinin-induced activation of cytosolic phospholipase A_2

6.2.1 Effect of protein kinase C inhibition upon bradykinin-mediated arachidonic acid release

Given the role of $cPLA_2$ in BK-stimulated AA release and prior knowledge of the mechanisms governing activation of this enzyme, experiments were next performed to reveal whether BK-receptor signalling events could be mimicked by PMA-induced activation of PKC and A23187-induced calcium mobilization. Results summarized in **Figure 6.5** illustrate that treatment of serum-starved RCCD cells with 100 nM PMA alone for 15 min failed to increase the release of AA as compared to the control situation whereas treatment with 100 nM A23187 did cause such a stimulation. Interestingly, it has been previously demonstrated that the rapid release of AA can be maximized when PKC activation and Ca^{2+} mobilization occur simultaneously (Chakraborti et al., 1992; Lin et al., 1992b; Slivka and Insel, 1988). Accordingly, when PMA was presented to RCCD cells together with A23187, there was a clear synergy of release above that caused by A23187 alone (compare 4.15±0.21 fold for A23187 to 9.08± for PMA + A23187). To determine whether BK-stimulated AA release was dependent upon PKC, cells were preincubated with Ro31-8220, a potent and specific PKC inhibitor of the bisindolylmaleimide family (Wilkinson et al., 1993), prior to stimulation with BK. As also seen in **Figure 6.5**, BK-stimulated AA release was inhibited 51% by 5 μM Ro31-8220. Higher concentrations of inhibitor did not result in any greater inhibition. A role for PKC in the synergistic release of AA was supported by the ability of Ro31-8220 to

Figure 6.5
Bradykinin-stimulated arachidonic acid release is protein kinase C-dependent

RCCD cells were labelled overnight with 0.3 μCi [^{3}H]AA in DMEM/F12 defined media containing 0.05% (w/v) BSA, then washed twice with HBSS+0.05% BSA followed by a 30 min pretreatment with 5 μM Ro-31-8220. Cells were subsequently stimulated for 15 min in the continued presence of inhibitor by 1 nM BK, 100 nM PMA, 100 nM A23187, or 100 nM PMA +100 nM A23187. The amount of label released was normalized by dividing by the total label incorporated into the cells and was expressed as the fold increase in [^{3}H]AA release compared to control. Results are expressed as means ±SEM of four independent experiments performed in duplicate. *P<0.05 compared to each respective control

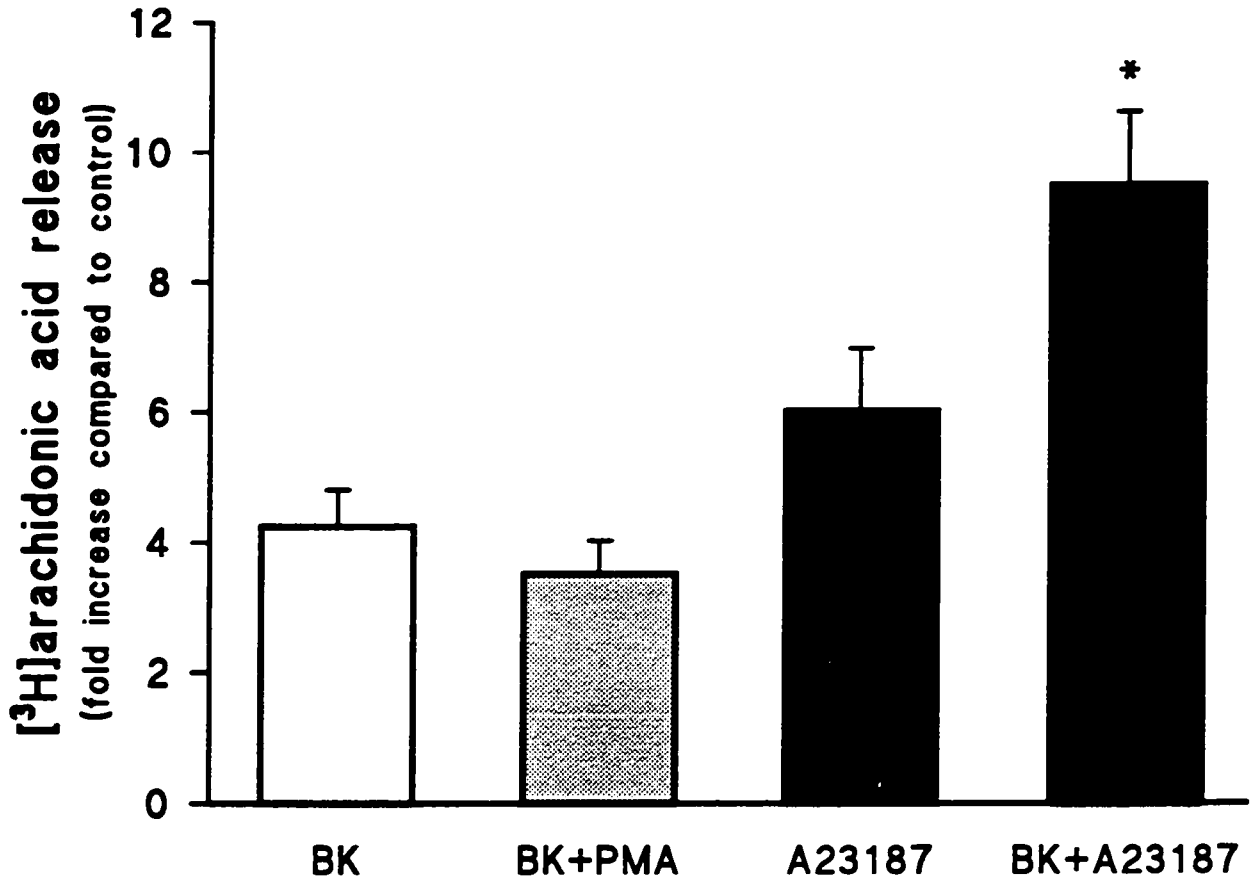

[3H]arachidonic acid release
(fold increase compared to control)
10
8
6
4
2
0
control
Ro 31-8220
*
*
*
basal
BK
PMA
A23187
PMA+A23187

reduce AA release to those levels seen for A23187 alone (compare 2.59±0.26 fold for A23187/Ro31-8220 to 2.91±0.05 fold for PMA + A23187/Ro31-8220). The agent, Ro31-8220, diminished AA release invoked by A23187 alone, thus suggesting that in addition to Ca^{2+} mobilization, the ionophore causes activation of PKC at least to some extent. A similar result was observed in CHO cells overexpressing $cPLA_2$ treated with the PKC inhibitor, staurosporine (Lin et al., 1992b), and may be a result of Ca^{2+}-mediated activation of PLC and subsequent DAG production leading to PKC activation (Billah and Anthes, 1990). Taken together, the results indicate that BK-stimulated AA release from RCCD is dependent on the synergistic action of PKC and Ca^{2+}.

It was previously reported by Slivka et al. (1988) that BK-stimulated AA release from MDCK cells can be potentiated by co-treatment phorbol ester. Results depicted in **Figure 6.6** however, reveal the inability of PMA treatment to potentiate the release of AA induced by BK in RCCD cells (compare 4.24±0.57 fold for BK to 3.51±0.51 for BK + PMA). Rather, it was observed that ionophore was able to potentiate the response to BK (compare 4.24±0.57 fold for BK to 9.48±1.14 fold for BK + A23187). Thus, it is possible that BK-mediated AA release is limited, not by activation of PKC, but rather by the size of Ca^{2+} signal engendered. Indeed, it was demonstrated by Kramer et al. (1995) that disparate $[Ca^{2+}]_i$ levels may contribute to attenuated AA release seen in human platelets treated with the thrombin receptor agonist peptide SFLLRN, versus when treated with thrombin itself.

Figure 6.6
Effect of phorbol ester and calcium ionophore treatment on bradykinin-stimulated arachidonic acid release

RCCD cells were labelled overnight with 0.3 μCi [^{3}H]AA in DMEM/F12 defined media containing 0.05% (w/v) BSA, then washed twice with HBSS+0.05% BSA followed by a 30 min preincubation. Cells were subsequently treated for 15 min stimulation with 100 nM BK, 100 nM BK + 100 nM PMA, 100 nM A23187, or 100 nM BK + 100 nM A23187. The amount of label released was normalized by dividing by the total label incorporated into the cells and was expressed as the fold increase in [^{3}H]AA release compared to control. Results are expressed as means ±SEM of five independent experiments performed in duplicate. *P<0.05 compared to BK alone.


[3H]arachidonic acid release
(fold increase compared to control)
12
10
8
6
4
2
0
*
BK
BK+PMA
A23187
BK+A23187

Since PMA was unable to potentiate the response to BK, experiments were performed to determine whether PKC was therefore similarily activated by both agonists. Interestingly, the lack of a PMA effect prevailed despite the observation that with respect to control, PMA caused a 93% increase in membrane PKC activity compared to only 42% for BK (**Figure 6.7**). Thus, PKC activation by BK does not appear to limit AA release. Subsequently, the effects of BK and PMA on *in vitro* $cPLA_2$ activity were examined to see if they corresponded accordingly to the different PKC activities achieved by each agonist. Even though the increase in PKC activity for PMA-treated cells was more than double the increase found for BK-treated cells, results shown in **Figure 6.8** demonstrate that there was no significant difference between the ability of either agonist to stimulate $cPLA_2$ activity (compare 30.9±2.97 $pmol \cdot min^{-1} \cdot mg^{-1}$ for BK to 36.3±8.08 $pmol \cdot min^{-1} \cdot mg^{-1}$ for PMA). The involvement of a PKC-dependent mechanism in the BK-stimulated activation of $cPLA_2$ was confirmed by inhibition of this response by 58% following Ro31-8220 treatment. This was similar to the 51% inhibition previously seen for BK-stimulated AA release (**Figure 6.5**).

6.2.2 Evidence that bradykinin-induced cytosolic phospholipase A_2 activation is dependent upon increased phosphorylation

Bradykinin-induced $cPLA_2$ activation may occur through the enhanced phosphorylation of this enzyme (Lin et al., 1993). To test whether this post-translational modification was responsible for the observed increase in $cPLA_2$ activity, cell lysates from both stimulated and unstimulated cells were treated with potato acid phosphatase prior to the

Figure 6.7
Effect of bradykinin and phorbol ester on protein kinase C activity

RCCD cells were serum starved overnight in DMEM/F12 defined media supplemented with 0.05% (w/v) BSA. Cells were preincubated with HBSS +0.05% BSA followed by a 2 min stimulation with 100 nM BK or 100 nM PMA. Membrane PKC activity was measured and is expressed as picomoles of peptide phosphorylated per minute per milligram of protein assayed. Values are presented as means ±SEM of 5 independent experiments performed in duplicate. *$P<0.001$ vs. basal, **$P<0.005$ vs. BK.

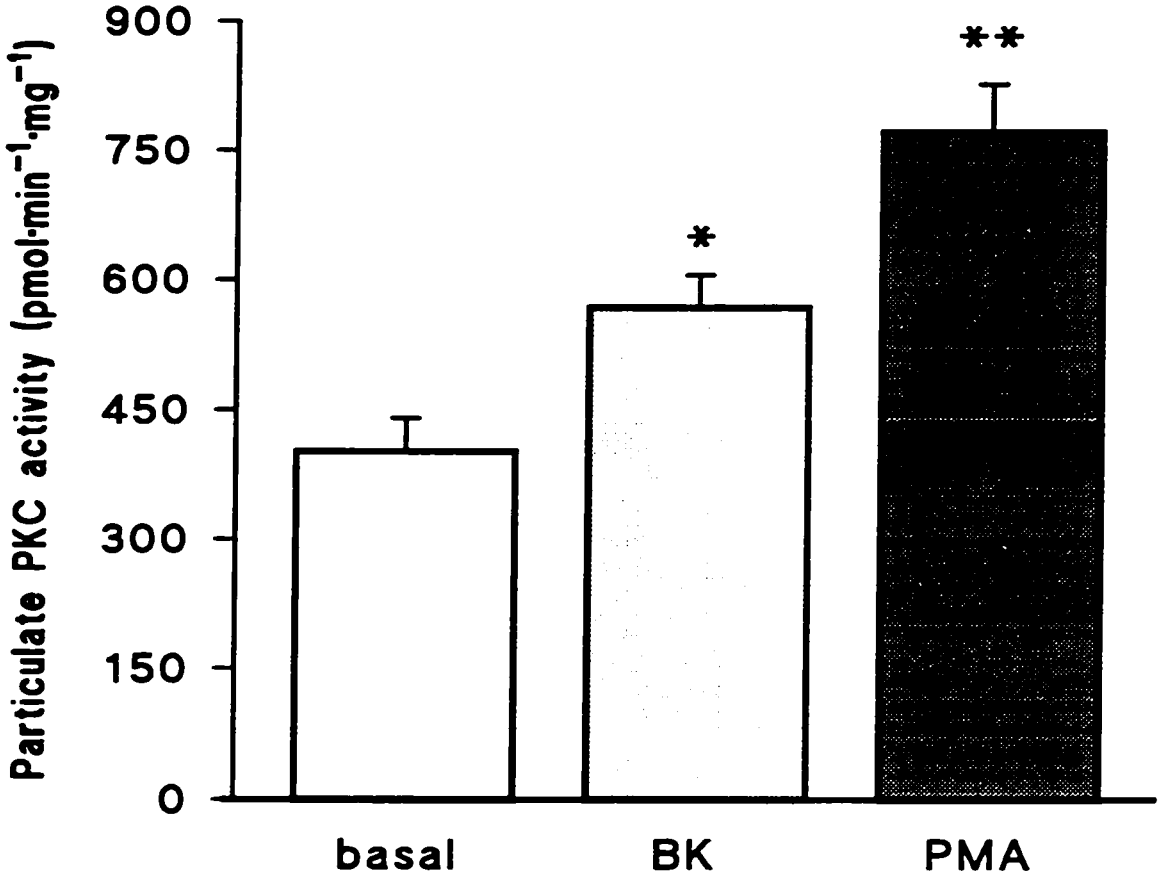
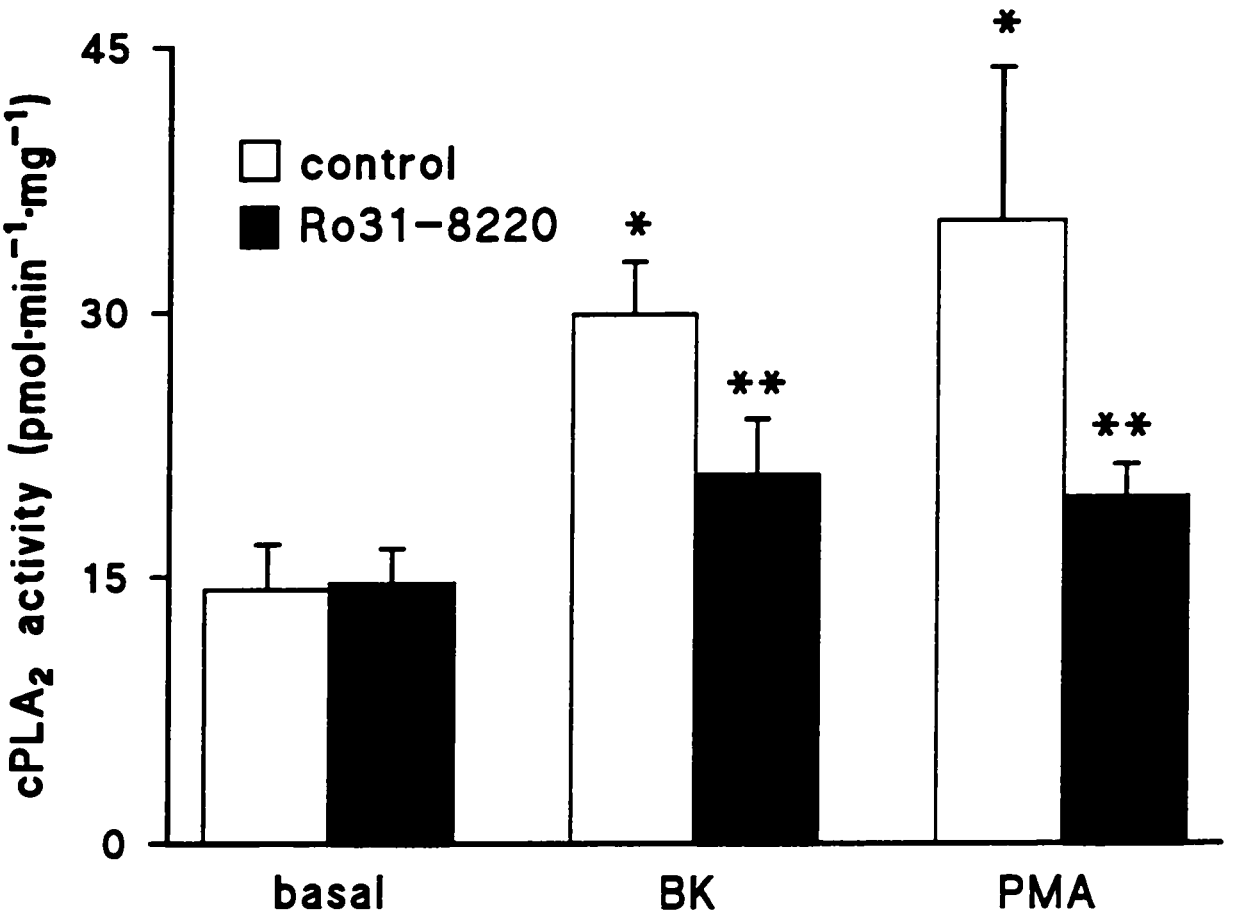

Particulate PKC activity (pmol·min⁻¹·mg⁻¹)
900
750
600
450
300
150
0
*
**
basal
BK
PMA

Figure 6.8
Protein kinase C is involved in the stimulation of phospholipase A_2 activity induced by bradykinin

RCCD cells were incubated overnight in DMEM/F12 defined media containing 0.05% (w/v) BSA. They were then preincubated with or without 5 μM Ro31-8220 for 30 min prior to stimulation with 100 nM BK or 100 nM PMA for 2 min. The total cell lysate PLA_2 activity was determined and results are expressed as the means ±SEM of four separate experiments assayed in duplicate. *$P<0.005$ vs. basal control, **$P<0.05$ compared to its respective agonist-treated control.


45
30
15
0
cPLA2 activity (pmol·min-1·mg-1)
control
Ro31-8220
*
**
*
**
basal
BK
PMA

assay. Results depicted in **Figure 6.9** illustrate that both BK- and PMA-induced activities were indeed a result of a phosphorylation event since phosphatase exposure completely abrogated these responses. Phosphatase treatment resulted in a slightly diminished basal $cPLA_2$ activity, but this was not statistically significant.

6.2.3 Demonstration that increased cytosolic phospholipase A_2 activity is a direct result of its enhanced serine phosphorylation following bradykinin and phorbol ester treatment

Using ^{32}P-labeled PLA_2 from mouse peritoneal macrophages, Qiu and co-workers (1993) demonstrated by two-dimensional phosphoamino acid analysis that $cPLA_2$ was phosphorylated exclusively on serine residues in both unstimulated and stimulated cells. Further evidence in support of this observation was provided by Lin and colleagues (1993) who demonstrated that treatment of cells with agents that stimulate AA release is concomitantly associated with an increase in serine phosphorylation and activation of $cPLA_2$. These investigators also noted that the phosphorylation event occurred at Ser-505 and was mediated by MAP kinase. To specifically determine whether BK treatment causes changes in the level of RCCD $cPLA_2$ serine phosphorylation, the enzyme was immunoprecipitated, separated by SDS-PAGE, and transferred to nitrocellulose membranes which were subsequently immunoblotted with a rabbit polyclonal antibody directed towards phosphoserine. Results summarized in **Figure 6.10A** show a representative blot, and those in **Figure 6.10B** the averaged optical density of scans from three such blots. While the upper

Figure 6.9
Effect of phosphatase treatment on bradykinin-stimulated phospholipase A_2 activity

RCCD cells were serum-starved overnight in DMEM/F12 defined media containing 0.05% (w/v) BSA, preincubated for 30 min, then stimulated with 100 nM BK or 100 nM PMA for 2 min. Cell lysates were prepared in lysis buffer (without phosphatase inhibitors) supplemented with 1 U/mL potato acid phosphatase prior to determination of PLA_2 activity. Results are expressed as the means ±SD of three separate experiments assayed in duplicate. $*P<0.05$ vs. basal control, $**P<0.05$ compared to its respective agonist-treated control.

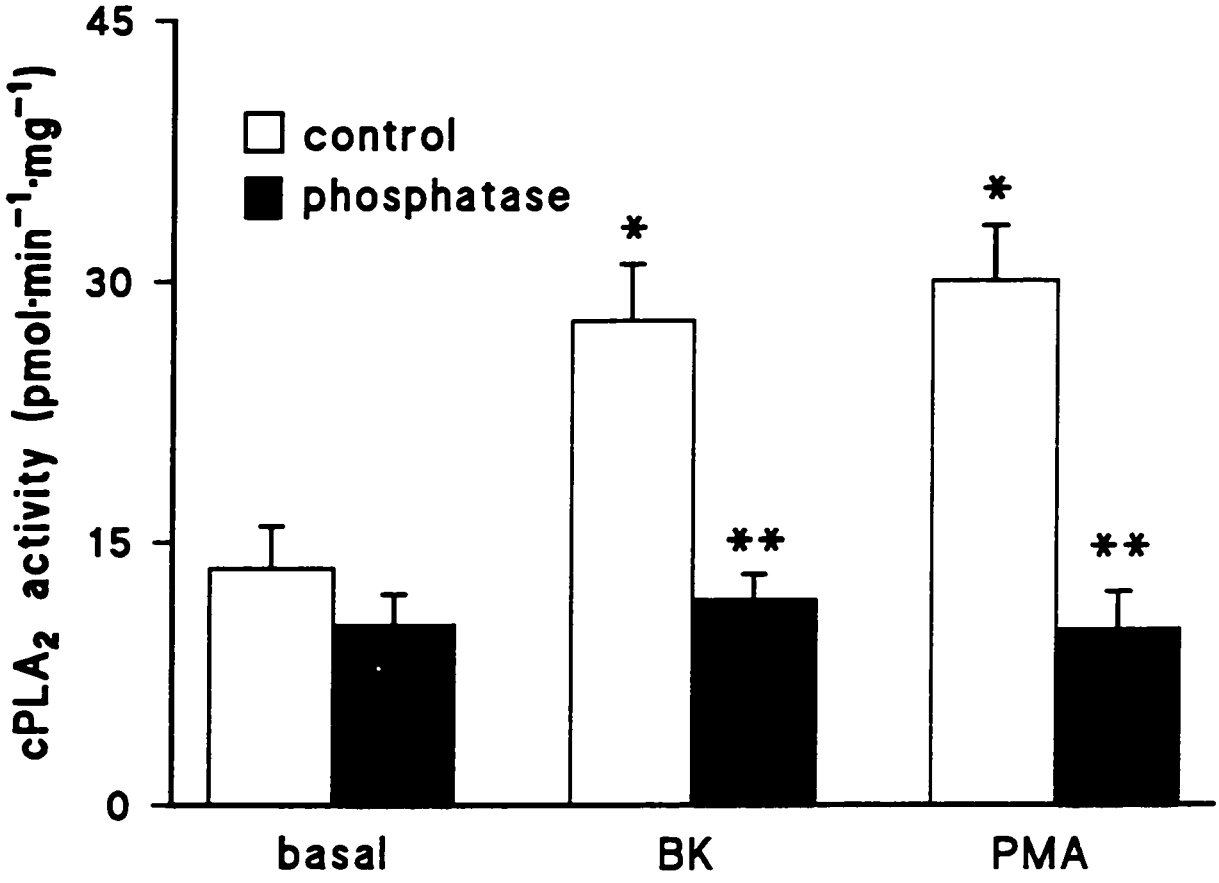
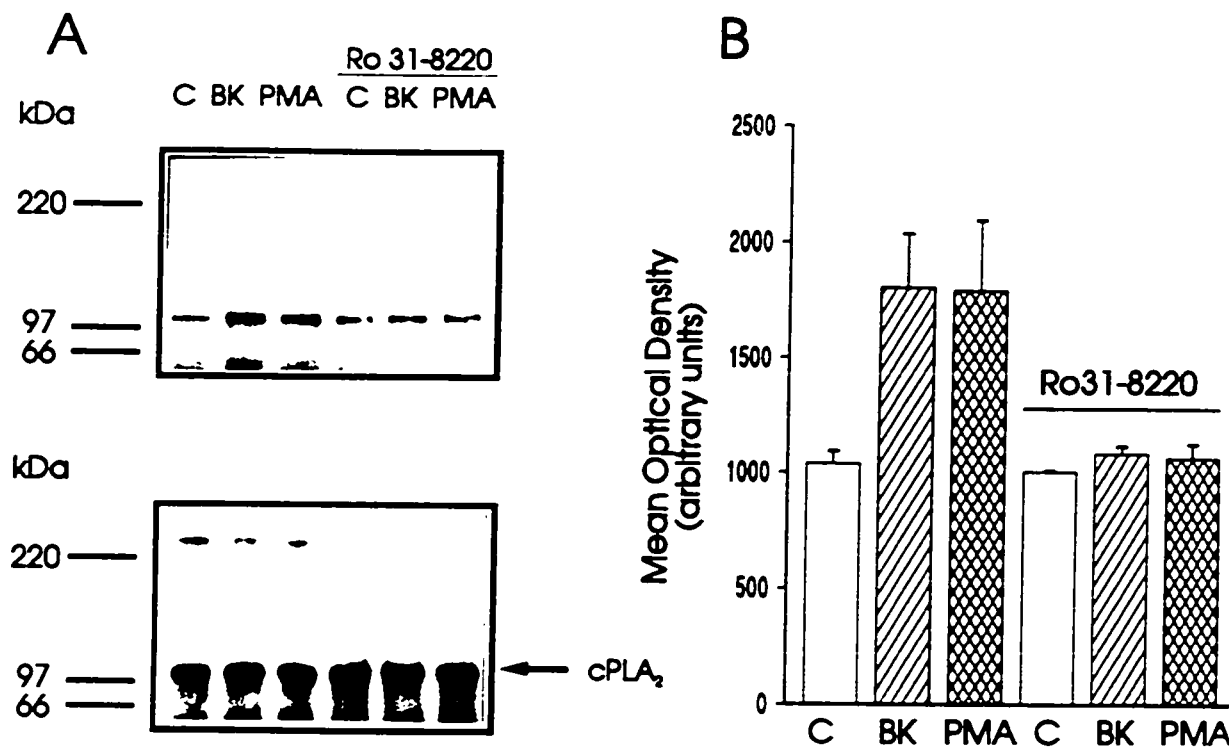

45
30
15
0
cPLA2 activity (pmol·min-1·mg-1)
control
phosphatase
*
**
*
**
basal
BK
PMA

Figure 6.10
Induction of cytosolic phospholipase A_2 serine phosphorylation following bradykinin or phorbol ester stimulation is reversed by Ro31-8220

RCCD cultures were incubated overnight in DMEM/F12 defined media containing 0.05% (w/v) BSA. The following day, cells were preincubated with or without 5 μM Ro31-8220 for 30 min prior to stimulation with 100 nM BK or 100 nM PMA for 2 min. $cPLA_2$ was immunoprecipitated using a polyclonal $cPLA_2$ antibody and was resolved on SDS-PAGE (7.5%). A. Serine phosphorylation was detected using a rabbit polyclonal antibody to phosphoserine residues (upper panel). Total $cPLA_2$ present in each lane was similar as detected by stripping the membrane and re-blotting with $cPLA_2$ antibody (lower panel). B. Average $cPLA_2$ phosphoserine content determined by densitometric analysis of three experimental blots. Position of molecular weight markers indicated to the left of each blot.


A
Ro 31-8220
C BK PMA C BK PMA
kDa
220
97
66
kDa
220
97
66
cPLA2
B
Mean Optical Density (arbitrary units)
2500
2000
1500
1000
500
0
Ro31-8220
C BK PMA C BK PMA

panel of **Figure 6.10A** displays phosphoserine levels, the lower panel demonstrates that equal amounts of $cPLA_2$ protein were present in each lane. Both BK and PMA stimulation caused an approximately two-fold increase in $cPLA_2$ phosphorylation, and as predicted, this phosphorylation was completely reduced to control levels by Ro31-8220 treatment. Although PKC may directly phosphorylate the 85 kDa PLA_2 (Nemenoff et al., 1993), evidence from numerous studies strongly favour MAPK as the major enzyme responsible for phosphorylation of this phospholipase (Kan et al., 1996; Sa et al., 1995; Xing and Insel, 1996). The virtually identical responses of both $cPLA_2$ activation and phosphorylation in reaction to BK or PMA treatment, considered together with the difference in activation of PKC achieved by either agonist, suggests that PKC need not be fully activated to allow the same result or that specific PKC isozymes are mediating the activation of $cPLA_2$.

6.2.4 Effect of inhibiting the mitogen-activated protein kinase cascade on bradykinin-stimulated arachidonic acid release

As mentioned above, the requirement for enhanced phosphorylation of $cPLA_2$ in response to certain agonists appears to be fundamental to activation of this enzyme. To address the question of whether MAPK is involved in the events leading to BK-stimulated AA release in RCCD cells, we measured the ability of PD98059, an inhibitor of the MAPK cascade, to reduce this response. This synthetic inhibitor prevents activation of MAPK and subsequent phosphorylation of MAPK substrates through the potent and selective inhibition of the MAPK-activating enzyme (MEK) (Alessi et al., 1995; Dudley et al., 1995). Treatment

of cells with 30 μM PD98059 was found to reduce BK-stimulated AA release by 54% while exhibiting an insignificant effect upon basal release (**Figure 6.11**). Xing and Insel (1996) also observed a similar inhibitory response in MDCK cells stimulated with the the α_1-adrenergic agonist, epinephrine. Moreover, these investigators found that PKC was likely involved in this process in a manner similar to what was observed in RCCD cells.

6.2.5 Combined effect of protein kinase C and mitogen-activated protein kinase kinase inhibition on bradykinin-stimulated arachidonic acid release and cytosolic phospholipase A_2 activity

Protein kinase C-mediated activation of the MAPK signalling cascade has been implicated in the release of AA from cells stimulated with various agonists (Clark and Murray, 1995; Lin et al., 1993; Xing and Insel, 1996). In contrast to these studies, there are also reviews reporting the dissociation of PKC and MAPK events in the activation of $cPLA_2$ (Araki et al., 1995; Hirasawa et al., 1995; Kan et al., 1996). Given that BK-stimulated AA release from RCCD cells is neither completely abolished by PKC inhibition with Ro31-8220 (**Figure 6.5**), nor MEK inhibition with PD98059, it was postulated that these kinases may act independently of one another to fully regulate the activation of $cPLA_2$. Results shown in **Figure 6.12** and **Figure 6.13** reveal the effects of single or combined inhibitor action on BK-stimulated AA release and $cPLA_2$ activity respectively. It was shown that inhibition of each of these responses following preincubation of cells with both inhibitors was similar to that following pretreatment with either one of the inhibitors alone, thus indicating the occurrence

Figure 6.11
Effect of PD98059 on arachidonic acid release

RCCD cells were labelled overnight with 0.3 μCi [^{3}H]AA in DMEM/F12 defined media containing 0.05% (w/v) BSA, then washed twice with HBSS+0.05% BSA followed by a 30 min pretreatment with 30 μM PD98059. Cells were subsequently stimulated with 100 nM BK in the presence of inhibitor for 15 min. Media was then removed and counted for [^{3}H]AA release by the cells. The amount of label released was normalized for the total label incorporated into the cells and is presented as fold increase compared to control. The data are expressed as means ±SEM of 4 independent experiments performed in duplicate. *P<0.05 vs. BK alone.

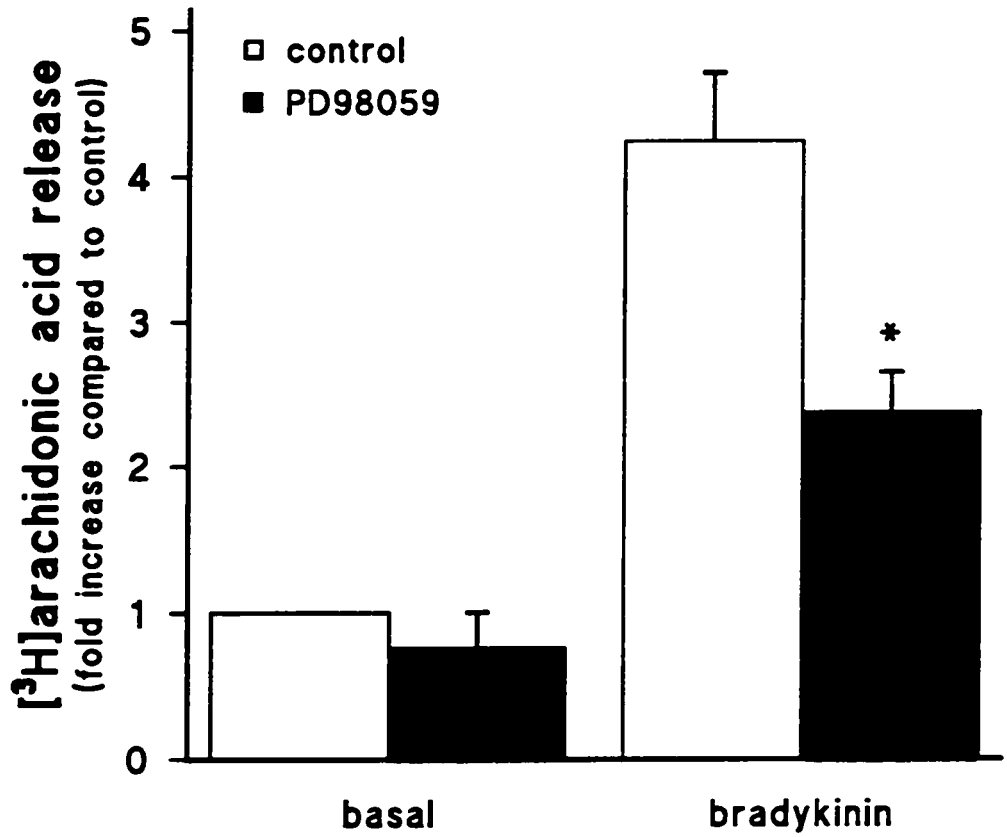

[3H]arachidonic acid release
(fold increase compared to control)
control
PD98059
5
4
3
2
1
0
*
basal
bradykinin

Figure 6.12
Combined effect of protein kinase C and mitogen-activated protein kinase inhibition on arachidonic acid release

RCCD cultures were labelled overnight with 0.3 µCi [^{3}H]AA in DMEM/F12 defined media containing 0.05% (w/v) BSA, washed twice with HBSS+0.05% BSA, and preincubated for 30 min with 5 µM Ro31-8220, 30 µM PD98059, or 5 µM Ro31-8220 + 30 µM PD98059. Cells were subsequently stimulated for 15 min with 100 nM BK in the continued presence of the inhibitors. [^{3}H]AA released into the media was counted and divided by the total label incorporated into the cells. The data are expressed as means ±SD of 3 independent experiments performed in duplicate. *P<0.05 vs. BK treatment.

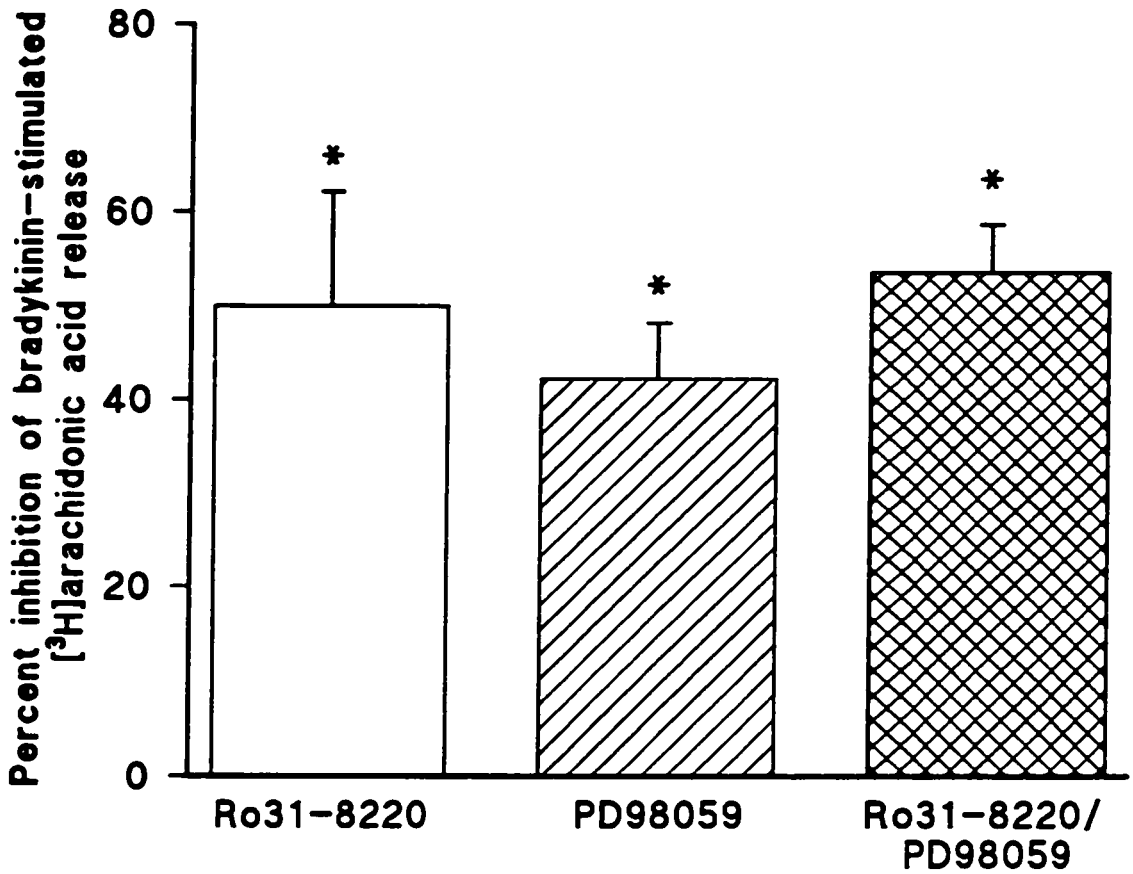
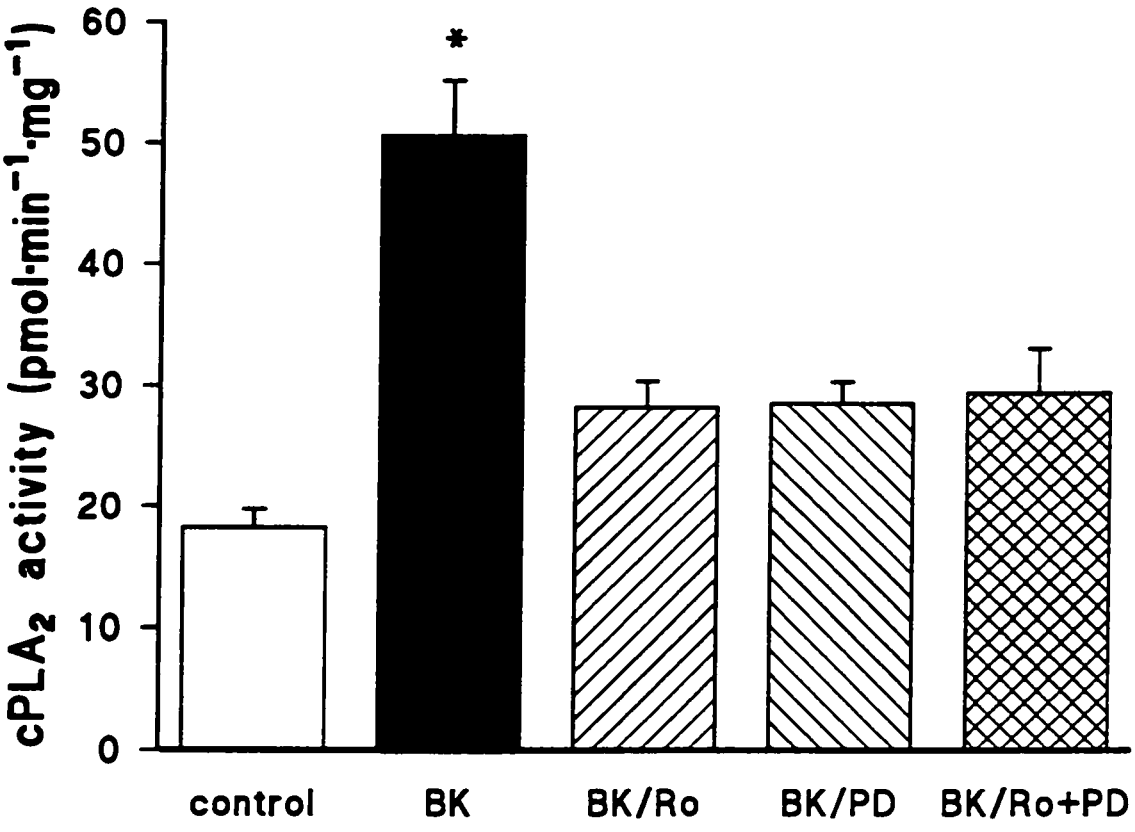

Percent inhibition of bradykinin-stimulated [3H]arachidonic acid release
80
60
40
20
0
*
*
*
Ro31-8220
PD98059
Ro31-8220/ PD98059

Figure 6.13
Bradykinin-induced activation of cytosolic phospholipase A_2 is dependent upon protein kinase C and mitogen-activated protein kinase

RCCD cells were incubated overnight in DMEM/F12 defined media containing 0.05% (w/v) BSA. They were subsequently preincubated with or without 5 μM Ro31-8220, 30 μM PD98059, or 5 μM Ro31-8220 + 30 μM PD98059 for 30 min prior to stimulation with 100 nM BK for 2 min. Total cell lysate PLA_2 results are expressed as the means ±SEM of four separate experiments assayed in duplicate. *$P<0.05$ vs. all other treatment groups.


cPLA2 activity (pmol·min-1·mg-1)
60
50
40
30
20
10
0
*
control
BK
BK/Ro
BK/PD
BK/Ro+PD

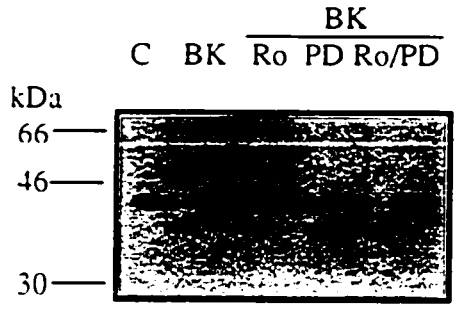
of PKC- and MEK-independent mechanisms. Although the other possible mediators accounting for RCCD $cPLA_2$ activation remain unexplored, it appears probable that the role of MAPK in the BK-mediated release of AA is determined initially by its stimulation via a PKC-dependent route.

6.2.6 Effect of Ro31-8220 and PD98059 on bradykinin-mediated mitogen-activated protein kinase activation as assessed by its level of phosphorylation

To confirm that MAPK activation takes place following BK treatment and that it is dependent upon the initial activation of PKC, cells were pretreated with Ro31-8220, PD98059, or both, and examined for the consequent effect upon MAPK activation. Activation of these kinases is a result of phosphorylation of threonine and tyrosine residues in a -TXY- motif (Seger et al., 1991), and can be visualized by a slight molecular weight-shift of the phosphorylated species to a more slowly migrating form following SDS-PAGE (Xing and Insel, 1996). Cells treated with BK showed such a mobility shift (**Figure 6.14**), and this response was confirmed to be a result of an increase in the level of phosphorylation of MAPK since phosphatase treatment was able to return the slower migrating band to its initial position in control cells (result not shown). Preincubation of cells with PD98059, an agent that prevents activation of MAPK through the inhibition of its phosphorylation on both tyrosine and threonine residues (Dudley et al., 1995), was able to completely restore MAPK to its faster migrating form.

Figure 6.14
Effect of Ro31-8220 and PD98059 on bradykinin-induced mitogen-activated protein kinase mobility shift

RCCD cells were incubated overnight in DMEM/F12 defined media containing 0.05% (w/v) BSA and preincubated with or without 5 μM Ro31-8220, 30 μM PD98059, or 5 μM Ro31-8220 + 30 μM PD98059 for 30 min prior to stimulation with 100 nM BK for 2 min. Protein samples (10 μg/lane) were then immunoblotted for MAPK by Western blotting. Shown is a representative blot. Position of molecular weight markers indicated to left of blot.


BK
C
BK
Ro
PD
Ro/PD
kDa
66
46
30

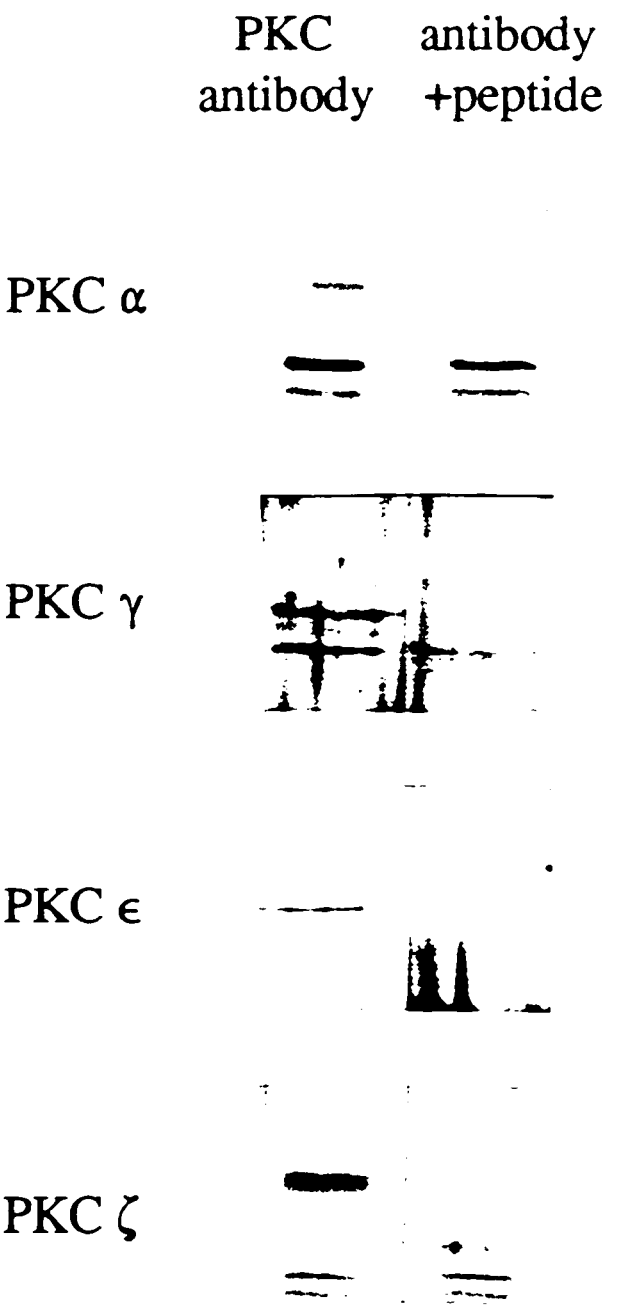
Moreover, as predicted, the PKC inhibitor, Ro31-8220, was also able to fully prevent the reduced mobility caused by BK stimulation. Thus MAPK activation following BK-receptor occupation appears to be a result of prior activation of upstream PKC.

6.2.7 Determination of protein kinase C isozyme expression in rabbit cortical collecting duct cells

Based upon the demonstration that PKC activation is a central component in the activation of $cPLA_2$, and upon previous results suggesting that BK-mediated regulation of this enzyme may be dependent on specific PKC isozymes, experiments were performed to establish whether such regulation was operational in RCCD cells. Indeed, increasing evidence is available to support the occurrence of a unique and specific pattern of PKC isozyme expression and activation in many cell types (reviewed by Hug and Sarre, 1993) including renal tissue (Östlund et al., 1995). Presently, the various PKC isozymes were screened by Western blotting strategies utilizing polyclonal antibodies to rabbit PKCα, β, γ, δ, ϵ, and ζ. Results depicted in **Figure 6.15** reveal the presence of four of the six PKC species tested for in RCCD whole cell lysates, namely, PKCα, γ, ϵ, and ζ. No signal was detected when cells were probed with antibodies to PKCβ and PKCδ. The efficacy of the latter two antibodies to detect their corresponding isozyme was confirmed by the identification of the predicted species in liver T51B cells, a cell line known to express these two PKC species. Although multiple bands are present in some of the blots to the various PKC entities, the distinct identity of each of the isozyme-specific signals was authenticated by incubating the antibody

Figure 6.15
Immunodetection of protein kinase C isozymes

RCCD cells cultured overnight in DMEM/F12 defined media supplemented with 0.05% (w/v) BSA were analyzed for the expression of PKC isozymes. Blots for PKCα, γ, and ε were loaded with 15 μg protein per lane, while blots for PKCζ were loaded with 3 μg protein per lane. The precise location of each of the various PKC isozymes was confirmed by the disappearance of the putative signal by the competing peptide.


PKC antibody
antibody +peptide
PKC α
PKC γ
PKC ϵ
PKC ζ

with its corresponding competing peptide. The disappearance of a band is associated with precise blockage of antibody recognition of the putative PKC isozyme, while those remaining signals are a result of non-specific binding.

6.2.8 Determination of protein kinase C isozyme involvement in bradykinin signalling

Three strategies were employed to determine whether BK-stimulated activation of $cPLA_2$ is dependent upon a distinctive pattern of PKC isozyme activation. The first examined the effect of long-term phorbol ester pretreatment upon BK-stimulated AA release, the second investigated the effect of PKC chemical inhibition upon this response, and the third assessed the pattern of PKC isozyme redistribution following BK and PMA stimulation.

Protein kinase C was first discovered as a protease-activated kinase, and only later was it proved that proteolytic cleavage actually occurred following its activation (Kishimoto et al., 1983). In many cell types, complete depletion of cellular PKC (down regulation) can be achieved following long-term incubation of cells with phorbol ester, and this technique is often used as a tool to uncover the role of PKC in intracellular signalling events mediated by a physiologically relevant (PKC-dependent) ligand. This strategy was employed in an attempt to reveal whether we could correlate a reduction in BK-stimulated AA release with the down regulation of specific PKC isozymes. However, it was observed that when RCCD cells were preincubated with PMA for 24 h prior to BK treatment, there was no significant effect upon agonist-stimulated AA release (**Table 6.2**). Shorter and longer preincubation times were also tested but were equally without significant effect. This was surprising given that PKC was shown to be involved in the activation of $cPLA_2$ by BK. Remarkably, Western blotting of

Table 6.2
Effect of long-term phorbol ester treatment on arachidonic acid release

RCCD cells were labelled overnight with 0.3 μCi [^{3}H]AA in DMEM/F12 defined media supplemented with 0.05% (w/v) BSA in the presence or absence of 100 nM PMA. Cells were then stimulated for 15 min with 100 nM BK. The amount of [^{3}H]AA released into the media was counted and divided by the total label incorporated into the cells. There was no significant difference in the incorporation of label for either PMA-treated or -untreated cells. Results are expressed as the means ±SD from 3 independent experiments.

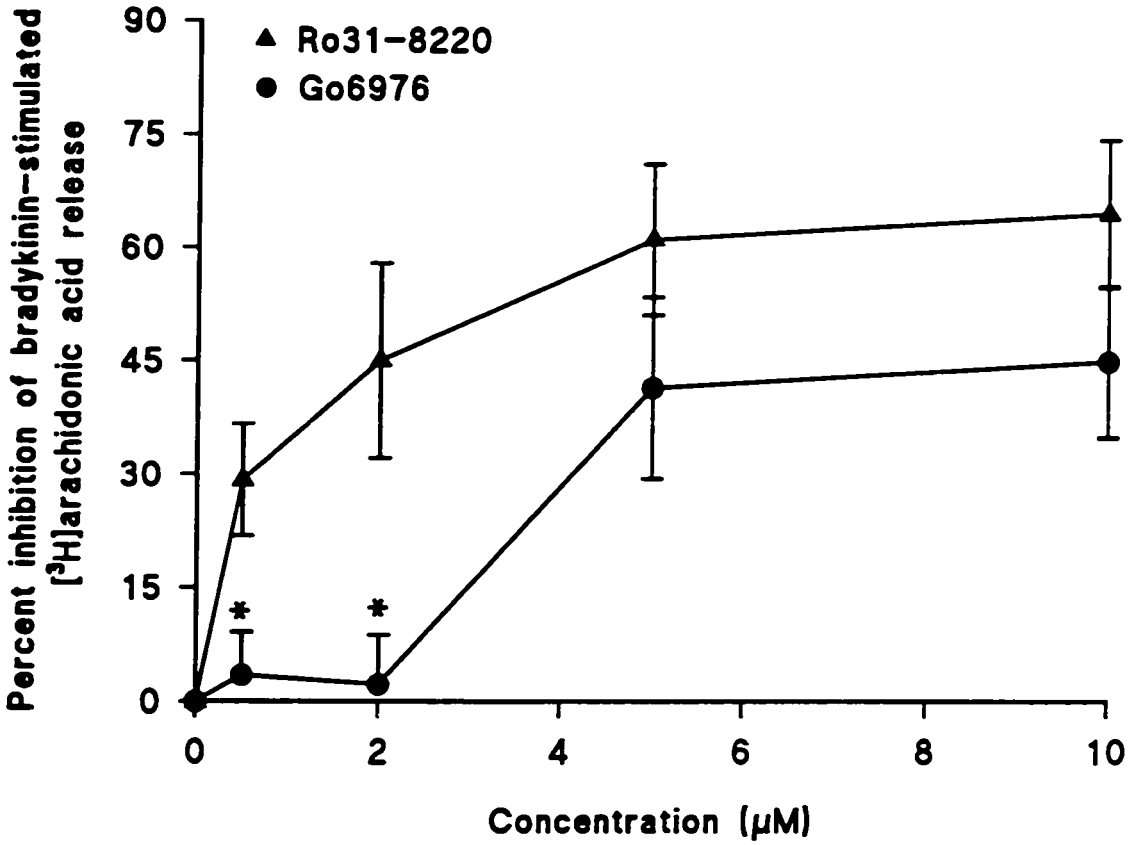
Treatment	-PMA	+PMA
Control	0.34±0.08	0.45±0.05
Bradykinin	2.26±0.09	2.41±0.10

PKC isozymes and measurement of PKC activity following long-term PMA incubation did not reveal any significant difference in either the expression pattern or magnitude of membrane bound activity when compared to control cells treated without PMA, thus indicating that PKC protein was not degraded. Based on these findings, it appears that PKC is resistant to long-term phorbol ester treatment or that a member of the DAG-insensitive aPKC group is responsible for mediating the observed BK responses.

Results from drug-based PKC inhibitory studies of BK-stimulated AA release provide some interesting possibilities regarding the signalling events occuring downstream of receptor occupation. The agent, Ro31-8220, is relatively specific for PKC but displays little selectivity with respect to its inhibition of the various PKC isozymes and reveals IC_{50} (nM) of 5, 24, 14, 27, and 24 to PKCα, β_1, β_2, γ, and ϵ respectively (Wilkinson et al., 1993). In contrast, another recently developed PKC inhibitor, Gö6976, displays selective inhibition of Ca^{2+}-dependent PKCα and PKCβ_1 [IC_{50} (nM) of 2.3 and 6.2, respectively] while revealing no effect upon the Ca^{2+}-independent PKC subtypes δ, ϵ, and ζ (Martiny-Baron et al., 1993). Results shown in **Figure 6.16** suggest that members of the cPKC group may not be involved in BK-stimulated AA release in RCCD cells since at low concentrations (from 0.5 µM up to 2 µM), BK-stimulated AA release is significantly blunted by Ro31-8220, and Gö6976 at similar concentrations, is ineffective in blocking this response. However, at concentrations above 2 µM, where Ro31-8220 inhibition plateaus, Gö6976 now abrogates the release of AA. This result is surprising given the above mentioned IC_{50} of PKCα (no data is available for PKCγ) for Ro31-8220 and Gö6976 [compare IC_{50} (nM) of 5 and 2.3 respectively]. It would stand to reason that Gö6976, if able to inhibit BK-stimulated AA release through its suppres-

Figure 6.16
Effect of two protein kinase C inhibitors on bradykinin-stimulated arachidonic acid release

RCCD cells were labelled overnight with 0.3 µCi [^{3}H]AA in DMEM/F12 defined media containing 0.05% (w/v) BSA. Cells were subsequently washed twice with HBSS+0.05% BSA and preincubated for 30 min with Ro31-8220 or Gö6976 at the concentrations indicated. They were then stimulated for 15 min with 100 nM BK in the continued presence of the PKC inhibitors. [^{3}H]AA released into the media and total cell label incorporated into cells was determined by scintillation counting. The amount of label released was divided by the total incorporation and was expressed as the fold increase in [^{3}H]AA release compared control. Results are expressed as means ±SEM of 4 independent experiments performed in duplicate. *P<0.05 vs. Ro31-8220.

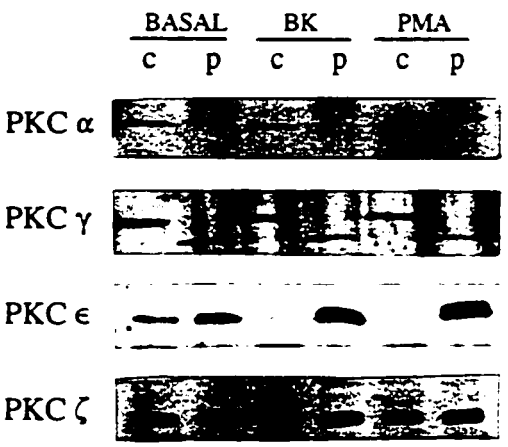

Percent inhibition of bradykinin-stimulated [3H]arachidonic acid release
90
75
60
45
30
15
0
▲ Ro31-8220
● Go6976
*
*
0
2
4
6
8
10
Concentration (μM)

sion of PKCα, would do so at a concentration equal to or less than that for Ro31-8220. Perhaps the agent, Gö6976, has a non-specific inhibitory effect upon AA release despite the claim of its PKC isozyme selectivity.

Although the results shown in **Table 6.2 and Figure 6.16** do not conclusively reveal the involvement of specific PKC subtypes in the BK-mediated regulation of $cPLA_2$, those results depicted in **Figure 6.17** provide conclusive information. The distribution of PKC isozymes within resting cells of various origins has been examined, and it is well established that treatment of cells with various stimuli (e.g. hormones, growth factors, phorbol esters) can cause a redistribution of PKC isozymes to cellular membranes, an event regarded as equivalent to the activation of the respective PKC isozyme. The question of whether a unique pattern of PKC isozyme translocation ensued BK treatment was investigated by Western blot techniques. Under resting conditions, when probed with antibodies to the PKC isozymes previously detected in RCCD cells (**Figure 6.15**), PKCα and PKCγ appeared mainly in the cytosolic portion whereas PKCε and PKCζ were found equally allocated between both cytosolic and particulate fractions. Following 100 nM BK treatment, PKCε translocated to the membrane fraction while there was no detected change in the distribution pattern of the other PKC isozymes examined (**Figure 6.17**). Since activation of PKC is widely accepted to occur following membrane association, only PKCε appears involved in events downstream of BK-receptor occupation. The PKC activator, PMA, mimics the action of DAG to activate PKC by its persistence in the cellular membrane and results depicted in **Figure 6.7** support the ability of phorbol ester to activate PKC in RCCD cells. When cells were exposed to this stimulus, the PKCα subtype was recruited to the membrane fraction in addition to PKCε.

Figure 6.17
Redistribution of protein kinase C isozymes to the cytosolic and particulate fractions following exposure to bradykinin or phorbol ester

RCCD cells were serum-starved overnight in DMEM/F12 defined media containing 0.05% (w/v) BSA. They were then exposed to 100 nM BK or 100 nM PMA for 2 min after which denatured proteins from cytosolic (c) and particulate (p) fractions were separated by 7.5% SDS-PAGE. Protein loading was 15 μg/lane for PKCα, γ, and ϵ and 3 μg for PKCζ. Following SDS-PAGE and transfer of proteins to nitrocellulose, immunodetection was performed using the appropriate anti-PKC antibody. As displayed in Figure 6.15, the signal corresponding to PKCγ is represented by the upper band.


BASAL
BK
PMA
c p c p c p
PKC α
PKC γ
PKC ϵ
PKC ζ

The expression pattern of PKCγ and PKCζ were unchanged and may indicate that these two isozymes were not activated by PMA, or that increased translocation could not be detected under the conditions employed. To confirm the specificity of the PMA-induced translocation, cells were treated with the inactive phorbol ester, 4α-phorbol-12,13-didecanoate (PDD). Exposure of cells to PDD failed to induce PKC isozyme redistribution, thus indicating that the response to PMA was not unspecifically lipid-mediated. These translocation results suggest that the epsilon form of PKC is the major isozyme activated in RCCD cells following BK treatment.

SECTION 7: DISCUSSION

7.1 Determination of the enzyme responsible for bradykinin-stimulated arachidonic acid release

7.1.1 Cytosolic phospholipase A_2 is activated following bradykinin treatment

Arachidonic acid release and the subsequent production of eicosanoids represent key signalling events for the maintainance of proper salt and water balance in the mammalian kidney. Accordingly, the nonapeptide, BK, is able to modulate kidney water balance largely through its ability to enhance AA availability and subsequent prostaglandin production in the collecting duct. Given the importance of AA mobilization to the function of numerous physiogical phenomena, investigation of the enzymes and pathways responsible for this response have been extensively examined.

Three major pathways are generally accepted to account for AA release, and they include the actions of either PLC/DAG lipase, PLD/PAP/DAG lipase, or PLA_2. Hydrolysis of AA by PLA_2 enzymes has received particular attention and several reports support the coexistence of members of different PLA_2 groups within the same cell or tissue, including those from the kidney. Aarsman and colleagues (1996), using whole tissue homogenates, have confirmed the presence of at least three PLA_2 activities in the rat kidney, namely those of group I, 14 kDa group II, and high molecular mass group IV ($cPLA_2$) enzymes. In addition to these Ca^{2+}-dependent PLA_2 forms, a novel 28 kDa Ca^{2+}-independent PLA_2, selective against arachidonylated-plasmalogen, was recently purified from rabbit kidney and

shown to represent the predominant PLA_2 activity measurable in freshly isolated proximal tubules (Portilla and Dai, 1996). The present study provides evidence indicating that cytosolic 85 kDa PLA_2 is the enzyme largely responsible for BK-stimulated AA release in immortalized RCCD cells (**cf. Figures 6.1-6.4 and Table 6.1**). Firstly, members of the secretory group of PLA_2 enzymes possess seven disulfide bridges, and are therefore sensitive to sulfhydryl reducing agents such as DTT (Seilhamer et al., 1989). A role for these enzymes in BK-stimulated AA release is not likely since no significant difference was apparent when PLA_2 activities were assessed in the presence or absence of DTT. Furthermore, $sPLA_2$ activity demonstrates an absolute requirement for millimolar Ca^{2+} and as shown in **Figure 6.1**, even following BK treatment, RCCD cells do not mobilize sufficient Ca^{2+} to induce such activation. Involvement of the known intracellular Ca^{2+}-independent PLA_2 enzymes in the events leading to AA release following BK stimulation was also precluded since HELSS, an inhibitor specific for $iPLA_2$ over Ca^{2+}-dependent $sPLA_2$s (Hazen et al., 1991b) and group IV Ca^{2+}-dependent $cPLA_2$ (Balsinde and Dennis, 1997), was without effect upon this response (data not shown). Additionally, while these data deny the involvement of $sPLA_2$ and $iPLA_2$ enzymes, the specific role of the 85 kDa $cPLA_2$ was confirmed by the inhibitory action of $AACOCF_3$ (**Figure 6.4**) and a polyclonal anti-$cPLA_2$ antibody (**Table 6.1**). The compound, $AACOCF_3$ functions as a transition-state analogue that gains access to the $cPLA_2$ active site and readily inhibits AA release and enzyme activity (Riendeau et al., 1994; Kennedy et al., 1996; Xing and Insel, 1996). However, it has also been demonstrated that this inhibitor and similar analogues readily inhibit 80 kDa $iPLA_2$ (Ackermann et al., 1995; Balsinde and Dennis, 1997). But since HELSS was without effect on the response to BK in RCCD cells, $iPLA_2$

is therefore not responsible for BK-induced AA release and $AACOCF_3$ is likely exerting its effect through distinct inhibition of $cPLA_2$.

Although the results presented reveal a strong connection between $cPLA_2$ and BK-induced release of AA, the presence of other PLA_2 group members in RCCD cells has not been precluded. Recently, Balsinde and Dennis (1997) proposed a composite model for AA metabolism in $P388D_1$ macrophages that involves the combined action of three PLA_2 subtypes. According to the model, following agonist treatment, both $sPLA_2$ and $cPLA_2$ forms are activated and hydrolyze different AA pools located at distinct cellular membranes, while regulation of $iPLA_2$ activity mediates a 'house-keeping' function via incorporation of AA at these two intracellular locations. Arachidonic acid metabolism is thus portrayed as a very dynamic process requiring the action of many PLA_2 members. Regarding the present experiments, while RCCD cells may possess such PLA_2 heterogeneity, an interplay of PLA_2 types is probably not a major mechanism in mediating BK-stimulated AA release since most of the PLA_2 of RCCD extracts is inactivated with anti-$cPLA_2$ antibody.

At this point, attention should also be drawn to the fact that RCCD cells were treated with agonists for only short periods of time. This is important because it appears that both $sPLA_2$ and $cPLA_2$ enzymes are subject to regulation by chronic stimulation. Treatment of mesangial cells and other cell types with proinflammatory cytokines such as IL-1 stimulates a delayed production of eicosanoids that is associated with an increased expression and activity of $sPLA_2$ (Pfeilschifter et al., 1991). This effect does not begin for at least a couple of hours. Thus, the potential role of this PLA_2 subtype is not revealed unless under the specific conditions of a maintained stimulation. Additionally, it has been shown that

prolonged treatment of the human lung fibroblast cell line, WI-38, with IL-1 results in the enhanced production and activation of $cPLA_2$ (without any change to $sPLA_2$ activity), a response which is closely correlated with PGE_2 release (Lin et al., 1992a) and may be involved in the inflammatory response. These observations thereby demonstrate that individual PLA_2 enzymes can be regulated by multiple pathways in different cell types depending on the particular agonist treatment. With RCCD cells not exposed to prolonged periods of stimulation, it is unlikely that changes in PLA_2 levels (either $sPLA_2$ or $cPLA_2$) account for AA release stimulated by 15 min BK treatment. This is supported by findings of Schaefers and co-workers (1996) who revealed that short term treatment of MDCK cells with PMA has no effect upon $sPLA_2$ levels or activity, but that 20 h phorbol ester incubation results in enhanced PGE_2 production dependent upon increased transcription and translation of $sPLA_2$.

Collectively, the observed results strongly support the conclusion that $cPLA_2$ activation is the major determinant of BK-stimulated AA release. In spite of the possibility that other PLA_2 isoforms may indeed be present in RCCD cells, they clearly are not responsible for the short-term response to this agonist. With this knowledge at hand, the focus of this study was next turned towards an examination of the mechanisms accounting for the activation of $cPLA_2$.

7.2 Signalling pathways involved in bradykinin-stimulated arachidonic acid release

7.2.1 Bradykinin treatment elicits calcium mobilization

It is well established that many calcium-mobilizing agonists trigger AA release, and that $cPLA_2$ requires Ca^{2+} for activity, but unlike $sPLA_2$ enzymes, this ion is necessary for $cPLA_2$ binding to membrane or phospholipid vesicles rather than for catalysis. Membrane binding of $cPLA_2$ is promoted by calcium concentrations of 0.3-1.0 μM and correlates with that concentration necessary to stimulate $cPLA_2$ catalytic activity *in vitro* using phospholipid vesicles as substrate (Clark et al., 1991). This property parallels that situation seen *in vivo* where $cPLA_2$ exists in the cytosol during resting conditions (50-100 nM calcium) but binds to membrane substrate following elevatation of $[Ca^{2+}]_i$ (Glover et al., 1995). Based upon the magnitude of the calcium signal generated after BK treatment (**Figure 6.1**), it is likely that RCCD $cPLA_2$ would become membrane-associated. The present investigation has not pursued this method of regulation, but $cPLA_2$ has been demonstrated by others to localize predominantly to endoplasmic reticulum and nuclear membranes (Glover et al., 1995; Schievella et al., 1995) and also to plasma membranes (Sierra-Honigmann et al., 1996) following an appropriate signal. It would be interesting to determine the site of $cPLA_2$ binding in RCCD cells in terms of prostaglandin production since PGHS-1 and PGHS-2 enzymes have also been localized to the endoplasmic reticulum and nuclear envelope (Schievella et al., 1995). In fact, the occurence of such a functional complex required for the production of eicosanoids has been suggested on the basis of 5-lipoxygenase, 5-lipoxygenase activating protein, and PGHS-2 colocalization (Serhan et al., 1996).

7.2.2 Protein kinase C is implicated in bradykinin-stimulated arachidonic acid release

In order to establish whether PKC was involved in the signalling events mediating BK-stimulated AA release and PLA_2 activity, the phorbol ester, PMA, was used to activate PKC, and the agent, Ro31-8220, was used to inhibit PKC activity. This inhibitor is a member of the bisindolylmaleimide class of PKC inhibitors and blunts the activity of a partially purified rat brain preparation containing PKCα, β_1, β_2, γ, δ, ϵ, and ζ with an IC_{50} of 23 nM. Bradykinin-stimulated AA release was at least partly dependent of PKC since treatment with Ro31-8220 resulted in a greater than 50% inhibition of this response (**Figure 6.4**). Activation of PKC with PMA alone was not successful in mimicking BK in its ability to release AA. However, when cells were treated with PMA + A23187 together, there was a synergistic increase in AA release. The inability of PMA to induce AA release on its own is not surprising since it does not generate a Ca^{2+} signal (Aboolian et al., 1989), a result that has been reported for other systems (Slivka and Insel, 1988; Lin et al., 1992b). Indeed, when $cPLA_2$ activity was determined in the presence of an assay buffer containing Ca^{2+}, PMA was able to activate $cPLA_2$ to a level comparable to that seen following BK treatment (**Figure 6.8**). The mechanism accounting for the observed PKC- and Ca^{2+}-dependent synergy of AA release is probably a result of PKC-mediated phosphorylation of $cPLA_2$ and its Ca^{2+}-dependent translocation to membrane lipids.

7.2.3 Bradykinin-induced arachidonic acid release appears limited by calcium, not protein kinase C activation

For the purpose of determining which of these two signals limits BK-stimulated AA release, cells were incubated with BK in the presence of either PMA or A23187 (**Figure 6.6**). In contrast to results on MDCK cells (Slivka and Insel, 1988), simultaneous addition of BK + PMA did not result in a potentiation of AA release. While these investigators did not pursue the mechanism through which this increased release occurred, they did observe that PKC can act as a negative regulator of signal transduction and that PMA pretreatment partially reduces BK-mediated IP_3 production (Slivka and Insel, 1988). In addition, this response has also been associated with a desensitization of BK-invoked Ca^{2+} transients (Aboolian et al., 1989; Luo et al., 1992). Thus, it appears that BK-stimulated AA release from MDCK cells does not require a full BK-invoked Ca^{2+} signal and that PMA must be potentiating the release of arachidonic acid through another mechanism. In the present scenario, it appears that PKC activation does not limit the ability of BK to cause AA release (since PMA was a more effective stimulus of PKC activation compared to BK, **Figure 6.7**), but rather that calcium may be limiting since BK + A23187 treatment resulted in a potentiation of the response to BK. Interestingly, the fold increase in AA release is analogous to that amount observed following PMA + A23187 treatment (compare **Figure 6.5 and Figure 6.6**). The question to be addressed in the following section then becomes one of why greater PKC activation, achieved by PMA versus BK, does not result in enhanced $cPLA_2$ activation and AA release.

Despite the conclusion that Ca^{2+} is critical to AA hydrolysis by $cPLA_2$, it should be

appreciated that A23187 treatment does not likely mimic the physiological release of calcium by agonists such as BK since in the prior case, Ca^{2+} from both extracellular and intracellular Ca^{2+} stores would be indiscriminantly liberated. It appears that the maintenance of an elevated $[Ca^{2+}]_i$ level (dependent upon extracellular sources) is specifically required to achieve optimal AA release in endothelial cells (Briand et al., 1996), mast cells (Ishimoto et al., 1996), and MDCK cells (Kennedy et al., 1997b). Such a similar prolonged elevation likely follows ionophore treatment. Another drawback to the use of calcium ionophores is that they have effects upon numerous cellular enzymes and may modify the integrity of the phospholipid bilayer. A change of this nature would likely alter $cPLA_2$ binding to its substrate (Peterson and Gruenhaupt, 1990). In the present series of experiments, A23187 treatment caused AA release not only through Ca^{2+} mobilization, but also via activation of PKC (**Figure 6.5**), a result similar to that described by Lin et al. (1992b). Based upon this discussion, although A23187 treatment induces a Ca^{2+} signal, it is difficult to firmly establish the absolute effect of calcium ionophore treatment on AA release.

7.2.4 Serine phosphorylation of cytosolic phospholipase A_2 accounts for enhanced enzyme activity in response to bradykinin treatment

It has been previously reported that $cPLA_2$ phosphorylation on serine residues is critical for its enhanced catalytic activity and ability to liberate AA (Lin et al., 1993; Qiu et al., 1993). In the current study a role for phosphorylation in BK-induced $cPLA_2$ activation was substantiated by the phosphatase-mediated inhibition of this response (**Figure 6.9**). Accordingly, Western blot techniques were subsequently developed to verify whether serine

phosphorylation was in fact occurring in BK- and PMA-stimulated RCCD cells. The results show that BK, like PMA, resulted in an almost two-fold increase in $cPLA_2$ serine phosphorylation compared to control, and that the response to either agonist could be completely reversed by Ro31-8220 (**Figure 6.10**). Consideration of this finding and the other results on AA release and $cPLA_2$ activity brings, to mind interesting possibilities regarding the regulation of this enzyme. Although Ro31-8220 completely reversed BK-induced $cPLA_2$ phosphorylation, this agent was unable to completely block the increase in $cPLA_2$ activity. Possible explanations that may account for this observed activity occurring independent of serine phosphorylation are agonist-induced effects upon a PLA_2-activating protein or PLA_2-inhibitory protein (annexin) (Croxtall et al., 1996; Mira et al., 1997), or changes in the level of tyrosine phosphorylation as has been seen in HeLa S3 cells activated by IFN-α treatment (Flati et al., 1996). Another explanation may perhaps be more likely, given the results of the serine phosphorylation experiments. In many cells, it appears that PKC activation can play a role in $cPLA_2$ activation by triggering a kinase cascade leading to MAPK activation and subsequent $cPLA_2$ phosphorylation on serine residues (Lin et al., 1993; Qiu and Leslie, 1994). While this phosphorylation event specific to Ser-505 can be blocked by PKC inhibition, it has recently been demonstrated in Sf9 insect cells overexpressing $cPLA_2$ that okadaic acid treatment results in the preferential phosphorylation of Ser-727, by a yet unidentified kinase (de Carvalho et al., 1996). Furthermore, in these same cells, there are also serine residues that maintain their level of phosphorylation in unstimulated cells. Thus, with respect to RCCD cell studies, where Ro31-8220-mediated inhibition of BK-induced PKC activity may prevent enhanced Ser-505 phosphorylation, the basal level of $cPLA_2$ serine phosphorylation

maintained at other sites may confer a slight activity to this enzyme under appropriate conditions. This maintained level of phosphorylation may be important given previous studies based upon gel-shift mobility assay and phosphatase treatment of $cPLA_2$ which reveal that unstimulated $cPLA_2$ occurs with varying degrees of phosphorylation (Ambs et al., 1995; Börsch-Haubold et al., 1995; Qiu et al., 1993). Under basal conditions (i.e., in the absence of BK stimulation), as seen in **Figure 6.10**, RCCD $cPLA_2$ appears to display a significant amount of phosphorylation. Thus, it is possible that some of the $cPLA_2$ could actually be primed for activation, just needing an appropriate calcium signal for translocation to membrane substrate. In fact, $cPLA_2$ may be at least partially active under basal conditions given the modest, yet statistically insignificant decrease in its activity following phosphatase treatment. It is likely that stimulation with bradykinin consequently engenders an appropriate Ca^{2+} signal that is able to enhance AA release via recruitment of basally phosphorylated enzyme as well as amounts of freshly phosphorylated enzyme to the membrane.

The results obtained thus far clearly demonstrate the involvement of PKC in the BK-induced activation of $cPLA_2$. Based upon the often described requirement for MAPK in the activation of $cPLA_2$, experiments were subsequenlty designed to reveal the potential role of this kinase in mediating AA release from RCCD cells stimulated with BK.

7.2.5 Mitogen-activated protein kinase is involved in bradykinin-stimulated arachidonic acid mobilization

The MAPK cascade has been implicated in the activation of $cPLA_2$ in many cell systems, but this is not always the case and evidence exists that different G protein-coupled receptors can also activate $cPLA_2$ through MAPK-independent pathways (Araki et al., 1995; Börsch-Haubold et al., 1995; Kramer et al., 1995; Winitz et al., 1995). However, as appears most commonly, when PKC is implicated in the events leading to $cPLA_2$ activation, MAPK is also involved. Under the present conditions, evidence is provided that the BK-induced activation of $cPLA_2$ is in fact MAPK-dependent (**Figure 6.11**) in addition to PKC-dependent. Although there is clear evidence supporting the occurrence of BK-mediated MAPK cascade activation (Clark and Murray, 1995), recent studies of BK-stimulated AA release in MDCK cells have either confirmed this conclusion (Kennedy et al., 1997a) or have indicated the process to be independent (Xing et al., 1997b) of extracellular signal-regulated kinase (ERK or MAPK) activation. Concerning the study of Xing and colleagues (1997b), BK-induced AA release was shown to occur despite the ability of BK to induce $cPLA_2$ activation through the increased phosphorylation of this enzyme. Where neither PKC nor MAPK cascade inhibitors were able to prevent BK-stimulated AA release, the authors provide evidence that a tyrosine kinase is involved in the activation of $cPLA_2$ by this agonist. Interestingly, these results with BK are in contrast to their previously published results regarding $cPLA_2$ regulation following α_1-adrenergic receptor stimulation of this same cell type (Xing and Insel, 1996). When using epinephrine as an agonist, these investigators found that α_1-adrenergic receptor stimulation regulates AA release and phosphorylation-dependent $cPLA_2$ activation

through a PKC- and MAPK-mediated process. Thus, within a single cell, agonist-unique regulation of $cPLA_2$ can occur, and with respect to the present study, a single agonist (BK) can invoke $cPLA_2$ activation through a unique pathway. The current evidence presented herein therefore provides an example of a cell-specific, BK-stimulated intracellular signalling cascade that results in $cPLA_2$ activation partially dependent upon PKC and MAPK.

7.2.6 Protein kinase C and mitogen-activated protein kinase are sequentially involved in bradykinin-stimulated cytosolic phospholipase A_2 activation and arachidonic acid release

Given the involvement of both of PKC and MAPK in the BK-mediated release of AA in RCCD cells and the partial inhibition imparted by both inhibitors (Ro31-8220 and PD98059), experiments were designed to determine whether these two kinases were involved independently or sequentially in the activation of $cPLA_2$. As discussed earlier, PKC-mediated activation of $cPLA_2$ is usually dependent upon a subsequent activation of MAPK. However, this proposed direct relationship appears dependent on the specific coupling betweeen receptor and G protein in native cells. In fact, there exist examples of MAPK-dependent $cPLA_2$ activation occurring independent of PKC activation (Hirasawa et al., 1995; Kan et al., 1996) as well as PKC-dependent/ MAPK-independent mechanisms of activation (Xing et al., 1997a). It is therefore possible that combined PKC and MAPK inhibition could fully blunt BK-stimulated AA release. The results presented herein however support the possibility that PKC and MAPK are involved sequentially in the regulation of the signalling cascade triggered downstream of BK-receptor occupation since inhibition of PKC and MEK did not reveal an additive reduction to either AA release or $cPLA_2$ activity (**Figure 6.12 and Figure 6.13**).

Moreover, the result displayed in **Figure 6.14** reveals that the BK-induced reduction in MAPK mobility (indicative of its activation) can be completely reversed by PKC inhibition with Ro31-8220. Thus, PKC activation takes place prior to, and is responsible for, MAPK activation following BK-treatment. The mechanism through which PKC mediates activation of MAPK has not been established in the current study, but has previously been shown to occur via prior activation of upstream kinases such as Raf-1, MEK, and MEKK (reviewed by Seger and Krebs, 1995). Based on the inability of Ro31-8220 and PD98059 to completely inhibit BK-stimulated AA release suggests that other routes leading to $cPLA_2$ activation are operational. The additional mechanisms by which this occurs remain uncharacterized, but as outlined in the previous section, they do not appear to be a result of changes in the level of $cPLA_2$ serine phosphorylation.

7.2.7 Rabbit cortical collecting duct cells express protein kinase C isozymes α, γ, ∈, and ζ

Since PKC activation represents a major step in BK-mediated $cPLA_2$ activation and AA release, experiments were designed to reveal whether this agonist exerted its effect through the induction of a specific pattern of PKC isozyme activity. Östlund and colleagues (1995) have demonstrated by Northern blot techniques that the rat kidney expresses mRNA transcripts coding for four PKC subspecies, PKCα, δ, ∈, and ζ, and they suggest that these isozymes maintain a unique distribution that may be representative of their specfic roles in the functional regulation of the mature kidney. Indeed, hormonal activation of PKC is believed to be a major signalling mechanism critical for regulating salt and water transport in the distal nephron (DeCoy et al., 1995; Hébert et al., 1990; Wilborn and Schafer, 1996). Accordingly,

DeCoy and colleagues (1995) reported that freshly immunodissected rabbit CCDs possess PKCϵ and PKCζ, and that PKC down regulation by chronic PMA treatment or down regulation of PKCϵ with antisense oligonucleotides abolishes the late inhibition of sodium transport induced by AVP. Surprisingly, these investigators found that when cultured, rabbit CCD cells express PKCγ in addition to the two species seen in the freshly isolated preparation. It remains unclear whether this is a result of a culture-induced dedifferentiation of these cells, or more simply, whether small undetected amounts of PKCγ are expressed *in vivo* and present in freshly isolated CCDs. Subsequent to this observation, another study performed by Wilborn and Schafer (1996) looked at the differential expression of PKC isoforms in fresh and cultured rabbit CCD. Western blot analysis of extracted proteins and reverse transcription-polymerase chain reaction (RT-PCR) analysis of extracted RNA demonstrated expression of PKCα, ϵ, and ζ in freshly microdissected CCD segments and in fresh and cultured immunodissected CCD cells. There was no apparent change in the level of expression of these isozymes when they were grown in primary culture. In addition to the above mentioned isozymes, and with same techniques, PKCη- and PKCθ-like isoforms were also identified (Wilborn and Schafer, 1996). However, in contrast to the maintained expression pattern seen for PKCα, ϵ, and ζ, they found that the η-like isoform was more heavily expressed in cultured CCD cells than in fresh CCD cells, and that the θ-like isoform was strongly expressed in fresh CCDs but only weakly expressed in cultured CCDs. Western blots presented herein, prepared with rabbit polyclonal PKC antibodies, show that the immortalized RCCD cell line expresses four PKC isozymes, namely, PKCα, γ, ϵ, and ζ (**Figure 5.15**). These results are comparable to the expression pattern discussed above, and

support the absence of PKCβ and PKCδ from rabbit CCD segments. Similar to that of the two studies, the conclusion of the present investigation indicates that PKCϵ and PKCζ are also present in RCCD cells. The occurrence of PKCα was expected since PKCα is the one isozyme regarded to be virtually ubiquitous in all tissues (reviewed by Hug and Sarre, 1993). With respect to PKCγ, it can be mentioned that although this isozyme appears typically restricted to neural tissue, its observed presence here is similar to that described by DeCoy et al. (1995). The aforementioned results thus make it apparent that culturing techniques can reduce or enhance the expression of individual PKC isozymes. Their physiological significance can be ascertained only to the extent that they are also found expressed *in vivo*.

7.2.8 Long-term phorbol ester pretreatment does not reduce bradykinin-stimulated arachidonic acid release

In many cell types, prolonged treatment with phorbol esters results in down regulation of cellular PKC. Since phorbol ester mimics the action of DAG to redistribute and activate PKC, translocated PKC is subsequently subject to proteolytic cleavage and inactivation which invariably results in decreased responsiveness of cells to subsequent stimulation with a physiologically relevant ligand. This technique is often used to demonstrate the involvement of PKC in signalling events and furthermore, based on the different sensitivities of the distinct PKC species to DAG, it offers a method to implicate the involvement of particular members of the PKC family. When RCCD cells were pretreated with PMA for 24 h, there was no effect upon BK-stimulated AA release (**Table 6.2**), PKC isozyme expression, or BK-stimulated PKC activity. This result is surprising since PMA induces PKCα and PKCϵ

(**Figure 6.17**) translocation and in itself suggests that RCCD PKC is not susceptible to phorbol ester-induced down regulation. This observation is not unprecedented, and PKCα and PKCε resistance to down regulation by prolonged PMA treatment has been previously reported (Akita et al., 1990; Strulovici et al., 1992). Regardless of this response, it should be appreciated that PKC down regulation offers only circumstantial evidence for the involvement of PKC. Prolonged phorbol ester treatment is likely to exert other non-specific effects which may complicate interpretation. In fact, PKC members are not the only receptors for phorbol esters and it has been observed that neuronal chimaerin is able to bind phorbol esters with high affinity and as such enables the regulation of $p21^{rac}$-GTPase activity which could conceivably lead to activation of the MAPK cascade (Ahmed et al., 1993).

7.2.9 Isozyme-selective protein kinase C inhibition and the characterization of the protein kinase C isozyme involved in bradykinin-stimulated arachidonic acid release

Over the past few years, a series of potent and highly selective inhibitors of PKC have been developed. These include the indolocarbazoles and bisindolylmaleimides such as Gö6976 (Martiny-Baron et al., 1993) and Ro31-8220 (Wilkinson et al., 1993) respectively. The agent, Gö6976, has been shown to effectively inhibit the cPKC isozymes while being relatively inactive against the Ca^{2+}-independent isozymes (nPKC and aPKC) (Martiny-Baron et al., 1993; Wenzel-Seifert et al., 1994). In the current study, the effect of this inhibitor on BK-stimulated AA release however, does not provide definitive evidence regarding specific PKC isozyme involvement. At low concentrations of Gö6976, a comparison of the effect of this inhibitor and Ro31-8220 on BK-stimulated AA release suggested either nPKC or aPKC

involvement. However, at higher concentrations this is no longer true (**Figure 6.16**) and as such brings doubt to the actual effect of Gö6976. Unfortunately, no better isozyme-specific PKC inhibitors are currently available, and the results obtained with the present inhibitor remain inconclusive.

7.2.10 Protein kinase Cϵ is selectively translocated to the particulate fraction following stimulation with bradykinin

Another approach used to reveal the involvement of individual PKC isozymes in signalling events downstream of BK-receptor occupation involved an examination of altered PKC species distribution following agonist stimulation. The pattern of PKC isozyme location from unstimulated cells revealed a significant portion of both PKCϵ and PKCζ in the particulate fraction. Despite translocation being regarded as equivalent to activation, in various cell types rather significant portions of certain PKC isozymes are constitutively present in the particulate fraction, and this is understood not to be indicative of permanent and persistent activation (Bazzi and Nelsestuen, 1988). More appropriately, it is a change in the distribution of individual PKC species induced by agonist stimulation that should be viewed as a better representation of their involvement and activation. Accordingly, Western blotting of cytosolic and particulate fractions with anti-PKC isozyme antibodies revealed that only PKCϵ redistributed to membranes following BK treatment (**Figure 6.17**). This as such suggests that PKCϵ is the only isozyme responsible for BK-stimulated activation of $cPLA_2$.

Prior evidence for PKC isozyme-selective participation in the activation of $cPLA_2$ is provided by studies using CHO cells (Clark et al., 1994), MDCK cells (Godson et al., 1990;

Godson et al., 1993) and FRTL-5 cells (Wang et al., 1996). In the above-mentioned cases, PKCα was the isozyme predicted to play an essential role in the release of AA whether the cells were stimulated with an extracellular ligand or artificially by phorbol ester. The results presented herein appear to represent the first observation linking PKCε to $cPLA_2$ regulation. Since it appears that only PKCε is activated by BK-stimulation, it is suggested here that this species may be responsible for the pending activation of MAPK which then ultimately results in the phosphorylation and activation of $cPLA_2$. In fact, a role for specific PKC isozymes in the phosphorylation and activation of MAPK has been previously reported. In the MDCK cell line, Xing and collaborators have shown that PKC isoforms, other than PKCα, are responsible for a portion of MAPK activation resulting in $cPLA_2$-mediated AA release following P_{2U}-receptor stimulation (Xing et al., 1997a). More specifically, in αT3-1 gonadotrope cells, PKCα and/or PKCε are implicated in MAPK activation by gonadotropin-releasing hormone (Sundaresan et al., 1996), and in fibroblasts it appears most likely that PKCε is the major isozyme involved in MAPK activation (Clark and Murray, 1995).

In addition to experiments performed to reveal PKC redistribution in response to BK, the effect of PMA treatment was examined. An interesting corrolate can be hypothesized when this result is tied to the observation that PMA, while being a more effective activator of PKC than BK (**Figure 6.7**), it is not a superior stimulus of $cPLA_2$ activation (**Figure 6.8 and Figure 6.10**). When RCCD cells were exposed to PMA, there was recruitment to the particulate fraction of PKCα in addition to PKCε, and thus is indicative of activation of both species. One possible scenario accounting for the greater membrane-associated PKC activity yet similar $cPLA_2$ activity in response to PMA is that both PKCα and PKCε contribute to the

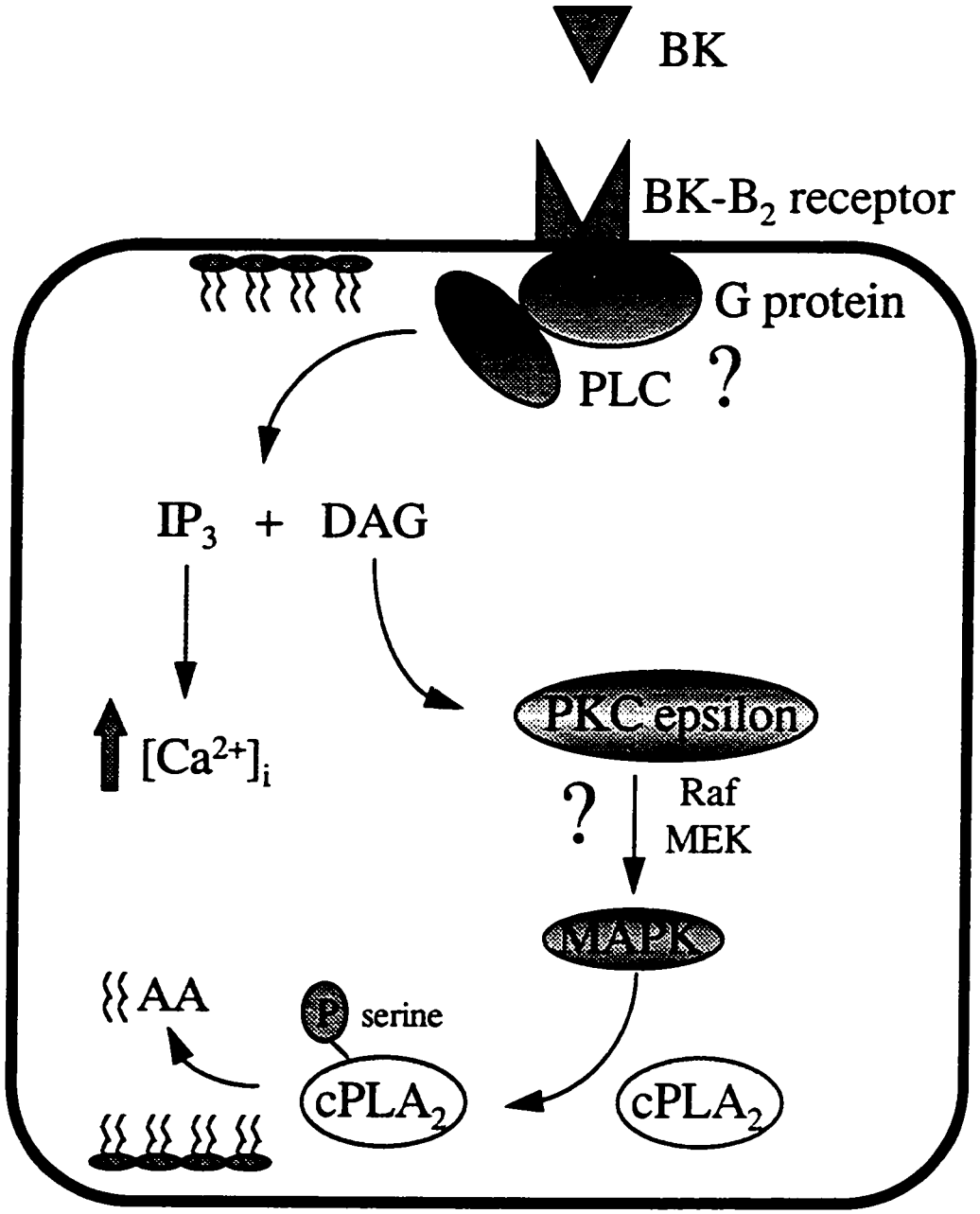
increase in PKC activity and only PKCε mediates the activation of $cPLA_2$. Since BK and PMA both equally augment the association of PKCε with membranes, both agonists are similarly effective in stimulating AA release. Additional PKCα activation does not have an effect upon this response, (**cf. Figure 6.6**). The reason why BK-receptor stimulation (which increases DAG) does not activate the same PKC subtypes as PMA (which mimics the action of DAG) is unknown, but such an occurrence has been previously documented. In fact, with respect to MAPK, MacKenzie and colleagues (1997) have recently shown that PKCδ and PKCε isoforms can couple to the MAPK pathway in 3T3-F442A preadipocytes treated with PMA, but that stimulation with growth hormone specifically involves only the PKCδ isoform. It is possible that PMA acts as a more widespread, indiscriminate activator of PKC isozymes, whereas the DAG generated upon ligand (BK)-receptor stimulation is unique and is suited for the activation of specific PKC species. Additionally, distinct receptor-mediated regulation of PKC isozyme activation may occur through differences in DAG affinity, cofactor dependence, substrate range, and localization.

7.3 Summary

Upon agonist-receptor occupation, a series of signalling events is initiated that dictates how the target cell will respond to a given stimulus. Results collected herein provide substantial evidence implicating the cytosolic 85 kDa PLA_2 as the enzyme responsible for AA release induced by BK treatment of RCCD cells. Regulation of enzyme activation is a complex process, and this thesis presents data that address a particular perspective in $cPLA_2$ activation. With respect to RCCD cells, activation of PKC occurs prior to that of MAPK, and together these kinases represent a major signalling cascade involved in BK-induced activation of $cPLA_2$. More specifically, enhanced serine phosphorylation of $cPLA_2$ is associated with agonist-stimulated AA release, and finally the PKCε isozyme appears to be uniquely involved in this process. These findings are combined and represented schematically in **Figure 7.1**.

At this point many questions still remain unresolved regarding BK action. A more thorough investigation of the role of PKCε is necessary to show that its translocation in response to BK is indeed associated with activation. Additionally, it would be interesting to pursue an investigation of the G protein-coupling associated with BK binding. Also, examination of the calcium pools implicated in $cPLA_2$ translocation would shed light on the regulation of this enzyme. Lastly, a demonstration of $cPLA_2$ allocation to particular membrane substrate sites following BK stimulation could reveal information on its physiological significance. These investigations all merit study, and the present study demonstrates that the RCCD cell line is a very convenient and useful model to pursue the regulation of $cPLA_2$.

Figure 7.1
Signalling pathways involved in bradykinin-stimulated arachidonic acid release from rabbit cortical collecting duct cells


BK
BK-B2 receptor
G protein
PLC ?
IP3 + DAG
[Ca2+]i
PKC epsilon
?
Raf
MEK
MAPK
AA
P
serine
cPLA2
cPLA2

SECTION 8: REFERENCES

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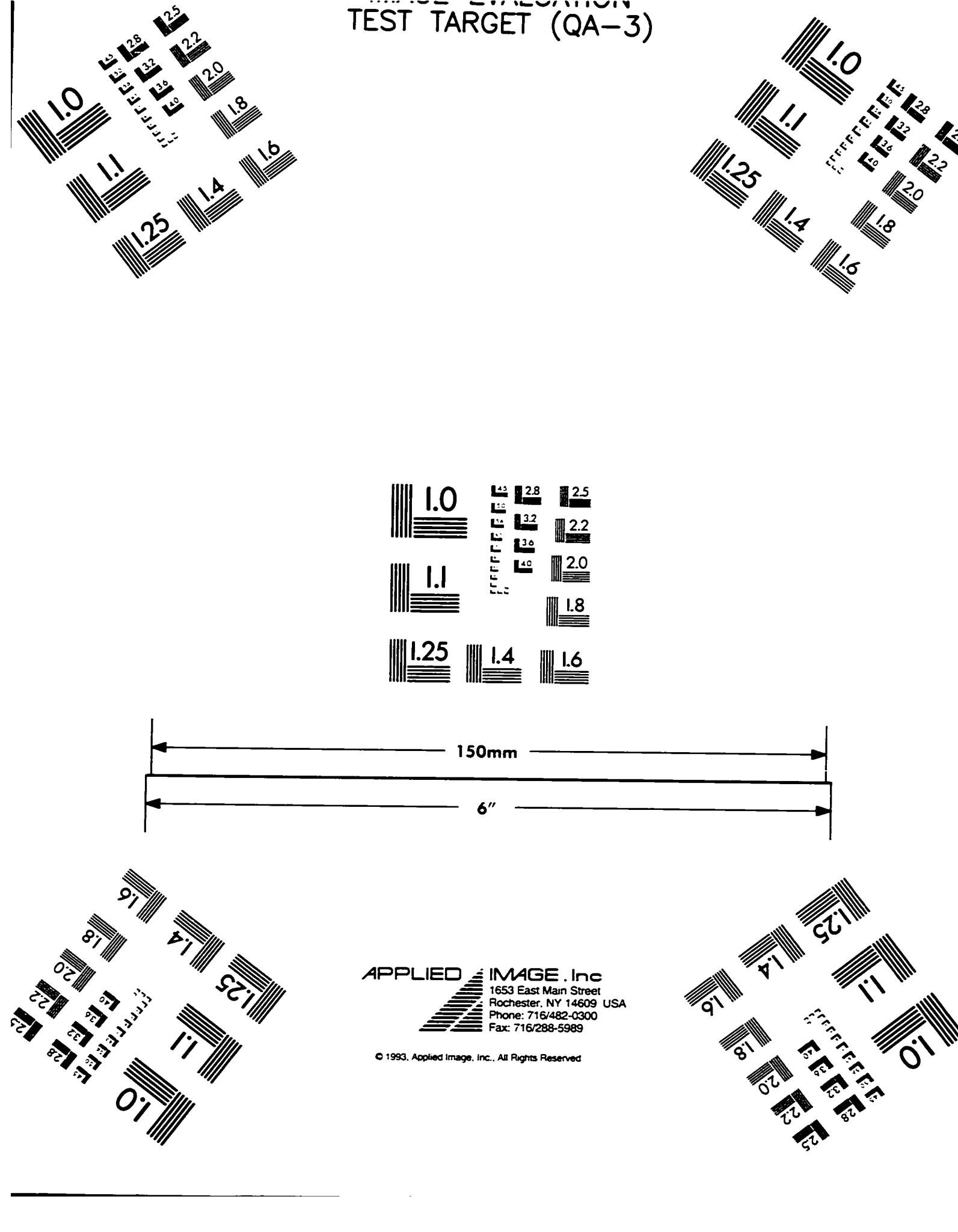
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