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SIGNALLING PATHWAYS OF BRADYKININ-MEDIATED ARACHIDONIC ACID RELEASE IN MDCK-D1 CELLS

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ABSTRACT

An investigation was undertaken to elucidate the signal transduction pathways involved in bradykinin (BK)- mediated release of arachidonic acid (AA) from the D1 clone of Madin-Darby canine kidney cells (MDCK-D1) which display distal tubule- and cortical collecting duct principal cell-like characteristics. Prostaglandins (PG), generated subsequent to BK stimulation, are known to modulate arginine-vasopressin (AVP)-stimulated water flow across this portion of the nephron.

The enzyme immediately responsible for AA release was determined to be the 85 kDa cytosolic phospholipase A_2 ($cPLA_2$), since Western blots revealed the presence of this enzyme and its specific inhibition by an arachidonate analogue completely blunted BK-stimulated AA release. Additionally, *in vitro* PLA_2 activity could be significantly reduced by preincubating cell lysates with an antibody to this enzyme. Lastly, this *in vitro* activity met all the requirements specific to $cPLA_2$, including activity at micromolar Ca^{2+} concentrations and dithiotreitol (DTT)-insensitivity.

The findings herein suggest the signalling route taken for BK-induced AA release involves phosphatidylcholine-specific phospholipase C (PC-PLC) as well as phospholipase D (PLD). Accordingly, production of *sn*-1,2-diacylglycerol (DAG) and to a lesser extent, phosphatidic acid (PA), both contribute to this release of AA by enhancing PLA_2 activity within the cellular membranes, whereas the activation of phosphatidylinositol-specific phopholipase C (PI-PLC) and subsequent inositol trisphosphate ($InsP_3$) production does not. While reports indicate the activation of protein kinase C (PKC) is required for epinephrine-mediated AA release in this cell line, inhibition of PKC failed to abrogate BK-stimulated AA release. On the other hand, down regulation of PKC levels via long-term incubation with phorbol ester (PMA) reduced both BK- and calcium ionophore (A23187)-induced AA release. However, both *in vitro* $cPLA_2$ activity and its phosphorylation , but not its expression, were significantly reduced subsequent to long-term PMA treatment, thereby demonstrating that this strategy falsely implicates immediate activation of PKC as being required for BK-mediated AA release. Extracellular calcium (Ca^{2+}) was also needed for AA release as blockade of receptor-operated Ca^{2+} channels significantly decreased BK-induced AA release.

In addition, a negative regulatory pathway in MDCK cells was demonstrated which diminishes BK-mediated AA release. Agents which cause elevations in adenosine-3',5'-cyclic monophosphate (cAMP) levels, such as AVP or forskolin (FSK) and 3-isobutyl-1-methylxanthine (IBMX), were found capable of significantly reducing BK-induced AA release and *in vitro* PLA_2 activity. This method of inhibition could represent a physiological mechanism of negative feedback promoted by agents which increase cAMP levels within the cells of the distal tubule and collecting duct. Possible targets for inhibition are suggested in light of results obtained in the present thesis and reported by others.

DEDICATION

To my loving wife Pamela, whose strength and faith provide for me, a source of encouragement and inspiration through all adversity.

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LIST OF ABBREVIATIONS

A23187	calcium ionophore
AA	arachidonic acid
ACSII	aqueous counting scintillant
ADH	anti-diuretic hormone
AG	alkylglycerol
AVP	arginine vasopressin
BCS	biodegradable counting scintillant
BIS	bisindolylmaleimide I
BK	bradykinin
BSA	bovine serum albumin
Ca^{2+}	calcium (ion)
CaLB	calcium-dependent lipid-binding domain
cAMP	cyclic-adenosine-3',5'-monophosphate
CCD	cortical collecting duct
Chol	choline
$cPLA_2$	cytosolic phospholipase A_2
DAG	*sn* 1,2-diacylglycerol
dBucAMP	dibutyrylcyclic-adensosine-3',5'-monophosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethylenebis(oxyethylenenitrilo)tetraacetic acid
EP	E-prostanoid receptor
EtOH	ethanol
FCS	fetal calf serum
FSK	forskolin
G-protein	guanine nucleotide-binding protein
GDP	guanosine diphosphate
G_i	inhibitory guanine nucleotide-binding protein
GroPIns	glycerophosphoinositol
G_s	stimulatory guanine nucleotide-binding protein
GTP	guanosine triphosphate
H-89	N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide·2HCl
HBSS	Hank's balanced saline solution
HELSS	haloenol lactone suicide substrate

HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
IBMX	3-isobutyl-1-methylxanthine
INF-γ	interferon-gamma
InsP	inositol monophosphate
$InsP_3$	inositol trisphosphate
L_p	hydraulic conductivity
MAPK	itogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MDCK	adin-Darby canine kidney cell
MW	molecular weight
PA	phosphatidic acid
PAP	phosphatidic acid phosphorylhydrolase
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PC-PLC	phosphatidylcholine-specific phospholipase C
Pchol	phosphorylcholine
PD 98059	2'-amino-3'-methoxyflavone
PE	phosphatidylethanolamine
PEt	phosphatidylethanol
PG	prostaglandin
PGE_1	prostaglandin E_1
PGE_2	prostaglandin E_2
PI	phosphatidylinositol
PI-PLC	hosphatidylinositol-specific phospholipase C
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PLA_2	phospholipase A_2
PLD	phospholipase D
PMA	phorbol-12-myristate-13-acetate
PMSF	phenylmethylsulfonylfluoride
PS	phosphatidylserine
S.D.	standard deviation
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
S.E.M	standard error of the mean
SK&F 96365	1-[β[3-(4-Methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole•HCl
$sPLA_2$	secreted phospholipase A_2
SSP	staurosporine

STMR	Seven-Transmembrane receptor
TBS	Tris-buffered saline
TTBS	Tween Tris-buffered saline
$[Ca^{2+}]_i$	intracellular calcium concentration

1 INTRODUCTION

Looking through his primitive instrument, the seventeenth century microscopist, Robert Hooke, may never have imagined that the tiny cork cell he was gazing at contained such incredible complexity. Indeed, the discovery of this basic functional unit underlying the later development of biology and biochemistry has led to an ever increasing knowledge of the cell. Yet, as more pieces of the puzzle are found, researchers come to realize how little we really know. Or perhaps an equally fitting remark would be- one question answered inevitably causes three more to arise. The intricacy of such organization found within the cell presents a formidable challenge to those who would attempt to unravel the specific relationships and functions which hold homeostatic processes together at the level of not only the cell, but within whole tissues and organisms.

One of the more intriguing aspects of cellular biochemistry is that which is concerned with how highly specific signals, originating from outside the barrier of the cell membrane, are transmitted interpreted and lead to an appropriate response within the cell. This response can induce changes not only within the targetted cell but also in other adjacent, or substantially distant cells. These interactions or communications form the basis of auto- para- and endocrine signalling between the cells, tissues, and organs of the body, providing the mechanisms required for adaptive responses to both environmental and internal stimuli. An excellent example of such organization can be found within the mammalian kidney, an elaborate organ

composed of a variety of cell types, each possessing capabilities of responding to specific stimuli, and each performing a vital role in maintaining homeostasis.

1.1 The nephron and the MDCK cell line

1.1.1 *Role of the nephron*

The kidney plays an important role in regulating homeostatic volume and composition of the extracellular fluid of the body. The basic functional unit which carries out this role is the nephron, a highly organized apparatus composed of the glomerulus, which is responsible for filtering plasma proteins, followed immediately by a luminal tube formed by a series of intricately organized epithelial cells, which vary in function and structure depending upon their particular roles. In the process of glomerular filtration, large quantities of a variety of electrolytes, water, and organic compounds are presented to the tubules. The nephron is responsible for ensuring that the proper amounts of these substances are either excreted along with the urine or reabsorbed for further circulation. Examples of such substances are sodium, water, glucose, uric acid, urea, amino acids, and phosphate. Specific areas along the nephron function to regulate the composition of the urine.

One of the more important roles played by the nephron is that of the reabsorption of water and salt. Since sodium is taken into the body as a part of diet, the kidneys must adjust the excretion of sodium to maintain a proper level so that extracellular fluid volume is retained. Additionally, sodium reabsorption is one of the major driving forces behind water reabsorption

since active salt transport creates an osmotic gradient by which water can passively follow. In fact, 70% of the filtered sodium and water are reabsorbed along the first section of the nephron, the proximal convoluted tubule. However, the final determination of the osmotic character of the urine is determined by one of the last sections of the nephron, the collecting duct.

The collecting duct plunges through the cortex into the outer- then inner-medullary regions of the kidney. As the luminal fluid flows along the lumen of the cortical collecting duct (CCD) in the presence of AVP, water is reabsorbed. This is because water moves along its osmotic gradient from the tubule fluid into the cortical interstitium, where it is carried away by a high blood flow. As the fluid moves down the length of the inner-medullary collecting duct, water is reabsorbed and the urine becomes isosmotic with the surrounding interstitium. Finally, urine of high osmolarity, a result of the urea-impermeant character of the collecting duct, and of low volume, due to the active water reabsorption, is excreted. This reabsorption of water by the collecting ducts accounts for only a small fraction of the total reabsorbed by the entire nephron. Nonetheless, it is this fraction which determines the final composition of the excreted urine, and allows for fine tuning of the osmolality of the extracellular fluid.

As mentioned above AVP plays a central role in controlling water excretion by enabling the normally water-impermeant collecting ducts to transport water from the lumen through the cell and finally across the basolateral membrane. The main stimulus for the release

of this hormone is cellular dehydration or increases in effective plasma osmolality followed by a subsequent decrease in arterial blood pressure. Stretch receptors on the left atrium and in the pulmonary veins are activated under such conditions and transmit fewer signals to the brain via the vagal afferents. However, it is within the collecting duct that AVP exerts its most important effects as it controls renal sodium and water reabsorption. Since this is the final segment of the nephron, the collecting duct or tubule has the last word in determining the final composition of urinary salt and water content. Upon binding to V_2 receptors on the basolateral membrane of the CCD, AVP causes the otherwise water-impermeable epithelium to become highly conductive of water transport. It is now well recognized that an AVP-mediated elevation of cAMP levels, followed by the activation of cAMP-dependent protein kinase (PKA), results in the recruitment and insertion into the apical membrane of the aquaporin-2 water channel (Deen et al., 1994) which normally resides in subapical membrane vesicles. Fusion of these vesicles with the apical membrane results in the insertion of the water channel. Once inserted, this channel allows water to enter the cell after which it is extruded through the basolateral membrane through exocytosis. The effect is that water reabsorption by the kidney is increased. The main targets for AVP are the principal cells of the collecting duct. The detailed studies of such cells have been made possible by the advancement of the isolated perfused tubule technique.

1.1.2 *The use of cultured cells to study renal epithelia*

The development of the isolated perfused tubule technique by Burg and his colleagues in the sixties (Burg et al., 1973) has provided the necessary tool by which researchers have gained considerable insights into the functional properties of individual segments of the renal tubule. Despite the many advantages provided by this procedure, including a means to study water and solute transport, voltage/resistance properties, and agonist-mediated calcium mobilization among many other characteristics, it does possess some limitations. For instance, its usefulness for elucidating specific intracellular events and signalling pathways is somewhat restricted by the rather nonmanipulative nature of the elaborate technique involved and the relatively short period of time within which the perfusion studies can be made for lack of tissue resistance. Alternatively, the use of cultured cells to study the structure, function and signalling properties isolated from individual segments of the nephron provides many advantages over the isolated perfused tubule technique.

A key benefit of this cell culture tool is its convenience, that is to say, large homogeneous populations of cells can become readily available without the time-consuming and technically demanding process of micro-dissection. Direct access to cellular monolayers is possible from both the apical and basolateral sides under well controlled conditions, making the study of metabolic requirements relatively uncomplicated. Another key advantage with cell cultures lies in the fact that a much broader scope of intracellular signalling pathways can be assessed, their products being more easily measured than in the case of isolated perfused

tubules. However in choosing the cell culture technique, an important consideration must be taken into account, namely, that the cultured cells should ideally retain most of the essential properties displayed by the tubular cells before culture. Therefore, every attempt should be made to characterize new cell lines, ensuring that aspects such as transport capabilities, responses to hormones and morphology are conserved. One such cell line, which has been extensively characterized and found to largely, if not completely conform to the above-stated criteria is the Madin-Darby canine kidney (MDCK) epithelial cell line.

1.1.3 *The MDCK renal epithelial cell line*

The MDCK cell line is a well characterized epithelial cell line displaying many properties which typify the renal distal tubule and early collecting duct. These cells were first isolated from the kidney of a healthy cocker spaniel by Madin and Darby in 1958 and have since been used as a model for epithelial cell transport, voltage, structure, and intracellular signalling studies (Madin and Darby, 1958). The precise origin of the cell type has never been unequivocally determined due to the ambiguity of the isolation procedure employed. However most of the evidence to date suggests that the characteristics displayed by this cell line resemble those of the principal cells of the distal tubule or CCD. Indeed, as in the collecting duct, MDCK counterparts are responsive to agents which raise intracellular cAMP such as glucagon, AVP, oxytocin, prostaglandin E_2 (PGE_2), prostaglandin E_1 (PGE_1), cholera toxin, and to a lesser extent, isoproteranol. In a similar manner to CCD cells, this cell line is indifferent to the following mediators with respect to cAMP production; parathyroid hormone, calcitonin,

angiotensin and prolactin. Studies have also demonstrated that MDCK cells are similar to CCD in their ability to respond to both muscarinic cholinergic agents and epidermal growth factor (EGF) (Taub et al., 1984). More recently, studies have shown that carbachol can induce a release of calcium from intracellular stores in rabbit CCD while this agent can increase inositol phosphate accumulation in MDCK cells (Schulz et al., 1989; Mohuczy-Dominiak and Garg, 1992). The kidney is noted for possessing abundant EGF receptor populations (Breyer and Cohen, 1990; Breyer et al., 1988). The collecting duct appears to be a target for EGF since it has been demonstrated that this growth factor can block AVP-stimulated water permeability. Similarly, MDCK cells are also responsive to EGF (Taub et al., 1984).

1.1.4 *MDCK morphology*

Most MDCK cells (>70%) exhibit properties associated with the principal cells of the CCD, including a lack of binding of peanut lectin, a marker for intercalated cells (Devuyst et al., 1994). A prime characteristic of an epithelial cell is polarity, that is, it possesses fully differentiated apical and basolateral membranes (Grenier, 1986). MDCK cells have been shown to possess both apical and basolateral membranes, including apically-located microvilli (Cereijido et al., 1980). These epithelial cells have the potential to form monolayers of densely packed cells when grown on an impermeable surface, such as plastic. In addition, MDCK cells grown upon permeable supports, such as nylon filters, retain many characteristics of epithelial cells such as brush borders, cell-cell tight junctions which seal the apical surface and lateral intercellular spaces (Simmons, 1981). Another interesting feature of these cells is that

they are able to regenerate kidney tubular-like structures when injected into baby nude mice. Such mice developed nodules containing both normal mouse fibroblasts and MDCK cells which formed epithelial sheets lining internal fluid-filled glandular structures suggesting a crude tubular arrangement (Rindler et al., 1979).

1.1.5 *Na^+ transport across MDCK cells*

In natural epithelia, Na^+ flux is achieved by a sequential process of Na^+ diffusive transport across the apical membrane, which is subsequently followed by active Na^+ transport across the basolateral membrane by the Na^+/K^+-ATPase. With MDCK cells, studies have conclusively shown the presence of ouabain binding sites exclusively on the basolateral membrane (Lamb et al., 1981). Furthermore, Na^+ ion diffusion across the apical membrane in MDCK effectively limits transepithelial Na^+ transport, as in natural epithelia (Simmons, 1981). This cell line is capable of both vectorial salt and water transport from the apical to the basolateral surface associated with a spontaneous voltage and electrical resistance. Indeed, MDCK cells express voltage and electrical resistance values within the range of those occurring in natural distal nephron segments. One difference however, is that maximum Na^+ transport rates, as estimated from short-circuit current, Na^+ pump sites and ATP hydrolysis, are considerably lower in MDCK cells (Simmons, 1981). This is most likely due to the comparatively lower degree of basolateral organization (Cereijido et al., 1980).

1.1.6 *MDCK water reabsorption capacity*

Both light and electron microscopy reveal the presence of microvilli and tight junctions similar to those of the intact CCD when the cells are grown as a monolayer (Leighton et al., 1969). However no basement membrane on the basolateral side is found. Furthermore, hemicysts may form when monolayers are grown on impermeable supports. These blister-like structures represent cells which are lifted off the plate by the hydrostatic pressure generated by the salt and water transport from the apical to basolateral surfaces (Leighton et al., 1970). Indeed, agents which cause elevation of intracellular cAMP, such as AVP, also lead to increased hemicyst formation implying an inherent functionally relevant water transport capability within MDCK cells (Valentich et al., 1976).

With all of these characteristics resembling those of the CCD, including the many relating to hormonal responsiveness, structural attributes, transport capacities and peanut lectin binding, MDCK cells appear as a reasonably valid model for the study of some of the more intricate details of cellular signal transduction found in this portion of the nephron.

1.2 The signal

1.2.1 *Bradykinin*

In the late 1940's, the Brazilian researcher, Rocha E. Silva observed that incubation of bothrops jara raca or trypsin with the pseudoglobulin fraction of plasma resulted in the formation of a potent vasodilator and smooth muscle-stimulating substance (Rocha E Silva,

1949). The peptide principle involved was termed bradykinin due to the slow (*bradys*) contraction it caused in the guinea-pig ileum relative to other factors such as histamine or acetylcholine. Accordingly, BK acts as a potent arteriolar dilator in the kidney and the gastrointestinal tract, an ability which is demonstrated by the fact that systemic infusion of BK leads to an increased renal blood flow as the vasculature relaxes. Over a decade passed before its exact amino acid sequence and structure were determined by Boissonnas et al, (1960) and Elliot et al. (1960), respectively. This peptide contains nine amino acids and is enzymatically-derived from a kinogen.

1.2.2 *Synthesis and degradation of bradykinin*

Synthesis occurs through the action of kallikrein, a serine protease, which converts the low molecular weight kinogen to the decapeptide, lysyl-bradykinin, which is further digested by an aminopeptidase to yield the nonapeptide, BK (Carretero et al, 1980). Schuster observed that high levels of circulating AVP could promote the activation of the kallikrein-kinin system (Schuster et al., 1984). Within the kidney, the cortex itself is a rich site for kallikrein, containing no less than 90 % of that found within the entire organ. The kidney is also a site of BK degradation. Indeed the proximal tubule contains a significant amount of kininase that ultimately allows this segment to restrict filtered BK from reaching the more distal regions of the nephron (Hall et al., 1976). Therefore, any urinary BK is probably formed at the distal tubule rather than originating upstream. Accordingly, kallikrein, the enzyme responsible for its production, has been localized to the distal convoluted tubule of the rat (Tomita et al., 1981).

Further evidence supporting this conclusion was obtained by Nasjletti and co-workers who infused BK into the renal artery. They observed that following this procedure, greater than 90 % of the BK infused was inactivated in one passage through the kidney, while less than 0.2 % appeared in the urine (Nasjletti et al., 1975). Additionally, since kininase II can be found in the lung, and thus destroys circulating BK, intrarenally-derived BK must act as a vasodilator only within the confines of the renal vasculature. The kallikrein/kinin system can be enhanced by aldosterone, the renin-angiotensin system and through the action of prostaglandins. Specifically, angiotensin-converting enzyme (ACE) inhibitors have been shown to increase renal BK levels, thereby demonstrating a role for ACE (kininase II) in the renal metabolism of BK (Campbell et al., 1993).

1.2.3 *Sites of action and roles for bradykinin*

Bradykinin is most certainly an auto- or paracoid and not a systemic agent since it is rapidly inactivated by ACE during its first passage through the lungs. Therefore its actions are confined to its site of release (Ferreira et al, 1967). However, despite its very limited ability to function as a hormone at sites distant from its lieu of production, this nonapeptide functions in a variety of tissues, playing several key roles in the central and peripheral nervous system and adrenal medulla, where it increases release of catecholamine. As mentioned above, BK also has a vasodilatory effect which participates in the maintainence of normal blood pressure. This is best demonstrated with the use of BK antagonists which, when infused, induce a consistent rise in blood pressure in rats. Bradykinin achieves this homeostatic result by countering the

pressor influences of other vasoactive mechanisms. Within the rabbit kidney, BK is able to reverse the water transport initiated by vasopressin. This inhibitory effect is blocked by pertussis toxin, thereby indicating the mediatory role of an inhibitory G-protein in this effect. Furthermore, inhibitors of cyclooxygenase activity reverse the effect of BK, implying that prostaglandins participate in this action (Schuster, 1985, Schuster et al, 1984).

This nonapeptide also inhibits Cl^- transport in rat CCD but not that of bicarbonate. This observation supports a role for BK in the regulation of extracellular fluid volume and precludes its direct participation in the regulation of acid-base balance (Tomita et al., 1986). In addition to its vasodilator effects in kidney, and in opposition to the action of the renin-angiotensin system, BK also induces natriuresis. This effect is due to the ability of BK to inhibit Na^+ reabsorption by the distal nephron. Intrarenally-derived BK causes natriuresis, diuresis and release of prostaglandins without changing the glomerular filtration rate (Campbell et al., 1993).

In summary, BK is a nonapeptide, derived from kinogen through the action of kallikrein. It is produced and acts at different sites in the body including the kidney vasculature where it induces vasodilation in opposition to the renin-angiotensin system. In the kidney BK functions as a negative regulator of both water and salt reabsorption. Bradykinin is able to initiate each of these intracellular responses through its interaction with a specific receptor located on the target cell.

1.2.4 *The bradykinin B_2 subtype of receptor*

It was first recognized by Regoli and co-workers in 1977 that at least two different BK receptor subtypes exist. Pharmacological data showed that the response to BK in tissues such as cat ileum or rat uterus differed significantly from that of rabbit aorta. These experiments demonstrated that the rabbit aortic strip was responsive to a truncated version of BK, [des Arg^9]-BK, while being entirely insensitive to BK itself. This truncated form of BK arises as a natural product in the vascular circulation through the action of kininase I. The receptor to the truncated form was termed B_1. In contrast, the cat ileum or rat uterus were highly responsive to BK, but failed to be stimulated by [des Arg^9]-BK. The receptor characteristic of these tissues was termed B_2 (Regoli et al., 1977). Thus the B2-receptor subtype prefers BK and does not respond well to [des Arg^9]-BK.

1.2.5 *G-protein coupling of the B_2-receptor*

The B_2 subtype is a member of the seven-transmembrane receptor (STMR) family and therefore elicits its actions through G-protein coupling. The recent cloning of the rat and human B_2-receptors confirms the presence of many of the characteristics of a G-protein coupled receptor(s) including the seven transmembrane alpha helices. The cloned rat B_2 gene has a predicted sequence which codes for 366 amino acids while that of the human, 364 amino acids. The predicted molecular weight is 41,696 Da and the sequence contains putative sites for PKC and PKA phosphorylation. There seems to be a fairly high degree of conservation

between species since the rat and human counterparts share 81 % homology at the gene level (Hess et al., 1992; McEachern et al., 1991). In isolated nephron segments of the rabbit, the major sites for BK binding are the cortical and medullary collecting tubules (Baylis et al., 1976). Depending on the tissue in question, the B_2-receptor may couple to an inhibtiory G protein (G_i) and perhaps also to a G_q subtype. In MDCK-D1 cells, the release of AA caused by BK was found to be pertussis toxin-insensitive thereby demonstrating that the B_2-receptor does not couple to G_i in this particular cell type clone (Slivka et al., 1988; Terman et al., 1987). However, in wildtype MDCK cells, BK-stimulated phospholipid hydrolysis was eliminated by pretreatment with pertussis toxin, thus indicating that B_2-receptors can also couple to G_i depending on the cell line investigated (Portilla et al., 1988). Evidence for the involvement of G_q proteins in mediating B_2-receptor activation of second messenger systems has been provided by Gutowski et al (1991). They were able to demonstrate that antibodies to the αq-subfamily of guanine nucleotide-binding regulatory protein α subunits attenuated the activation of phosphatidylinositol 4,5-bisphosphate hydrolysis by BK in NG108 cells. It would appear therefore that the B_2-receptor can couple to more than one G-protein subtype, thereby activating a variety of second messenger pathways in different cell types. In neuronal and smooth muscle cells, the activation of the B_2-receptor is associated with intracellular Ca^{2+} mobilization (Reiser et al., 1990; Bleakman et al., 1990; Fasolato et al., 1990; Boyajian et al., 1991). The B_2-receptor, through G-protein coupling, has also been implicated in the production of cGMP in endothelial cells (Boulanger et al., 1990; Schini et al., 1990). It will be appreciated from subsequent sections of this introduction that, BK, acting through the B_2-

receptor and several G-proteins, can signal by recruiting pathways which induce the activity of phospholipases C, D and A_2 in addition to that of PKC.

1.3 Cross-talk between bradykinin, vasopressin, and prostaglandin E_2

1.3.1 *Bradykinin induces synthesis of prostaglandin E_2 required for water flow modulations*

Bradykinin was first thought to counteract the ability of AVP to initiate water transport following the observation that direct infusion of this nonapeptide into the renal artery of the dog was capable of increasing urine flow and free water clearance even in the presence of AVP (Heidenreich et al., 1964; Barraclough and Mills, 1965). This nonapeptide is known to increase intracellular calcium levels, stimulate the production of PGE_2, and activate PKC in cultured CCD cells (Dixon et al., 1989). In order to elucidate the signalling mechanism of BK-mediated inhibition of AVP-stimulated water reabsorption, PMA was used to activate PKC, while calcium ionophore (A23187) served to increase intracellular Ca^{2+} levels. In one particular case, both PMA and A23187 each maximally blunted the hydraulic conductivity (L_p) induced by AVP in rabbit CCD (Ando et al., 1987; Ando et al., 1988). However, only PMA, and not A23187, was able to reverse L_p induced by a cAMP analogue, 8-CPTcAMP, thereby demonstrating that PKC-mediated inhibition likely occurs at a yet to be determined site distal to cAMP generation, while that brought about by Ca^{2+} alone probably targets cAMP accumulation itself. The inhibition of AVP-stimulated L_p by A23187 could be reversed by cyclooxygenase blockers while PKC inhibition failed to counteract the ionophore effect (Ando et al., 1988). In a similar manner to that of calcium ionophore, BK pretreatment was shown to

be capable of inhibiting AVP- but not 8-bromo-cAMP-induced water permeability in the rabbit CCD. These observations imply that even though BK activates PKC in rabbit CCD (Dixon et al., 1989), the result does not guarantee an effect similar to that of phorbol ester, a phenomenon that will be seen in MDCK-D1 cells in the present study. Furthermore, the effects of BK are reversible by indomethacin, suggesting that, like A23187, BK inhibits water permeability through an endogenous cyclooxygenase metabolite, most likely PGE_2. Evidence favours the idea that PGE_2 mediates its effects upon AVP-stimulated cAMP generation through its interaction with an E-prostanoid receptor (EP) coupled to a G_i protein, since the inhibitory effects exerted by this prostanoid can be reversed by pertussis toxin treatment (Sonnenburg and Smith, 1988).

This matter is complicated however, since others have demonstrated that BK can block cAMP formation independent of PGE_2 production. The postulated signalling mechanism involves the activation of PKC. In rabbit cortical collecting tubule cells in culture, BK was shown to increase PKC activity and decrease AVP-stimulated cAMP formation (Dixon et al., 1989). This effect was not blocked by mepacrine, an inhibitor of PLA_2. Furthermore, in MDCK wildtype cells, activation of PKC with either BK or a tumor-promoting phorbol ester was able to partially reduce the formation of cAMP initiated by AVP, but not that of PGE_2 or isoproteronol (Friedlander and Amiel, 1987). This inhibitory effect was retained despite the presence of the cyclooxygenase inhibitor, indomethacin, and serves to indicate that the effect is PKC-dependent and may target the AVP receptor itself since the ability of glucagon to elevate

cAMP levels, acting through a separate receptor, was in contrast, not impaired by prior PMA or BK treatment. Yet results previously described indicate that PKC acts at a step following cAMP formation. This discrepency of results is not easily explained but may be due to a lack of proper expression of G_i-coupled EP receptors in MDCK following minor dedifferentiation. Indeed, the loss of pertussis toxin-sensitive inhibition of cAMP formation in primary rabbit CCD cultures has been reported (Regoli and Barabé, 1980, Dixon et al., 1989).

1.3.2 *Negative feedback of cyclic adenosine monophosphate upon arachidonic acid release*

Among the targets of PKA could be those which lie within the signalling pathway responsible for AA release in MDCK cells. There have been a number of reports indicating that AA release and/or prostaglandin production is diminished by elevations of intracellular cAMP within MDCK wildtype cells (Hassid, 1983) and rat inner medullary collecting tubule cells (Teitelbaum et al., 1986). This method of inhibition could represent a physiological mechanism of negative feedback promoted by agents which increase cAMP levels within the cells of the distal tubule and collecting duct. Accordingly, vasopressin might utilize such a pathway to block the production of eicosanoids such as PGE_2, which as mentioned earlier, play an inhibitory role with respect to AVP-stimulated water and salt transport. *However, the sites of PKA inhibition were, at the start of the present investigation, not well defined for BK-stimulated AA release in MDCK cells. Therefore, by looking at the various products of phospholipases in MDCK-D1 cells, those enzymes affected by elevations of intracellular cAMP could be identified as targets of PKA activity, and AVP control.*

In any case, the wealth of results overwhelmingly favour a role for BK in maintaining a negative regulatory effect upon AVP's ability to stimulate water transport in the collecting tubule of the nephron. In addition, this negative feedback pathway exists, allowing agents such as AVP, which raise intracellular cAMP levels, to block the production of AA release and subsequent PG synthesis by these epithelial cells. However, the specific targets involved remain elusive and require further investigation. Furthermore, an elucidation of the signalling route utilized by BK leading to the provision of AA required for the generation of PGE_2 would be useful in developing a more adequate portrait of the overall functioning of this section of the nephron. First, it must be noted that a major determinant in prostaglandin formation is the availability of arachidonic acid since there is no evidence to suggest that prostaglandins are stored (Morrison and Needleman, 1979). Therefore, one of the main questions that has been addressed throughout the last ten years has been that of how arachidonic acid is produced.

1.4 Arachidonic acid and phospholipase A_2

1.4.1 *Arachidonic acid, precursor to eicosanoids*

Arachidonic acid is found stored predominantly in position *sn*-2 of phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylinositol (PI). This polyunsaturate can be released by phospholipase action and in the free form can serve very important functions as precursor to the eicosanoids (the prostaglandins, leukotrienes, thromboxanes and prostacyclin) and as a second messenger by itself since it has recently been implicated in the activation of PKCζ (Muller et al., 1995).

1.4.2 *Mechanisms of arachidonic acid release*

The liberation of AA from membrane phospholipids is thought to occur via three enzymatic mechanisms: i) through the sequential action of phospholipase C and DAG lipase. The former enzyme uses either phosphatidylinositol-4,5-bisphosphate or phosphatidylcholine as substrate to produce DAG, whereas the second releases fatty acids from DAG at either position *sn*-1 or -2, arachidonate included (Chau and Thai, 1988; Hee-Chong et al., 1989; Florin-Christenson et al., 1993). ii) through the action of phospholipase D (PLD), which prefers PC as substrate. The action of this phospholipase generates two products, PA and choline. Phosphatidic acid can then be hydolyzed by the activity of phosphatidic acid phosphorylhydrolase (PAP) which yields DAG as its major product (Billah and Anthes, 1990). The newly formed DAG can then be subjected to DAG lipase, as described above. iii) finally, AA can be released from membrane phospholipids through the action of PLA_2, which unlike

DAG lipase, releases fatty acids exclusively from position *sn*-2 where arachidonate is abundant. The action of DAG lipase has been shown to be an important route in platelets (Chau et al., 1988), cardiac monocytes (Hee-Cheong et al., 1989) and fibroblasts (Florin-Christenson et al., 1993). The combined action of PLD and PAP seems to be a predominant route of phospholipid breakdown in A10 cells and cultured aortic smooth muscle cells stimulated by various contractile agonists (Lassegue et al., 1993; Lassegue et al., 1991,); however, that of PLA_2, probably dominant in many cell types, is complicated somewhat due to the fact that several forms of this enzyme exist and the signalling mechanisms which regulate their recruitment and activation remain incompletely resolved.

1.4.3 *The phospholipase A_2 family: Groups I, II, and III*

The PLA_2 acylhydrolases are a family of enzymes responsible for a diversity of cellular activities including host defense, phospholipid digestion, and signal transduction. As mentioned above, PLA_2 action produces free arachidonate as well as the precursor for platelet-activating factor when the *sn*-1 position of the phospholipid contains an alkyl ether linkage. This family of enzymes is widely diverse with respect to function, structure, localization, mechanism of action. genetic origin and therefore can be subdivided into several major groups. Group I PLA_2 enzymes have a mass of 14 kDa, are released as proenzymes from the pancreas yet may be present in the kidney (Hanasaki et al., 1992). Group II PLA_2 is also 14 kDa in size but is associated with specific cell types such as platelets and is secreted in response to proinflammatory mediators such as interleukin-1, interleukin-6. and tumor necrosis factor

(Vadas et al., 1985; Nakano et al., 1990). Group III PLA_2 was first isolated from bee venom and is structurally similar to both group I and II phospholipases except that it displays a mass of 16 to 18 kDa. Since groups I, II, and III PLA_2 counterparts are generally released into the extracellular space, they are usually referred to as 'secreted PLA_2' ($sPLA_2$) and all contain seven disulfide bonds. These phospholipases require millimolar amounts of calcium for activity and therefore are not likely to be involved in the intracellular release of AA initiated by extracellular agents since, at best, only micromolar levels of intracellular Ca^{2+} are ever attained following stimulation by these Ca^{2+}-mobilizing agonists. Additionally, the $sPLA_2$ enzymes display no preference for AA over other fatty acids at the *sn*-2 position, therefore their involvement in the signalling cascade leading to prostaglandin production is less likely.

1.4.4 *Ca^{2+}-independent forms of phospholipase A_2*

Gross and co-workers (Wolf and Gross, 1985; Hazen et al., 1990) have purified from canine myocardium an intracellular PLA_2 that is Ca^{2+}-independent, prefers arachidonyl-containing plasmalogen phospholipids, is activated by ATP, and has a molecular weight of 40 kDa. Little is known regarding the specific events surrounding the activation of this PLA_2, although it is experimentally provoked by anoxia, and thus its role in signal transduction of heart and other cells cannot be ascertained at this time. Again, most recently, a Ca^{2+} - independent activity localized within the proximal tubule has been identified (Portilla et al., 1994; Portilla and Creer, 1995). It is a 28 kDa form which prefers arachidonate at position *sn*-2 from diradylglycerophospholipids and is optimally active at neutral pH (Portilla and Dai,

1996). These workers noted that the most of the PLA_2 activity within the cytosol of rabbit proximal tubule cells was Ca^{2+} -independent and recruited during hypoxia, preceded cell death and was accompanied by hydrolysis of endogenous plasmalogen substrates, leading to the generation of AA and accompanying phospholipid catabolism (Portilla et al., 1994; Portilla and Creer, 1995). Again, it is difficult to situate this enzyme in terms of a possible role in signal transduction.

1.4.5 *Cytosolic phospholipase A_2*

The type IV PLA_2 has been recently cloned in human, rat, mouse, fish and rabbit species and displays a high degree of inter-species conservation, (i.e., > 90 %), and no homology with the $sPLA_2$ counterparts (Clark et al., 1991). This enzyme was first purified by Leslie and co-workers from RAW 264.7 mouse macrophages (Leslie et al., 1988) and later cloned by Sharp et al (1991). The human cDNA comprises 2880 nucleotides and encodes a protein of 749 amino acids. It was found to have a predicted M.W. of 85 kDa but migrates between 100-110 kDa on SDS-polyacrylamide gels, perhaps due to a proline rich C-terminal sequence. The $cPLA_2$ promotor region contains AP-1 and NfkB transcription factor recognition sequences but has no TATA box and is therefore considered a regulated TATA-less promotor.

This phospholipase was termed cytosolic PLA_2 ($cPLA_2$) because of its predominantly cytosolic presence in resting cells. Unlike the $sPLA_2$ enzymes, the $cPLA_2$ counterpart contains

no known disulfide bridges and therefore is resistant to reducing agents such as DTT. Additionally, it displays a 5-10 fold preference for *sn*-2-arachidonyl-containing phospholipid substrates with the following phospholipid preference: PC=PI>PE>PA>PS (Hanel et al., 1993).

1.4.6 *Calcium requirement of cytosolic phospholipase A_2*

A role for intracellular calcium in $cPLA_2$ activation could be subsequently inferred from studies which demonstrated that the addition of calcium ionophore to cells caused the release of arachidonate (Hassid, 1981). Indeed, the amino acid sequence contains a stretch of 68 amino acids which encompass a domain with striking similarity to the Ca^{2+} lipid-dependent binding domain (CaLB) or C2 region of the conventional Ca^{2-}-dependent PKC isoforms, PLC and GTPase-activating protein (Clark et al., 1991; Sharp et al., 1991). The binding of Ca^{2+} to this region likely facilitates the enzyme's association with lipid membranes since such association with membranes has been demonstrated *in vitro* at calcium concentrations between 300 and 700 nM (Nalefski et al., 1994). Translocation of the enzyme to membrane also occurs *in vivo* as can be appreciated from the fact that, for example, certain stimuli such as thrombin and INF-γ while initiating Ca^{2-} mobilization, cause a redistribution of the $cPLA_2$ from the cytosol to the membrane fraction in human platelets (Kramer, 1994) and bronchial epithelial cells (Wu et al., 1994), respectively. The role played by calcium with respect to $cPLA_2$ contrasts that for the $sPLA_2$ enzymes. For the $sPLA_2$ family, Ca^{2+} acts as a catalytic cofactor by binding to the conserved Ca^{2-}-binding loop, thereby stabilizing an important transition state.

In contrast, Ca^{2+} does not appear to be necessary for catalysis by $cPLA_2$, but instead functions to increase the enzyme's affinity for the phospholipid. This is supported by the observation that high salt concentrations result in greater activity even in the absence of Ca^{2+} (Reynolds et al., 1993). It was recently demonstrated that Ser228 on the $cPLA_2$ enzyme is required for catalysis (Huang et al., 1996; Kramer et al., 1994). More recently, the N-terminal CaLB domain of $cPLA_2$ has been shown to be essential in this process of association with membranes since a mutant lacking this portion of the enzyme, when overexpressed in CHO cells, failed to bind to membranes and remained in the cytosol (Nalefski et al., 1995).

1.4.7 *Phosphorylation requirement of cytosolic phospholipase A_2*

The catalytic activity of $cPLA_2$ can be enhanced through phosphorylation of specific serine residues such as Ser505 which lie within a mitogen-activated protein kinase (MAPK) phosphorylation consensus sequence (Lin et al., 1993). This property was suggested by Lin and Wartmann (1993) who examined overexpressed mutant forms of $cPLA_2$, in which Ser505 was replaced by Ala. Despite displaying a basal *in vitro* phospholipase activity, these forms failed to show increased activity in cell lysates derived from cells stimulated with agents which activate MAPK. Further evidence for phosphorylation-induced activation was obtained by Kramer et al (1993) who observed an increased phosphorylation of this phospholipase in thrombin-stimulated platelets. This modification resulted in an increased V_{max} while substrate affinity (K_m) and Ca^{2+}-requirement remain unchanged.

In summary, it can be concluded from the evidence at hand that it is $cPLA_2$ and not a $sPLA_2$ or other PLA_2 which is involved in receptor-initiated AA release. In fact, when $sPLA_2$ was overexpressed in CHO cells, there was no increased responsiveness to receptor activation while those cells overexpressing $cPLA_2$ increased their ability to release AA in response to both thrombin and ATP (Lin et al., 1993). The salient features of $cPLA_2$ which make this enzyme a suitable effector in signal transduction are its positional specificity, its ability to translocate to the membrane in response to a Ca^{2+} signal, and its sensitivity to increased phosphorylation following elevated stimulation of the cell.

1.5 Signalling elements mediating bradykinin-stimulated arachidonic acid release

1.5.1 *Early evidence for phospholipase A_2 activity in MDCK cells*

At the time this project was initiated, studies by Daniel and co-workers had implicated, in MDCK cells, the involvement of a yet undetermined subtype of PLA_2 in the release of AA induced by either phorbol ester or calcium ionophore. This conclusion was based primarily upon evidence showing a PMA-enhanced release of AA from phospholipids (i.e., PE and PC), with comparatvely little release of palmitate (Daniel et al., 1981). It will be recalled that AA resides mostly in position *sn*-2, whereas palmitic acid is found mainly in position *sn*-1. More recently, Slivka and Insel (1988) demonstrated that in MDCK-D1 cells, BK can increase the production of both lyso-PC and lyso-PE, confirming the involvement of a PLA_2 activity in stimulated cells. *However, the identity of the specific PLA_2 subtype directly responsible for AA release subsequent to BK stimulation of MDCK-D1 cells was still uncertain prior to*

initiation of this research project.

1.5.2 *The involvement of the phosphoinositide pathway*

The specific signalling pathways initiated by various agents interacting with their putative receptors which ultimately culminate in the release of arachidonate are only now being defined. That which is initiated through the interaction of BK with the B_2-receptor, has not been unequivocally determined. Many of the early studies dealing with AA release were based on the assumption that the Ca^{2-} signal generated subsequent to $InsP_3$ production was absolutely required for agonist-mediated AA release. However, work by Slivka and Insel (1988) produced evidence indicating that BK-stimulated PIP_2 hydrolysis and AA release are mediated through two non-interdependent pathways in MDCK-D1 cells since neomycin sulfate, an inhibitor of PI-PLC, failed to block BK-mediated AA release. Furthermore, while short-term PMA pretreatment effectively blunted BK-mediated $InsP_3$ production, its presence potentiated the ability of BK to release AA in MDCK-D1 cells. On the other hand, preliminary evidence obtained by the present author had indicated that the PLC inhibitor, U73122, could blunt BK-enhanced release of AA. *Thus the question as to the involvement of PI-PLC remained incompletely resolved. Also the possibility existed that other phospholipases stimulated by BK may provide second messengers required for signalling pathways leading to AA release.*

1.5.3 *Phospholipase D*

Phospholipase D is an enzyme which acts upon its phospholipid substrate to generate PA as one of its products. Phospholipase D was first recognized as a major enzyme involved in signal transduction when it was discovered that not only did accumulation of PA sometimes preceed that of DAG, but also that many agonists caused rapid activation of transphosphatidylation (Exton, 1990; Billah and Anthes, 1990). This latter reaction, which does not occur under normal conditions, results in the transfer of the phosphatidyl group of phospholipids to a primary alcohol such as ethanol added to the medium. It has been conveniently utilized as an indicator of PLD activity, through the measurement of phosphatidylethanol (PEt) production. The detailed study of this enzyme has been hampered by the fact that it has yet to be purified to homogeneity and is consequently not cloned. However, many of its characteristics have been elucidated through *in vitro* observations. The accumulated data suggest that different PLD isozymes exist, and that these vary in their pH optima, responses to cations, subcellular distribution, and substrate specificity. In MDCK wildtype cells, two forms of PLD have been identified, a PI-selective form, and a PC-specific form (Huang et al., 1992). The mechanisms by which receptor agonists activate PLD are poorly understood. Evidence existing for the involvement of G-proteins includes the formation of PEt in response to non-hydrolyzable GTP analogues in cell-free systems (Kusner et al., 1993; Agwu et al., 1989). In contrast, phorbol esters are known to increase PLD activity in intact cells, thereby suggesting a role for PKC. Indeed, work by Balboa et al (1994) has demonstrated that PLD activity within purinergically-stimulated MDCK-D1 cells is dependent

upon PKC, specifically, the α isoform. More recently, the substrates which are hydrolysed by PLD following G-protein coupling and PKC activation were found to differ (Huang et al., 1995). Specifically, phorbol ester-stimulated PLD activity preferentially targeted ether glycerophospholipids while G-protein regulatory PLD activity preferred hydrolysis of diacyl ester subclasses. Thus activation of PKC- and G-protein-mediated phospholipases D likely supply distinct forms of PA and subsequently DAG (i.e., if the PAP pathway is involved) for further signalling within the cell. Interestingly, the alkyl-DAG species are ineffective towards PKC while acyl-DAG analogues are significantly active (Daniel et al., 1993). Finally, extracellular Ca^{2+} appears to be important for BK-stimulated PLD activity in MDCK wildtype cells as reported by Huang et al (1992).

The role of PLD with regard to AA liberation has recently been examined in other cell types. Indeed, the product of PLD, PA itself, may be involved in mediating AA release as demonstrated in both macrophages and platelets (Fernandez et al., 1994; Sato et al., 1992). Alternatively, the PA produced by PLD activation can be converted by PAP to DAG, as demonstrated in rat peritoneal mast cells can be further degraded by DAG-lipase to yield free fatty acids, including AA (Ishimoto et al., 1994). *The fate and mode of action of PA in MDCK-D1 cells were not clear at the time this project was initiated, and similarly, the role played by PLD in response to BK, in contributing to AA release had not yet been explored in this cell line.*

1.5.4 *Phosphatidylcholine-specific phospholipase C*

Phosphatidylcholine-specific phospholipase C catalyzes the hydrolysis of the glycerophosphoryl bond of PC to yield both DAG and phosphorylcholine (Pchol) as products. The PC-PLC enzyme has been implicated in cholecystokinin's ability to generate DAG from longitudinal intestinal smooth muscle cells (Murthy and Makhlouf, 1995). The generation of Pchol was detectable within 5 sec of stimulation of these cells. The activation of PC-PLC may depend upon either direct G-protein coupling or PKC recruitment since Barnett et al (1993) were able to demonstrate a PKC-independent production of Pchol following angiotensin stimulation of mesangial cells, while Cybulsky and Cyr (1993) presented data showing that complement C5b-9 -mediated PC-PLC activity is blunted by down regulation of PKC by phorbol esters. In light of studies with epinephrine as stimulus, a role for PC-PLC in providing the DAG for activation of PKC in MDCK-D1 cells was demonstrated (Slivka et al., 1988), while the role of this phospholipase in mediating BK's effects remains unexplored. *The PLC form (i.e., PI-PLC vs. PC-PLC) responsible for generating DAG during the time frame of AA release in MDCK-D1 cells in response to BK remained to be identified.*

The importance of generating DAG in signalling has been understood so far mainly in terms of its ability to activate PKC. Reports indicated however that DAG can increase *in vitro* PLA_2 activity (Bass et al., 1987; Kramer et al., 1987). *Thus the role of PC-PLC and its product , DAG, remained to be elucidated especially in MDCK cells where very little was known in this respect prior to commencement of the present studies. Stated in general terms,*

an important study to be undertaken was to determine whether or not PLC and PLD were involved in mediating BK-stimulated AA release in MDCK cells and to elucidate the mechanisms of such involvement.

1.5.5 *The role of protein kinase C, unresolved*

Originally discovered in 1977 by Nishizuka and coworkers as a histone protein kinase from rat brain, PKC represents a large family of serine/threonine kinases. All PKC isoforms can be structurally defined as possessing regulatory and catalytic domains separated by a variable hinge region. The various isoforms within this family of serine/threonine kinases are subdivided into 3 major categories; 1) conventional isoforms (α, βI,βII, and γ) which require Ca^{2-}, PS, and DAG for membrane association and catalysis; 2) non-conventional isoforms (i.e., novel) (ε,η,θ,σ,μ and δ) which lack the second conserved (C2) region, responsible for Ca^{2+} -binding; and 3) atypical PKC isoforms (ζ,λ) which lack part of the C1 domain in addition to the C2 region, rendering this enzyme both DAG- and Ca^{2+}-independent. MDCK-D1 cells express an abundance of both α and βII PKC, and a lesser amount of PKC βI, while no γ isoform has been detected (Godson et al., 1990). Balboa et al (1994) have also demonstrated the presence of δ and ε forms of PKC. Phorbol esters are efficient and potent activators of both conventional and novel PKC isoforms, and have been frequently used to probe the involvement of PKC in a variety of signalling pathways. These activators are believed to bind to the cysteine residues along the C1 region of the PKC enzyme and to act by mimicking the action of DAG (Gschwendt et al., 1991). The binding of intracellular Ca^{2-} to the C2 region of the

conventional PKC isozymes causes the translocation of these proteins to the plasma membrane where they are activated following binding to DAG and phosphatidylserine (PS). It is interesting to note however, that Godson et al (1991) have shown that greater than 40% of PKC activity in MDCK-D1 cells is independent of Ca^{2+}.

As mentioned earlier, there exists substantial data showing that agents which mimic the action of DAG (i.e., PMA) and thereby activate PKC, can increase AA release in MDCK after incubations > 15 min, as well as in other cell types. However, evidence for the involvement of PKC in BK's ability to induce AA release is conflicting. Studies using a variety of rather non-specific chemical inhibitors of PKC have yielded opposing conclusions regarding the involvement of this enzyme. For example, H7 and staurosporine, failed to abrogate BK-stimulated AA release in MDCK-D1 cells (Weiss et al., 1989), while high concentrations of sphingosine only partially blocked this effect (Robinson et al., 1995). In contrast, Godson and co-workers (1993) reduced the expression of PKCα by an antisense strategy resulting in a significantly decreased PMA-stimulated release of AA. This study therefore implicates the α isoform in the PLA_2 signalling pathway initiated by phorbol esters. However, the ability of BK to elicit AA release from these antisense-transfected cells was not tested, and therefore leaves unresolved, the role of PKC α with respect to BK-mediated AA release. This question of PKC involvement has been further complicated by studies which employ long-term incubations with phorbol ester to down regulate PKC. The mechanism of down regulation proceeds as the sustained presence of PMA causes PKC to remain associated with the cell membranes; this is

followed by progressive proteolytic cleavage of the enzyme until insignificant levels of PKC remain. Godson et al (1990) showed that this method effectively blocked greater than 50% of the BK-stimulated AA release, thereby suggesting a role for PKC in the nonapeptide's ability to release AA in MDCK-D1 cells. *With these two opposing conclusions at hand, a more in depth investigation was required to accurately assign a role for PKC with respect to BK-stimulated AA release in MDCK-D1 cells.*

2 HYPOTHESIS

Bradykinin-stimulated AA release is contingent upon the recruitment of $cPLA_2$ to the membranes of MDCK-D1 cells following the influx of extracellular Ca^{2+} as well as upon the formation of DAG and PA by PC-PLC and PLD respectively. These products enhance the ability of the acylesterase enzyme to access its substrate. Additionally, activation of PKA leads to a reduction of AA release by lowering $cPLA_2$ activity.

3 OBJECTIVES

In MDCK-D1 cells:

1) to identify the enzyme required for BK-induced AA release

2) to elucidate early steps in BK-initiated signal transduction pathway upon which the subsequent release of AA is contingent.

3) to reveal more clearly possible cross-talk mechanisms involving the action of protein kinases on AA release.

4 APPROACH

Several strategies were employed in order to accomplish the objectives.

A) To identify the enzyme responsible for AA release in MDCK-D1 cells, *in vitro* PLA_2 activity of cell lysates was assayed over a range of Ca^{2+} concentrations in the presence or absence of DTT, or following immuno-depletion of these lysates with a polyclonal $cPLA_2$ antibody. Furthermore, BK-stimulated cells were assessed for the increased presence (i.e., translocation) of $cPLA_2$ within the membrane fraction.

B) To determine the role of phospholipase and lipase activities suspected of involvement in the signalling pathway for AA release cells were treated with selective inhibitors and effects, upon BK-stimulated AA release in cell cultures, were assessed.

C) To determine the role of PKC in modulating $cPLA_2$ activity the effect of PKC inhibitor, phorbol ester and PKC down regulation on BK-enhanced release of AA was examined.

D) To determine potential targets of PKA, intracellular cAMP levels were elevated with either AVP or FSK, an activator of adenyl cyclase plus IBMX, a phosphodiesterase inhibitor and the resulting effects, upon various products of BK-stimulated enzymes were, determined.

5 MATERIALS AND METHODS

5.1 Materials

Phosphatidyl choline-1-stearoyl-2-arachidonyl, neomycin sulfate, BK, bovine serum albumin (BSA), FSK, IBMX, A23187, dBucAMP, (1-stearoyl-2-arachidonyl) phosphatidic acid (PA), 1-stearoyl-2-arachidonyl-*sn*-glycerol, 3-heptanone, sodium tetraphenylboron, sodium fluoride (NaF), sodium orthovanadate ($NaVO_3$), and staurosporine (SSP) were obtained from Sigma Chemical Co. (Mississauga, ON)

Radiochemicals, [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid, Sp. Act.7.81 TBq/mmol, D-myo-[^{3}H]inositol, Sp. Act. 3.89 TBq/mmol, D-myo-[^{3}H]inositol 1,4,5-trisphosphate, Sp. Act. 2.2 TBq/mmol, [methyl-^{3}H]choline chloride, L-3-phosphatidylcholine,1-stearoyl-2-[5,6,8,9,11,12,14,15-^{3}H]arachidonyl, and adenosine 5'-[α-^{32}P]triphosphate, along with biodegradable counting scintillant (BCS) and aqueous counting scintillant (ACSII) in addition to the Western blotting materials, Hyperfilm, ECL reagents, Hybond nitrocellulose and anti-rabbit Ig horseradish peroxidase-linked secondary antibody were purchased from Amersham Life Science Co. (Oakville, ON) . [9,10-^{3}H] Palmitic acid was purchased from Dupont Canada Ltd. (Mississauga, ON). Protein A-agarose was obtained from Boehringer Mannheim Canada (Laval, QB).

Bisindolylmaleimide I (BIS), and SK&F 96365 (1-{β-[3-(4-

methoxyphenyl)propoxyl]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride) were purchased from Calbiochem-Novabiochem International (San Diego, CA).

All other organic compounds and solvents were procured from BDH Co. (Toronto, ON). The rabbit polyclonal antibody to $cPLA_2$ as well as the purified $cPLA_2$ standard were kind gifts of the Genetics Institute, Boston, MA. The rabbit polyclonal antibody to phosphoserine was a product of Dimension Laboratories Inc. (Mississauga, ON). Phorbol-12-myristate-13-acetate (PMA), phosphatidylethanol, (±) propranolol, {N-[2-((3-(4-bromophenyl)-2-propenyl)-amino)-ethyl]-5-isoquinolinesulfonamide dihydrochloride}(H-89), $AACOCF_3$, RHC80265, and D609 were supplied by Biomol Research Laboratories (Plymouth, PA). Whatman HPTLC plates were obtained from Chromatographic Specialties Inc. (Brockville ON).

5.2 Methods

5.2.1 *Cell culture*

The MDCK-D1 cell line used throughout was a generous gift of Dr. Paul Insel, University of California, La Jolla, California. The D1 cells were used because they represent a well-characterized clone isolated from wildtype MDCK cultures which are believed to contain more than one cell type (Meier et al., 1985). Cells were cultured in 150mm Petri dishes or flasks containing 20mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% antibiotic/antimycotic solution (GIBCO), 15mM HEPES and 3.7g/L sodium bicarbonate. Stock cultures were maintained at 37°C in an atmosphere of 5% CO_2 and were passaged before reaching confluence.

For experiments, cells were diluted 1:100 and added to either 6-well Falcon dishes with permeable inserts (for measurement of InsP , $InsP_3$, or DAG), or to regular 12-well cluster dishes (for measurement of AA, choline (Chol) or Pchol release), or to regular 6-well dishes (for determination of PLD products, PA or PEt), or to 100 x 20mm Petri dishes (for PKC assays, PLA_2 activity assays). The latter dishes were also used for preparing cell culture lysates required for Western blotting or immunoprecipitations. Finally, diluted cultures were also applied to 20 mm glass coverslips for Ca^{2+} signal measurements. Except when experiments involved inositol phosphate determinations, cultures were used prior to reaching confluency. Due to the low amount of inositol phosphate production in this clone of MDCK, Falcon inserts were used to optimize the access to the basolateral surface where B_2-receptors are also

located (Simmons, 1992).

5.2.2 *Measurement of inositol phosphate production*

In this case cells grown in Falcon inserts were used after 1 day of culture when approximately 50% confluency had been reached. After washing 3 times with medium containing no inositol, the cells in the upper chamber were subsequently incubated for up to 72 hours with inositol-free medium containing 10% dialyzed FCS and 7.5 μCi of [^{3}H]myo-inositol (5μCi/mL) (Slivka and Insel, 1987). Two mL of inositol-free DMEM, without labelled precursor, was added to the lower chamber. Upon completion of the incubation, confluency had been attained and cultures were washed 3 times with DMEM containing inositol but no serum and preincubated for either 0, 20, or 60 min. with selected agents (10μM FSK + 0.25-0.5mM IBMX, 1mM dBucAMP, 1μM AVP, or 30μM H-89). After preincubation, cells were incubated another 40 min in DMEM containing 50mM LiCl and appropriate agents added individually or in combination (FSK + IBMX, dBucAMP, AVP, H-89, 1μM BK, or 10μM A23187). Controls contained equal amounts of vehicle (i.e., < 0.2% DMSO or ethanol). Reactions were terminated by aspiration of the medium, excision of the Falcon insert and its immersion into a safelock 1.5 mL microfuge tube containing an ice-cold mixture of chloroform- methanol- H_20 (0.5 mL:0.5 mL:0.4 mL). In initial studies, inositol phosphates were separated by conventional ion exchange column chromatography as described herewith but in similar studies involving much shorter stimulation periods, separations relied on high performance liquid chromatography (HPLC) as described in the next section. After Vortex

mixing and centrifugation at 5000*g* for 2 min, the aqueous upper phase was applied to a 1 mL slurry of AG 1-X8 resin (100-200 mesh, formate form) contained in a 10 mL BIO RAD polypropylene column. The column was drained and washed with 30 mL of H_2O (to remove free [^{3}H]myo-inositol). Labelled glycerophosphoinositol (GroPIns) was eluted with 10mL of 5mM disodium tetraborate/60mM sodium formate while radioactive inositol monophosphate (InsP) was recovered with the addition of 200 mM ammonium formate/100 mM formic acid (Garg et al., 1988). A 2 mL aliquot was taken from each eluate and counted in 10 mL of BCS, (Amersham), with a Beckman LS 1701 spectrometer. The number of counts in the released products was normalized for total incorporation of the tritiated label into each culture following solubilization of the cells in 5% SDS for 2 hrs at 37°C.

Measurement of $InsP_3$ production was performed after 10 sec of stimulation with BK. The inositol phosphates were extracted as outlined above. These extracts were then frozen at -85°C, lyophilized and redissolved in 20 μL of 10 mM ammonium phosphate. Inositol trisphosphate was separated on a Whatman Partisil SAX High Performance Liquid Chromatography (HPLC) column with a stepwise elution gradient of ammonium phosphate as described (Dean and Beaven, 1989). The flow rate was maintained at 1 mL/min with a maximum pressure of 1 Kpsi. The $InsP_3$ fraction was well separated from other inositol phosphates and eluted in a period between 39 and 43 min as identified with authentic tritiated 1,4,5-$InsP_3$ standard. One mL aliquots of eluate were counted in 10 mL of BCS.

5.2.3 *Measurement of diacylglycerol*

Cells were grown on Falcon cell culture inserts in DMEM containing 10% FCS. Confluent monolayers were washed 3 times with serum-free DMEM and subsequently preincubated in this medium for 0, 20 or 60 min with selected agents 10 μM FSK + 0.5 mM IBMX (20 min), 30 μM H-89 (60 min) or with vehicle alone. Following preincubation, monolayers were treated 40 min. with either FSK + IBMX, H-89, BK, various combinations of these agents or vehicle control. Reactions were terminated by aspiration of the medium, excision of the Falcon insert and its immersion into a 15 mL polypropylene centrifuge tube containing 3.8 mL of an ice-cold mixture of chloroform- methanol-1.0 M NaCl (1:2:0.8 v/v/v). Tubes were then vigorously mixed for 30 sec, after which time 1 mL of chloroform and 1 mL of 1.0 M NaCl were added to separate the phases. Following centrifugation at 5000*g* for 2 min, the lower organic phase was removed and dried under nitrogen. The lipid extract was redissolved in 20μL of chloroform-methanol (95:5 v/v) and analyzed for DAG by the method of Kennerly et al (1989) as adapted from Preiss et al (1986), using an Amersham kit. Accordingly, the DAG was converted to [^{32}P]PA with DAG kinase and the separation of [^{32}P]PA was achieved by high performance thin layer chromatrography (HPTLC) on activated Whatman 60 plates developed with chloroform: methanol: acetic acid (65:15:5). An autoradiogram of the HPTLC was taken to locate radiolabelled PA (identified with authentic PA as standard) which was subsequently scraped into scintillation vials and counted in 10 ml ACS II (Amersham).

5.2.4 *Measurement of cyclic adenosine monophosphate*

Cells were grown as described for DAG measurement; however, 12-well cluster dishes were used instead of the Falcon membrane system. Incubations were carried out for 20 or 60 min in serum-free DMEM containing 0.5 mM IBMX and other agents as specified. Reactions were terminated with the addition of ice cold 10% trichloroacetic acid and after 30 min at 4°C, each sample was washed four times with 4 volumes of water-saturated diethyl ether and subsequently brought to pH 7.4 with 1M Tris base. The cAMP was then determined with an Amersham kit making use of a specific cAMP-binding protein (Brown et al., 1971). Each sample was normalized for cellular protein content by solublizing the cells with 0.8 N NaOH overnight at 37°C. Immediately following, the solublized protein was determined using a modified Lowry method with BSA as standard.

5.2.5 *Measurement of arachidonic acid release*

Subconfluent MDCK-D1 grown in 12 well cluster dishes were washed 3 times with 0.05% BSA (w/v) in DMEM and labelled with 0.3μCi [^{3}H]AA for 24 hrs in DMEM containing either 0.05% BSA or 0.5% fetal calf serum. Following this labelling period, cells were washed 3 times with Hank's Balanced Saline Solution (HBSS) containing 0.05% BSA. When the effect of short term PMA treatment was to be tested, the cells were preincubated with a 100 nM concentration of this phorbol ester in HBSS for 10 min at 37°C, or alternatively, when the effect of an inhibitor on AA release was to be tested, the cells were preincubated with appropriate concentrations of inhibitor in HBSS for the appropriate period of time at 37°C,

after which the medium was aspirated and replaced with fresh HBSS containing 1 μM BK, 10μM A23187 and a similar concentration of either PMA or inhibitor. Control conditions without activating agents and /or inhibitor were similar. After 10 min of incubation, the medium was removed, centrifuged at 5000*g* for 5 min to pellet dislodged cells and a 0.5 mL aliquot was routinely taken for counting to determine total [^{3}H]eicosanoid released. The remaining 0.5 mL of media was acidified to pH 3.0 with phosphoric acid and extracted 3 times with an equal volume of ethylacetate. The combined extracts where dried under a stream of nitrogen, redissolved in 20 μL of ethylacetate and the AA metabolites, separated on HPTLC in a solvent system consisting of ethyl acetate : acetic acid (99:1 v/v) (Hassid, 1983). Arachidonic acid and PGE_2 were identified by cochromatography with authentic standards. The bands containing AA and PGE_2 were scraped into scintillation vials and counted in 10 ml of ACSII. Approximately 20% of the counts released into the medium were identified as PGE_2, while greater than 60% were associated with the arachidonate fraction. The remaining 20% of the counts were unidentified metabolites. The changes in production of PGE_2 paralleled those of the AA released but are not reported herein.

In early experiments dealing with AA release, conditions differed slightly from those of later experiments. In the former case, there was the use of DMEM as opposed to HBSS for both preincubations and incubations. Also, incubations carried out in DMEM were performed at room temperature instead of 37°C. It was subsequently determined that only the quantitative but not the qualitative aspects of the results were altered by adopting the later

conditions. These details are noted in the figure legends of the results section, where appropriate.

5.2.6 *Measurement of phosphatidic acid and phosphatidylethanol production*

Cells grown in 6-well cluster dishes were washed 3 times with DMEM and labelled with 2.0μCi [^{3}H]palmitic acid for 24 hrs in DMEM containing 0.5% fetal calf serum. Following the labelling period, cells were washed 3 times with HBSS containing 0.05% BSA and preincubated for 30 min with the appropriate inhibitor in HBSS as required. Preincubation medium was then aspirated and replaced with fresh HBSS containing 2% ethanol, 1μM BK and inhibitor. Ethanol was added to assess the transphosphatidylation activity of PLD as measured by PEt production. Alternative conditions without BK and/or EtOH were set up in a similar manner. After 1 min of incubation at 37° C, the medium was aspirated and the cells were scraped off the dish in 1.5 mL of ice-cold methanol containing 1% HCl. The lipids were extracted according to the method of Bligh and Dyer (1959) and [^{3}H]PA and [^{3}H]PEt were separated by HPTLC with a solvent system obtained from the upper phase of ethyl acetate/isooctane/acetic acid/water (130:20:30:100 v/v) (Liscovitch and Amsterdam, 1989). The products were identified by cochromatography with authentic standards and counted in 10 mL of ACSII.

5.2.7 *Measurement of labelled choline products*

MDCK-D1 cells grown in 12-well cluster dishes were washed 3 times with DMEM

and labelled with 1.0 μCi [methyl-^{3}H]choline chloride for 48 hrs in DMEM containing 1% fetal calf serum. After labelling, cells were washed 3 times with HBSS containing 0.05% BSA and preincubated for 30 min with the appropriate inhibitor in HBSS as required. Preincubation medium was then aspirated and replaced with fresh HBSS containing the desired inhibitors and 1μM BK. Control medium was free of BK and/or inhibitor. Following 1 min of incubation at 37°C, the medium was removed and centrifuged at 5000 g for 5 min to pellet dislodged cells. An aliquot of the supernatant was taken and extracted in a biphasic solvent mixture to separate labelled Pchol and Chol, the products of PC-PLC and PLD respectively, according to Fonnum (1969). Briefly, 400 μL of supernatant was removed and extracted with 200 μL of distilled water and 600 μL of tetraphenylboron in 3-heptanone (75mg/mL). The upper organic phase, containing [^{3}H]Chol, was dried under a stream of nitrogen to reduce quenching and counted in 10 mL of BCS. The lower aqueous phase, containing [^{3}H]PChol was counted in 10 mL of BCS. HPTLC was used to assert the identity of the products in each phase, demonstrating that contamination of phases was less than 10%, while >95% of the counts released into the medium were accountable by [^{3}H]Chol and [^{3}H]PChol.

5.2.8 *Protein kinase C activity assay*

Cells grown to approximately 75% confluency on 100 x 20mm Petri dishes were washed 3 times with DMEM and incubated for 24 hrs with DMEM containing 0.5% fetal calf serum. The following day the cells were again washed, 3 times with HBSS containing 0.05% BSA (w/v), and preincubated for 30 min with or without 50μg/mL of D609. After

preincubation, medium was aspirated and cells were stimulated for 1 min at 37°C with 1μM BK in the presence or absence of D609. Stimulation was terminated by aspirating the medium and washing the cells 2 times with ice-cold phosphate-buffered saline (PBS). They were then scraped off the plate with a rubber policeman in 1 mL of PBS and centrifuged at 1000*g* for 3 min to pellet the cells. The PBS was removed and the pellets were run through 3 cycles of freeze/thawing at -85°C to rupture cell membranes. Cells were then resuspended in lysis buffer containing 1 mM $NaHCO_3$, 10 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF, 20 μg/mL leupeptin, 5 mM NaF, 5 mM benzamidine and 10 mM $NaVO_3$ and vortexed for 2 minutes. Tris HCl, pH 7.5, and ethylenebis(oxyethelenenitrilo)tetraacetic acid (EGTA) were added to a final concentration of 50 mM and 5 mM, respectively. This suspension was then centrifuged at 1000*g* for 10 min to pellet nuclei and unbroken cells. The supernatant was removed and centrifuged at 100,000*g* for 60 min to obtain cytosolic and membrane fractions. The high-speed pellet was resuspended in lysis buffer containing Tris/EGTA, pH 7.5, and the protein determined using the BIO RAD microassay procedure with BSA as standard. The membrane PKC activity was determined with an Amersham kit, the methodology of which is based on the phosphorylation of a PKC-specific peptide in the presence of Ca^{2+}, phosphatidylserine, and phorbol ester, and is a modification of a mixed micelle assay. The assay was performed at 37°C for 15 min with 1μg of protein from each sample. The results are expressed as the number of pmoles of phosphate transferred to the peptide per mg of protein assayed per min. The amount of endogenous phosphorylation under each condition in the absence of added peptide was used as a control and is subtracted from each experimental value. Under these conditions, the α,β,

and γ isoforms of PKC could be assayed for activity, if present in the MDCK-D1 cell line.

5.2.9 *Phospholipase A_2 activity assay*

To obtain a total cell lysate containing PLA_2 activity, MDCK-D1 cells were cultured on 150 x 20mm plastic dishes and subsequently washed twice with ice-cold 100mM Tris HCl, pH 7.4, containing 250mM sucrose and 1mM EGTA. Cells were then scraped off the plates, centrifuged at 1000*g* for 5 min and the pellet was resuspended in sonication buffer (100 mM Tris HCl, pH 7.4, 250 mM sucrose, 10 μg/ml leupeptin, 1 mM PMSF, 10 mM $NaVO_3$, 50 mM NaF, 10 mM sodium pyrophosphate, 5 μM phosphoserine, 1 mM DTT, 1 mM EGTA, and 1 mM EDTA). The cell suspension was then sonicated on ice 3 times for 5 sec with 15 sec intervals in between each pulse to maintain ice-cold conditions. For this purpose, an Ultrasonics cell disruptor fitted with a small probe was used with a setting of 5. Cell lysates were then centrifuged for 5 min at 1000*g* to pellet unbroken cells. Supernatants were normalized for protein determined by BIO RAD micro-assay with BSA as standard.

To determine the effect of long-term incubation with PMA on PLA_2 activity, cells were grown as described above, but incubated for 20 hrs with or without 100 nM PMA in DMEM containing either 0.05% BSA or 0.5% FCS. This procedure was shown to down regulate PKC in MDCK-D1 cells (Godson et al., 1991). Incubations were terminated by washing the cells 2 times with ice-cold Tris buffer and cells were processed as described above.

To obtain a PLA_2-containing cytosolic fraction in which the $[Ca^{2+}]$ could be precisely controlled, MDCK-D1 cells were cultured in 150 x 20mm plastic dishes and subsequently washed 2 times with ice-cold PBS. Cells were then scraped off the plates, centrifuged at 1000*g* for 5 min and the pellet was resuspended in sonication buffer consisting of 100 mM Tris HCl pH 7.4, 250 mM sucrose, 10 μg/ml leupeptin, 1 mM PMSF, 10 mM $NaVO_3$, 1 mM DTT, 1 mM EGTA, and 1 mM EDTA. The cell suspension was then sonicated on ice 3 times for 30 sec with 15 sec intervals in between each pulse to maintain ice-cold conditions. The cell sonicate was then centrifuged for 10 min at 1000*g* to pellet unbroken cells. The supernatant was removed and centrifuged for 1 hr at 100,000*g* to obtain cytosolic and membrane fractions. The cytosolic fraction was subsequently dialysed 2 times for 18 hrs against 1 L of 10 % glycerol (w/v) in 100 mM Tris HCl, pH 7.4 to remove EDTA/EGTA. This step subsequently allowed the preparation of incubation media with a precise Ca^{2+} concentration. The cytosolic extract was finally adjusted to contain 30 % glycerol (w/v) to assure a better preservation of the enzymatic activity during prolonged storage at -85°C. Samples each contained a protein concentration of 0.225 mg/mL.

To determine the effect of BK on PLA_2 activity, cells were grown as described previously, but incubated for 2 min with or without 1 μM BK in HBSS. Incubations were terminated by washing the cells 2 times with ice-cold PBS. After scraping from the plate, the cells were centrifuged at 1000*g* for 5 min. Pellets were resuspended in a solution containing 50 mM Tris-HCl, pH7.4, 250mM sucrose and protease inhibitors at the aforementioned

concentrations. The suspensions were then homogenized 2 times by exerting 10 strokes in a glass homogenizer fitted tightly with a teflon pestle. After centrifugation of the homogenate at 100,000*g* for 1 hr, the pelleted fraction was resuspended in homogenization buffer containing 10 mM n-octyl-β-D-glucopyranoside and incubated for 30 min at 4°C to allow extraction of membrane proteins. The extract was then centrifuged for 1 hr at 100,000*g* and the resulting supernatant was assayed for PLA_2 activity (Paglin et al., 1993).

The PLA_2 activity was determined according to a procedure described by Leslie (1990). Briefly, the cell lysate, dialysed cytosolic fraction, or the octylglucoside membrane extract was incubated at 37°C for 1 hr with 30 μM 1-stearoyl-2-arachidonyl phosphatidylcholine as substrate (with 55,000 dpm 1-stearoyl-2-[^{3}H]arachidonyl phosphatidylcholine as tracer) in the presence or absence of 5 mM DTT. The substrate was prepared, first by removal of solvent under nitrogen followed by sonication for 3 min on ice, in incubation buffer containing 100 mM Tris HCl, pH 7.4, 250 mM sucrose, 0.5 mg/ml BSA. A range of stock calcium solutions in Tris-EGTA were prepared essentially as described by Tsien and Pozzan (1989). The incubation was terminated with the addition of 2.5 mL of Dole reagent consisting of 2-propanol/heptane/0.5 M H_2SO_4, 20:5:1 (v/v/v) (Dole and Meinertz, 1960) followed by the addition of 1.5 mL heptane containing 20 μg unlabelled AA as cold carrier. Separate phases were obtained by the addition of 1 mL of H_2O and the top phase containing free AA was removed and further purified by silicic acid column chromatography. Two mL of diethyl ether was used to wash the columns and the eluent collected in scintillation vials was

dried under nitrogen and then counted in 10 mL BCS by liquid scintillation spectrometry.

5.2.10 *Western blotting of the 85 kDa and type I forms of phospholipase A_2*

Cells grown in 100 x 20 mm Petri dishes were washed 3 times with serum-free DMEM containing 0.05% BSA and subsequently incubated overnight (approx. 20 hrs) with or without 100 nM PMA. Immediately following, cells were washed 2 times with ice-cold 50mM Tris HCl buffer, pH 7.4, containing 150 mM NaCl and 1 mM EGTA. Cells were then scraped off the plates and pelleted by centrifugation for 5 min at 1000*g*. Pellets were resuspended in lysis buffer containing 50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% Nonidet P40, 0.1% sodium deoxycholate, 1 mM PMSF, 10 mM $NaVO_3$, 50 mM NaF, 10 mM sodium pyrophosphate, 10 μg/mL leupeptin, 10 μg/mL aprotinin and 5 μM phosphoserine). Suspensions were placed on ice for 20 min and then subjected to vigorous vortex mixing for 2 min to ensure complete lysis of the cells.

For Western blotting, either 2 x Laemmli buffer, or β-mercaptoethanol-free Laemmli buffer was added until the protein content was 0.5 mg/mL. Samples were run for approximately 45 min on either 7.5% SDS-PAGE gels, or non-denaturing gels with a Bio Rad Mini Protean II apparatus set at 200 V, 60 mA, until the Coomassie blue tracking dye left the gel. Protein was transferred to Hybond nitrocellulose membranes according to the manufacturer's instructions. Blots were blocked overnight using 5% skim milk in Tris-buffered saline (TBS) and subsequently incubated for 1 hr with rabbit polyclonal $cPLA_2$ antiserum

(1:2000 dilution), or with a rabbit polyclonal type I $sPLA_2$ antibody purchased from Boehringer (1:1000 dilution) dissolved in TBS containing 0.1% Tween-20 (TTBS) and 2% skim milk. Following this, the blots were repeatedly washed with TTBS and then incubated for 45 min with an anti-rabbit horseradish peroxidase-linked secondary antibody (1:2000 dilution). The blots were then washed with TTBS as stated above, and developed using Amersham's Enhanced Chemiluminescence (ECL) system according to the manufacturer's specifications.

5.2.11 *Western blotting of $cPLA_2$ within membrane extracts derived from bradykinin-stimulated cells*

Cells grown of 100 x 20 mm Petri dishes were washed 3 times with serum-free DMEM containing 0.05% BSA and subsequently serum-starved overnight (approximately 20 hrs). Subsequently, cells were preincubated for 10 min with HBSS containing 0.05% BSA, followed by further incubation for 2 min in the presence or absence 1μM BK at 37°C. Immediately following, they were washed 2 times with ice-cold 50mM Tris HCl buffer, pH 7.4, containing 150 mM NaCl, scraped off the plates and pelleted by centrifugation for 5 min at 1000*g*. Pellets were resuspended in lysis buffer containing 50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 10 mM $NaVO_3$, 10 mM sodium pyrophosphate, 10 μg/mL leupeptin, 10 μg/mL aprotinin and 1 mM DTT. The suspensions were then homogenized 2 times by applying 10 strokes in a glass homogenizer fitted with a teflon pestle. After centrifugation of the homogenate at 100,000*g* for 1 hr, the pelleted fraction was resuspended in homogenization buffer containing 10 mM n-octyl-β-D-glucopyranoside and incubated for 30 min at 4°C to

allow extraction of membrane proteins. The extract was then centrifuged for 1 hr at 100,000g and the resulting supernatant was normalized for protein content determined by the BIO RAD microassay. Ten μg of protein from each sample was then used for Western blotting as described above.

5.2.12 *Immunoprecipitation of phosphorylated $cPLA_2$*

For immunoprecipitations, cells were prepared and lysed as described just previously. In this case however. 200 μg of cell lysate diluted into 0.5 mL of lysis buffer was precleared for 3 hrs with 25 μL of the homogeneous protein A-agarose suspension at 4°C on a rocking platform. Subsequently, 10 μL of the rabbit $cPLA_2$ antiserum was added and allowed to bind antigen for an additional 3 hrs, after which time, 25 μL of the protein A-agarose suspension was added. The incubation was continued overnight (approximately 20 hrs) at 4°C. The immuno-complexes were then collected by centrifugation, washed 2 times with lysis buffer. 2 times with lysis buffer containing 500 mM NaCl, 0.1% Nonidet P40, and 0.05% sodium deoxycholate, and finally washed once with lysis buffer containing no NaCl. The last traces of the final wash were removed with strips of filter paper, and 45 μL of gel loading buffer was added to the agarose pellet. The proteins were then denatured by boiling for 5 min in a water bath, and the 25 μL of supernatant was subjected to SDS-PAGE. Western blotting was performed as described in the previous section with the exception that a rabbit polyclonal antibody to phosphoserine was used as the primary antibody.

5.2.13 *Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$) in MDCK-D1 cells*

Calcium measurements were performed essentially as described previously by Hébert et al. (1990) with some modifications. Briefly, subconfluent, serum-starved MDCK-D1 cells grown on 20 mm glass coverslips were loaded with 5 μM acetoxymethyl ester of fura-2 (fura-2/AM) (Molecular Probes, Eugene,OR) for 1 hr at 37°C. Intracellular fura-2 fluorescence intensity was measured by use of continuous rapidly alternating excitation from dual monochromators set at 340 and 380 nm, respectively. The monochromator output was coupled to the inverted microscope. The corrected emission intensity ratio, measured from 340 and 380 nm excitations (340:380 ratio, R), was monitored continuously. After fura-2 loading and equilibration, a baseline reading of 50-100 sec was taken. The cells were then incubated with or without 10 μM SK&F 96365 for 200 sec followed by stimulation with 1 μM BK. At the end of each experiment an *in situ* calibration of $[Ca^{2-}]_i$ was performed. The HBSS bath was changed to a Ca^{2+}-and Mg^{2+}-free solution containing 2 mM EGTA and 5 μM A23187. After a stable 340:380 ratio (minimum ratio, R_{min}) was achieved, the bath was renewed with normal medium (1.8 mM Ca^{2-}) and 5 μM A23187 and the ratio was again allowed to stabilize (maximum ratio, R_{max}). 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 equal to 224 nM at 37°C.

5.3 Statistics

Student's *t*-test for unpaired data was used when only two unrelated treatment groups

were compared. To determine the statistical significance of differences between more than two groups, analysis of variance (ANOVA) and the Student-Newman-Keuls (SNK) multiple comparison test was used. Differences of $P < 0.05$ were considered statistically significant. Data are averages of duplicate determinations from individual experiments presented as means ± S.E. for groups of values where $n \geq 4$ or means ± S.D. for groups of values where $n=3$.

6 RESULTS

Part I Phospholipases and bradykinin-stimulated arachidonic acid release

6.1 Cytosolic phospholipase A_2 involvement in MDCK-D1 cells

6.1.1 *The 85 kDa form of phospholipase A_2 responsible for the in vitro phospholipase A_2 activity and for bradykinin-mediated arachidonic acid release*

The enzyme responsible for arachidonate release in response to BK stimulation in MDCK-D1 cells had not been definitely characterized. To clarify this point, the following three approaches were chosen. Firstly, the effect of $cPLA_2$ antibody on PLA_2 activity of cytosol was tested. It should be mentioned that under the conditions used for isolating this cytosolic fraction (i.e., in the presence of calcium chelating agents) more than 95% of the total PLA_2 activity could be recovered therein and negligible amounts in the membrane fraction. Results summarized in **Table 6.1** indicate that PLA_2 activity of the cytosol fraction could be blunted by some 80% with the concentration of antibody used. This polyclonal antibody is directed toward an epitope of amino acids 42-58 located within the CaLB domain of the enzyme (Clark et al., 1991). Therefore binding of the antibody could hinder Ca^{2+} -dependent association of $cPLA_2$ with the lipid substrate or the simple bulk of the antibody could interfere with the enzyme's ability to carry out its catalytic function. Regardless, on the basis of the inhibition obtained, the greater part of the PLA_2 activity recoverable in cytosolic fractions, could be attributed to the 85 kDa cytosolic form. In agreement with this is the fact that a group I $sPLA_2$ antibody had no effect on the activity (**Table 6.1**) and Western blot analysis of cell extracts failed to reveal

Table 6.1 **Effect of cytosolic phospholipase A_2 and secreted phospholipase A_2 antibodies upon *in vitro* phospholipase A_2 activity**

Cell lysates (22.5 µg of protein) were assayed for PLA_2 activity according to Leslie (1990). The values are the means ± S.E.M. of 4 independent experiments performed in duplicate. [a]$P < 0.001$ vs control.

Conditions	PLA_2 activity ± S.E.M. (pmoles x mg^{-1} x min^{-1})
Control	49.3 ± 6.5
anti-$cPLA_2$	8.7 ± 1.1[a]
anti-$sPLA_2$	48.2 ± 8.8

group I $sPLA_2$ in this cell line **(Figures 6.1 and 6.2)**. Furthermore, inclusion of DTT in the PLA_2 activity assays performed had no effect on the results. Secondly, a specific inhibitor of $cPLA_2$ was used to test its effect on BK-induced AA release *in vivo*. The agent, $AACOCF_3$, is an inhibitory arachidonate analogue which displays strong preference for the cytosolic form of PLA_2 over the 14 kDa secreted form (Riendeau et al., 1994). As shown in **Figure 6.3**, $AACOCF_3$ caused an inhibition of BK-stimulated AA release of greater than 95%. It can be added that the involvement of DAG lipase in AA release, an enzyme which in the course of this study was detected in MDCK-D1 preparations, appeared unlikely since the specific inhibitor, RHC80267, was without effect on BK-stimulated AA release. This last finding agrees with that of Slivka and Insel (1988). A role for the 40 kDa, Ca^{2-} -independent PLA_2 also seemed remote since HELSS, a potent inhibitor of this enzyme (Hazen et al., 1991), was without effect on BK-induced AA release. In order to detect or further preclude the presence of a Ca^{2+}-independent form(s) of PLA_2, lysates derived from MDCK-D1 cells were tested for Ca^{2-} requirement of their activity. Cytosolic extracts were prepared and dialysed to remove lysis buffer EGTA after which precise concentrations of calcium were added to the assay medium to obtain a range of $[Ca^{2-}]$. As shown in **Figure 6.4**, maximal PLA_2 activity (i.e., 50.4 ± 0.8 pmoles x mg^{-1} x min^{-1}) was obtained with 1μM Ca^{2+} while very low activity was observed with EGTA re-added without calcium (i.e., 10.5 ± 4.4 pmoles x

Figure 6.1 Western blotting of cytosolic phospholipase A_2 and type I secreted phospholipase A_2 of MDCK-D1 cell lysates

MDCK-D1 cell lysates were processed using a 7.5 % non-denaturing PAGE gel, and probed with a rabbit polyclonal $cPLA_2$ antiserum (lanes 1-3) or a rabbit polyclonal type I $sPLA_2$ antibody (lanes 4-7). The blots were visualized using the Amersham ECL system. Lanes 1-3 were loaded respectively with 5, 7.5, and 10 µg, of cell lysate protein. Lanes 4-6 were loaded with the same samples as reported for lanes 1-3, while lane 7 contained 50 ng of standard porcine group I $sPLA_2$.

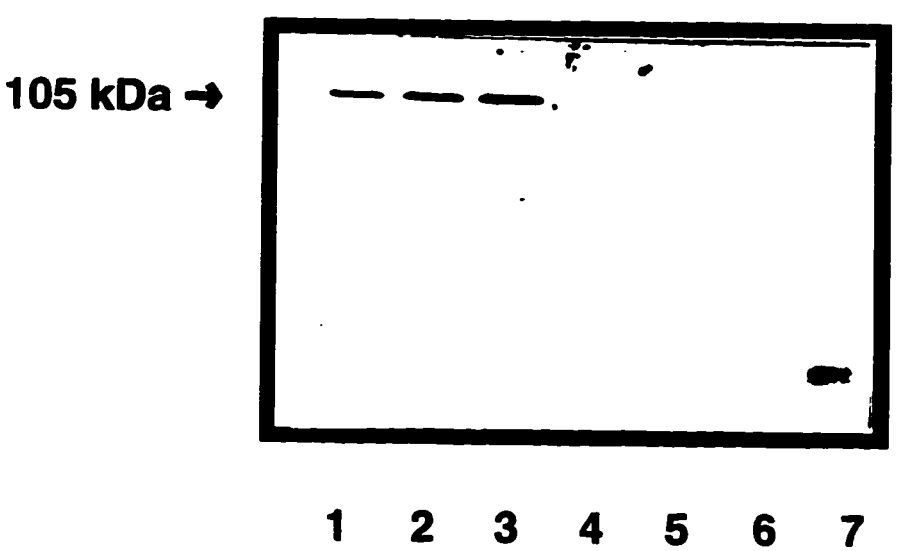

105 kDa
1
2
3
4
5
6
7

Figure 6.2 Western blotting of cytosolic phospholipase A_2 in MDCK-D1 cell lysate

MDCK-D1 cell lysates were run on a 7.5 % SDS-PAGE gel and probed with a rabbit polyclonal $cPLA_2$ antiserum. The blot was visualized using the Amersham ECL system. Lanes 1-3 were loaded with 5 µg of cell lysate protein, lanes 4-6 were loaded with 10µg of cell lysate protein, while lane 7 was loaded with 50 ng of standard $cPLA_2$.

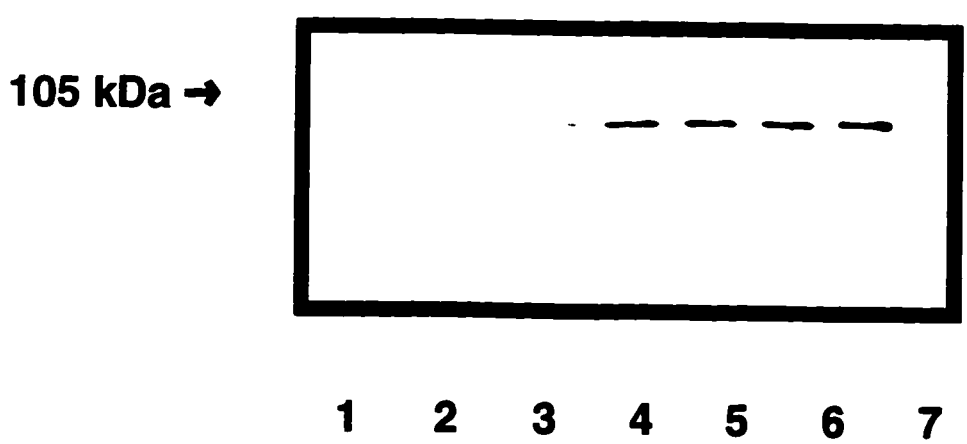

105 kDa
1
2
3
4
5
6
7

Figure 6.3 The effect of RHC80267, an inhibitor of diglyceride-lipase, HELSS, an inhibtor of Ca^{2+}-independent phospholipase A_2, and AACOCF, an inhibitor of cytosolic phospholipase A_2, on bradykinin-stimulated arachidonic acid release

Cells labelled overnight with [^{3}H]AA were preincubated in HBSS (without BSA - since albumin binds $AACOCF_3$) for 10 min with 10 µM RHC80267, for 10 min with 20µM HELSS, or for 1 min with 50 µM $AACOCF_3$ and subsequently stimulated with 1 µM BK for 10 min at 37°C in the presence or absence of these same inhibitors. Arachidonate released into the media was then determined. The release was expressed as the percent of the total label incorporated into the cells. The stimulation obtained with BK in the absence of inhibitors was counted as 100%. Control counts for [^{3}H] AA released were 0.67 ± 0.06% of total dpm incorporated, while BK accounted for 1.36 ± 0.11% of dpm incorporated. Values are the means ± S.D. of 3 separate experiments performed in duplicate. * $P < 0.0001$ vs BK alone.

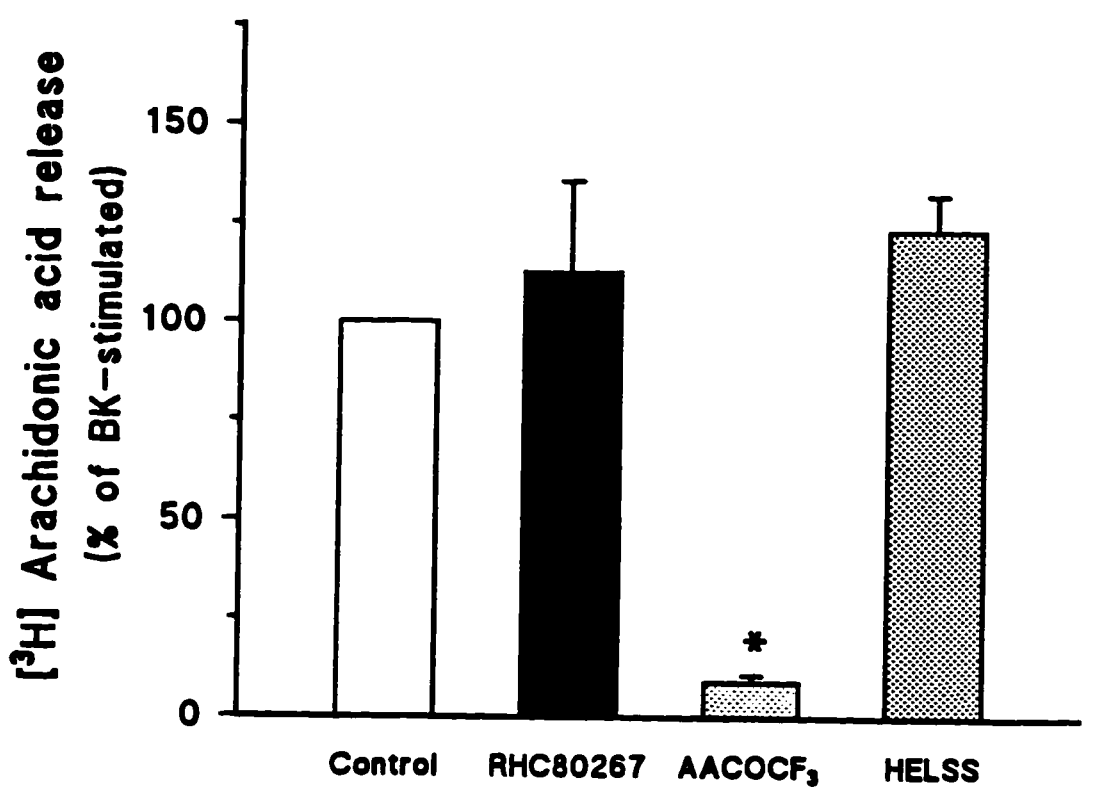
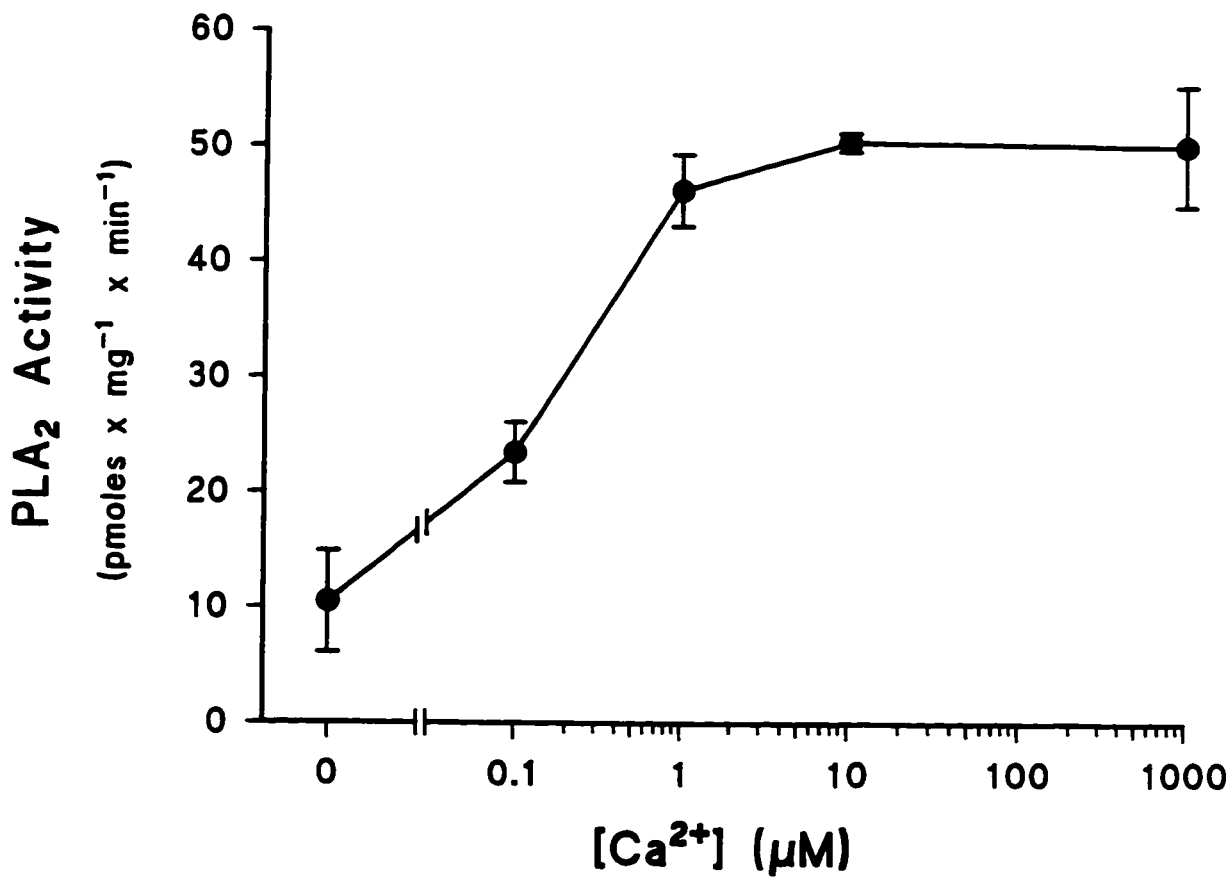

[3H] Arachidonic acid release
(% of BK-stimulated)
150
100
50
0
*
Control
RHC80267
AACOCF3
HELSS

Figure 6.4 The Ca^{2+}-dependency of *in vitro* phospholipase A_2 activity derived from MDCK-D1 cytosolic extracts

MDCK-D1 cells were grown to near confluency, incubated overnight in DMEM-containing 0.5% FCS, and subsequently lysed with Tris buffer, pH 7.4 by sonication. Lysate was then centrifuged at 1000*g* to pellet unbroken cells and the supernatant centrifuged for 1 hr at 100,000*g* to separate cytosolic from membrane fractions. The supernatant (cytosolic fraction) was then dialysed overnight at 4°C against Tris buffer, containing 10 % glycerol to remove EGTA. The resulting fraction was subsequently assayed for PLA_2 activity with precise [Ca^{2+}] being maintained employing a Tris/EGTA /Ca^{2+} buffer system. Results are expressed as the number of pmoles of AA hydrolysed per mg of protein per hour and represent the means ± S.E.M. (n=4) experiments.


PLA2 Activity
(pmoles x mg-1 x min-1)
60
50
40
30
20
10
0
0
0.1
1
10
100
1000
[Ca2+] (μM)

mg^{-1} x min^{-1}) reminiscent of the activity obtained following immunodepletion of the cytosolic extracts with the polyclonal antibody to $cPLA_2$. It would appear therefore that most of the PLA_2 activity in MDCK-D1 cellsrequires sub-micromolar Ca^{2+} concentrations while Ca^{2+}-independent activity accounts for only a minor component.

6.1.2 *Bradykinin-induced association of cytosolic phospholipase A_2 with MDCK-D1 membranes*

To further show the involvement of $cPLA_2$ in BK-enhanced release of AA, cells were stimulated with 1 μM nonapeptide for 2 min and the resulting PLA_2 activity was measured *in vitro* in the membrane fraction. Stimulation of the cell and the resulting increases in intracellular Ca^{2+} are known to cause the activation and translocation of $cPLA_2$ to the membrane where its arachidonyl-containing substrates are located. An octylglucoside extract of the membrane fraction was prepared as described by Paglin et al (1993). Analysis of this preparation revealed an increase of activity from 27.9 ± 1.4 pmoles/min/mg under control conditions, to 50.8 ± 8.01 pmoles/min/mg upon stimulation with BK (**Table 6.2**). This 1.8-fold increase was substantially reduced by addition of $cPLA_2$ antibody to the membrane sample 1 hr before the assay. The evidence at hand strongly indicated that BK-stimulated arachidonate release is second to increased $cPLA_2$ activity in the membrane and no other types of phospholipase A_2 are significantly involved at this site.

Next examined was whether this increased activity was due to an actual translocation

Table 6.2 The effect of bradykinin upon membrane phospholipase A_2 activity

Cells were treated for 2 min with or without 1 μM BK and the membrane fractions were isolated and extracted with 10 mM octyl glucoside. Samples were then assayed for PLA_2 activity according to Leslie (1990) . The values are the means ± S.E.M. of 5 independent experiments performed in duplicate.[a] $P < 0.05$ vs. control not stimulated with BK.

Conditions	PLA_2 activity ± S.E.M. (pmoles x mg^{-1} x min^{-1})
Control	27.9 ± 1.4
BK	50.8 ± 8.0[a]
anti-$cPLA_2$	5.6 ± 1.1
anti-$cPLA_2$ + BK	7.2 ± 1.8

of the enzyme to the membrane as opposed to an increased activation of the enzyme already present at the membrane. Accordingly, serum-starved cells were incubated with 1μM BK for 2 min, then washed at 0°C to halt the reaction and lysed in buffer without calcium chelators. Lysates were then processed to obtain a detergent-extracted membrane fraction as described in the methodology section. **Figure 6.5** shows this phospholipase increased dramatically within the membrane fraction.

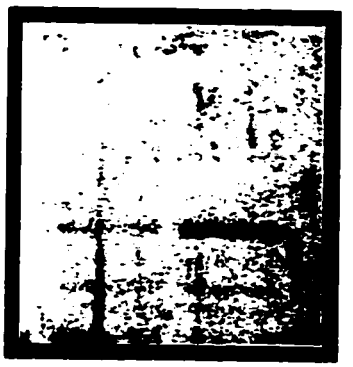
Therefore BK-mediated elevation of membrane $cPLA_2$ activity can be correlated with an increased level of this phospholipase at this site. These results do not preclude the possibility that at least some of the increased membrane activity is due to increased phosphorylation of the enzyme on its serine residues. Regardless, it would appear that BK is capable of inducing intracellular changes which favour the association of $cPLA_2$ with cellular membranes.

6.1.3 *Bradykinin-stimulated arachidonic acid release: dependence upon extracellular Ca^{2+}*

In order to determine whether release of AA in response to BK is dependent upon Ca^{2+} originating from intra- or extracellular sources, the effect of inhibitors, neomycin sulfate and SK&F 96365 were tested. Neomycin is a compound which selectively binds to phosphatidylinositol-containing phospholipids thereby preventing PI-PLC from accessing and hydrolysing its substrate. The action of this agent leads to an inhibition of receptor-induced production of $InsP_3$, the second messenger required for the release of Ca^{2+} from intracellular stores. In contrast, SK&F 96365 is a synthetic inhibitor of receptor-mediated extracellular

Figure 6.5 Western blot analysis of cytosolic phospholipase A_2 levels within membrane extracts derived from unstimulated and bradykinin-stimulated MDCK-D1 cells

Serum-starved cells were incubated for 2 min with or without 1 µM BK in HBSS containing 0.05 % BSA (w/v) at 37°C. The membrane fraction proteins (10 µg) from control (C) and bradykinin- (BK) stimulated cells were separated on a 7.5 % SDS PAGE gel and transferred to a nitrocellulose membrane. The blot was probed with a rabbit polyclonal $cPLA_2$ antibody and detected by means of the Amersham ECL system.



← 105 kDa

C BK

Ca^{2+} entry. The use of this compound was successful in blocking thrombin-stimulated Ca^{2+} entry in human platelets (Merritt et al. 1990). As shown in **Figure 6.6** , 100 μM neomycin failed to block BK-mediated AA release while 10 μM SK&F 96365 reduced the effect of the nonapeptide from a 2.82 ± 0.30 fold increase compared to control to 1.32 ± 0.14 fold, P <0.05. When combined together, neomycin and SK&F 96365 did not achieve any greater inhibition than that obtained using SK&F alone (compare BK + SK&F, 1.32 ± 0.14 fold increase compared to control, vs BK + SK&F + neomycin, 1.27 ± 0.12 fold compared to control, n.s.). These results strongly indicate that it is extracellular Ca^{2-} which is required for BK-mediated AA release in MDCK-D1 and not that which is mobilized from the endoplasmic reticulum through an $InsP_3$-dependent process.

As shown in **Figure 6.7A**, BK was able to elicit a significant increase in $[Ca^{2+}]_i$ which was completely eliminated in the presence of 10 μM SK&F 96365 (cf **Figure 6.7B**). However, preincubation of the cells with this agent caused a small but significant elevation of $[Ca^{2-}]_i$. Merrit et al. (1990), noted a similar phenomenon with human platelets where concentrations of 20 μM SK&F 96365 were sufficient to cause transient increases in $[Ca^{2+}]_i$ of 100-200 nM, not unlike that observed in the present study. The authors attribute this effect to a release of calcium from intracellular stores since the presence of EGTA in the medium failed to eliminate the increase. Despite this particular effect of the inhibitor, in its presence, BK was unable to induce an increase in $[Ca^{2+}]_i$ which suggests a minimum contribution of internal calcium stores in the generation of the BK response.

Figure 6.6 The effect of SK&F 96365 and neomycin on bradykinin-mediated arachidonic acid release from MDCK-D1 cells

[^{3}H]AA-labelled cells were washed 3 times with HBSS containing 0.05 % BSA and then incubated with either vehicle, 10 µM SK&F 96365, 100 µM neomycin, or both inhibitors together, for 10 minutes at 37°C. Cells were then stimulated in the presence of these agents with 1 µM BK for 10 min at 37°C and then the media was taken for determination of [^{3}H]AA released. The control conditions are those under which cells were incubated in the absence of BK and other agents. The release is normalized for the total label incorporated into the cells. Results are the means ± S.D. of 3 experiments (n = 3) performed in duplicate and are expressed as the fold increase compared to control. *, $P < 0.05$ vs BK alone.

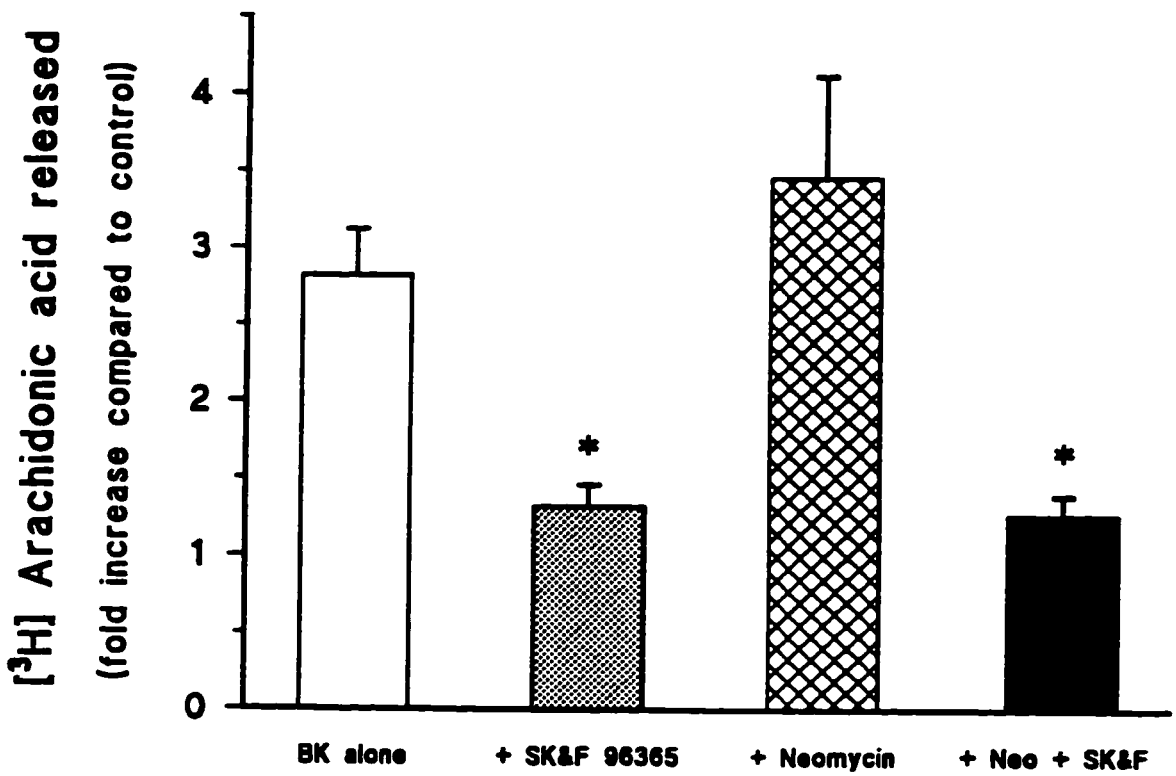
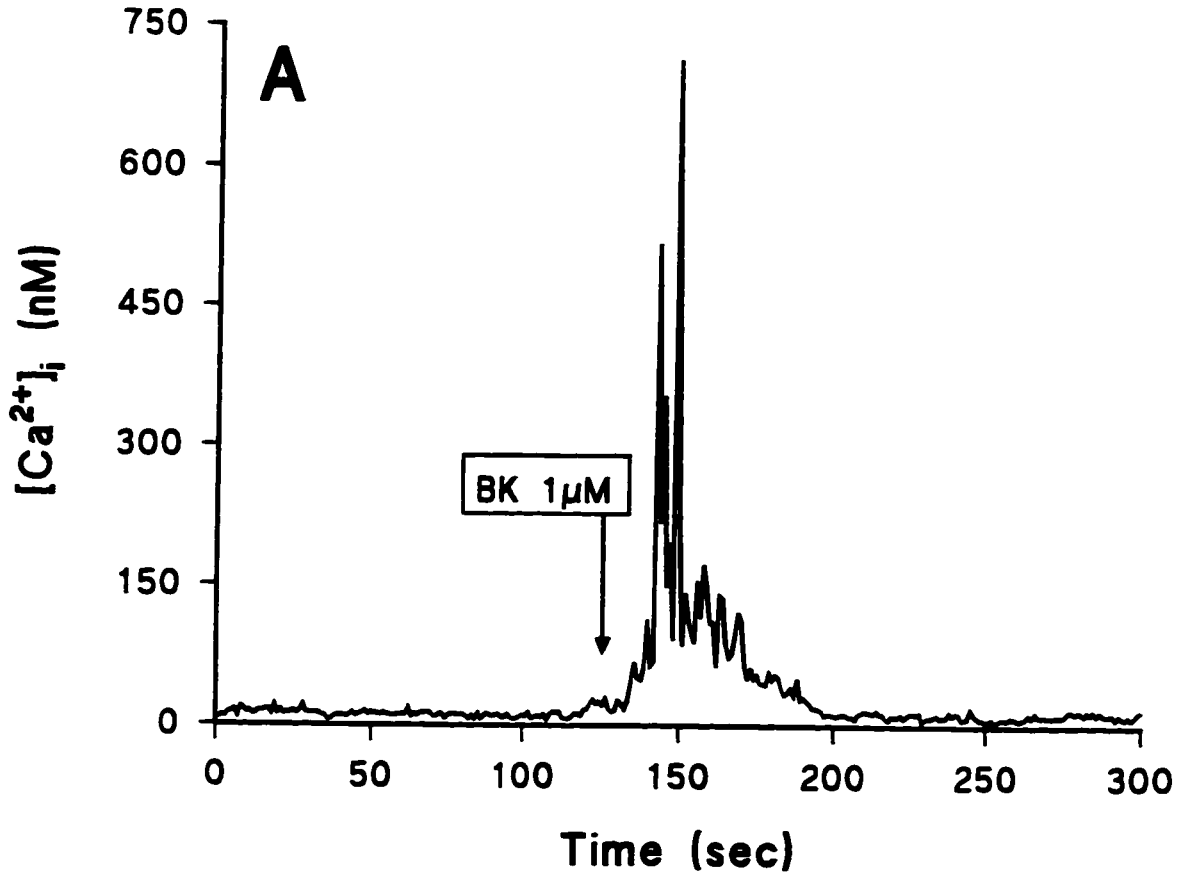
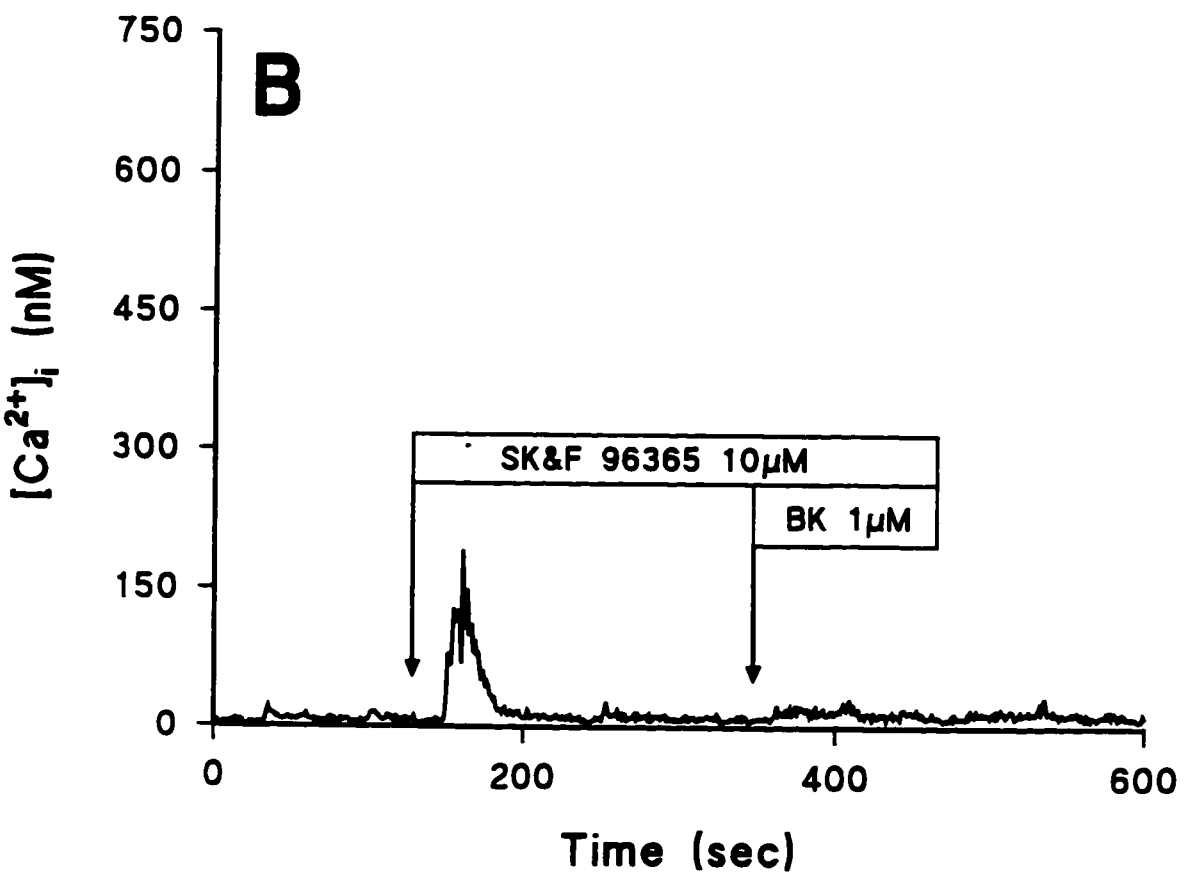

[3H] Arachidonic acid released
(fold increase compared to control)
0
1
2
3
4
*
*
BK alone
+ SK&F 96365
+ Neomycin
+ Neo + SK&F

Figure 6.7 The effect of SK&F 96365 upon bradykinin-stimulated $[Ca^{2+}]_i$

Cells grown on 20 mm glass cover slips were serum-starved for 24 hrs and subsequently loaded with 5µM fura-2 for 1 hr. Subsequently, cells were either stimulated at 37°C with 1 µM BK (A) or preincubated with 10 µM SK&F 96365 for 200 sec followed by BK treatment (B) and the $[Ca^{2+}]_i$ measured. The result shown is representative of two independently performed experiments.


A
750
600
450
300
150
0
$[Ca^{2+}]_i$ (nM)
BK 1μM
0
50
100
150
200
250
300
Time (sec)


B
750
600
450
300
150
0
$[Ca^{2+}]_i$ (nM)
SK&F 96365 10μM
BK 1μM
0
200
400
600
Time (sec)

Taken together, these results provide strong evidence for a mechanism of AA release requiring the activity of $cPLA_2$ contingent upon its extracellularly-derived-calcium-dependent translocation from the cytosol to membrane(s) subsequent to BK.

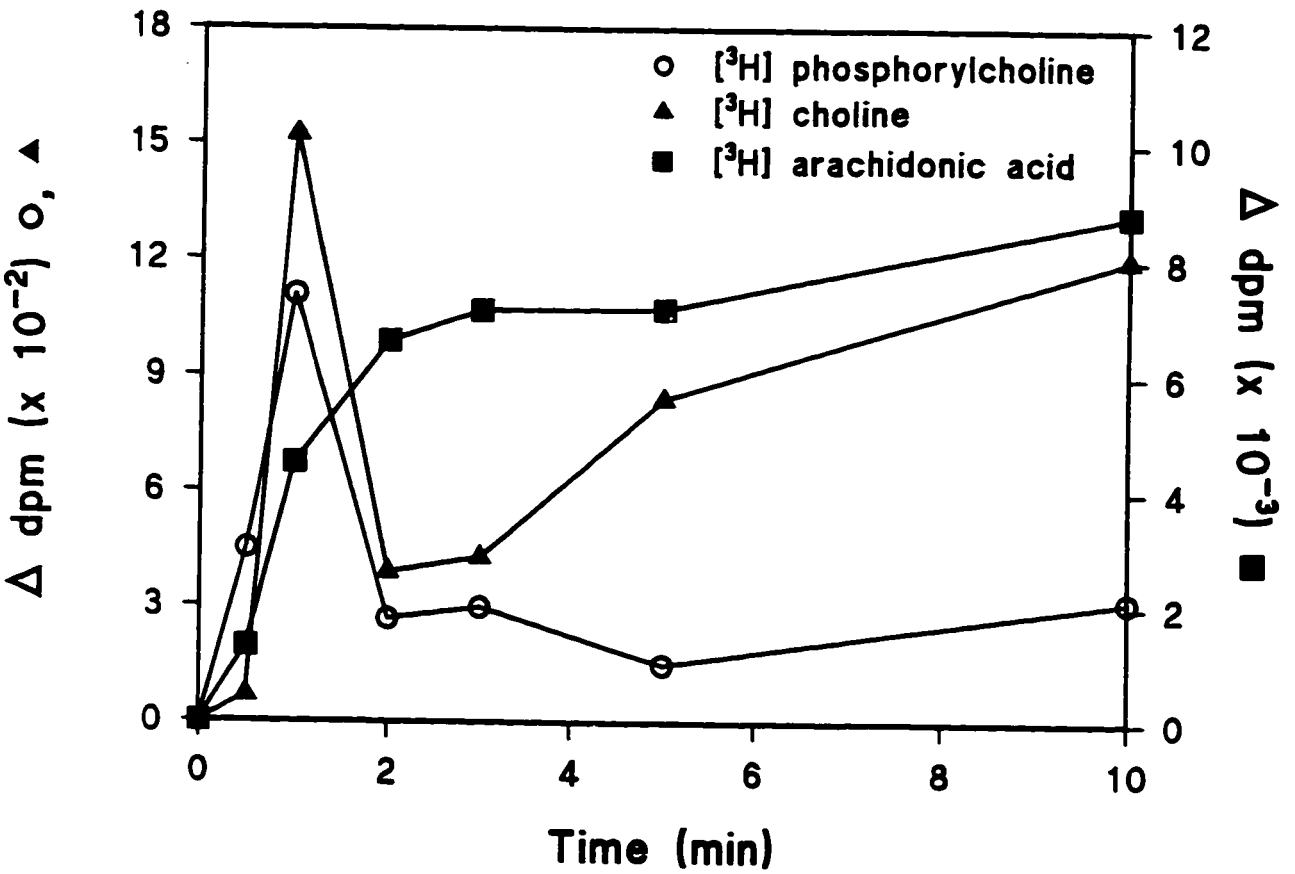
6.2 The role of phosphatidylcholine-specific phospholipase C and phospholipase D in mediating bradykinin-stimulated release of arachidonic acid

6.2.1 *The time course for phosphatidylcholine-specific phospholipase C and phospholipase D activation in response to bradykinin*

Studies have shown that BK-stimulated arachidonate release is a rapid event, beginning within the first min following agonist interaction with the cell (Weiss et al., 1989). With cells equilibrium-labelled with [^{3}H]arachidonate and stimulated with 1 μM BK, there was a rapid release of AA which plateaued at approximately 2 min, as shown in **Figure 6.8**. If either PC-PLC or PLD were implicated in PLA_2 activation and subsequent AA release, one would expect an at least equally rapid release of Pchol (a water soluble product of PC-PLC activation) and Chol (a water soluble product of PLD activation). To determine whether the time frame of PC-PLC and/or PLD activation is compatible with AA release in response to BK, the kinetics of Pchol and Chol release were studied. With cells [^{3}H]Chol-labelled for 48 hrs and then stimulated with 1 μM BK, there resulted a marked increase in products of both PC-PLC and PLD within the first min of stimulation. The production of radioactive Pchol was transient, peaking at 1 min, while that of [^{3}H]Chol was biphasic, displaying both a transient peak at 1 min and a sizeable, steady increase after 2 min. Thus the time frame for PC-PLC activation and aspects of the kinetics of PLD activation, are compatible with the mediatory involvement of

Figure 6.8 Time course of bradykinin-stimulated choline, phosphorylcholine and arachidonate release

[Methyl-^{3}H]choline-labelled cells were stimulated at 37°C with 1 μM BK and the medium was collected at the times indicated. Cells were also labelled with [^{3}H]AA as usual. The number of dpm for each product was normalized for the amount of label incorporated into the cells and the unstimulated control values at each time point were subtracted. The data are the means of duplicate determinations taken from one representative experiment which was repeated 3 times.


[3H] phosphorylcholine
[3H] choline
[3H] arachidonic acid
Δ dpm (x 10-2) o, ▲
■ (x 10-3) Δ dpm
Time (min)
0
2
4
6
8
10
0
3
6
9
12
15
18
0
2
4
6
8
10
12

these enzymes in the rapid, enhanced AA release in response to BK.

6.2.2 *Further characterization of phosphatidylcholine-specific phospholipase C, phospholipase D and their involvement in bradykinin-stimulated release of arachidonic acid*

The other product of PC-PLC activity is DAG. The levels of this product were previously shown to increase in MDCK-D1 cells in response to stimulation by BK (Weiss and Insel, 1991) a finding which was confirmed in the present study (cf **Figure 6.24**). The results illustrated in **Figure 6.9** indicate that BK is also able to significantly increase membrane PKC activity from 257.4 ± 15.0 pmoles/min/mg to 565.0 ± 19.9 pmoles/min/mg, $P<0.001$ which is an indication of increased DAG production from PC-PLC activation. However, the DAG could have also originated from the sequential action of PLD and PAP or from PI-PLC. The present results indicate that under conditions of assay, the specific PC-PLC inhibitor, D609 (Schutze et al., 1992), could completely abolish the stimulatory effect of BK on labelled Pchol production (**Figure 6.10**) and on PKC activity (**Figure 6.9**) while inhibiting by 70%, the BK-enhanced release of labelled AA (**Figure 6.11**). The other product of PLD is PA, which also increased following stimulation with BK in the absence of EtOH (**Figure 6.12**). The specificity of D609 can be appreciated from the fact that it had no effect on BK-stimulated production of either radioactive PA (**Figure 6.12**) or labelled Chol (**Figure 6.13**) and consequently did not act on PLD. In addition D609 had no effect on PLA_2 activity assayed *in vitro* (results not shown). These data strongly suggest once again that it is PC-PLC that is mainly responsible for producing the DAG mediator and not PI-PLC which is also present in MDCK-D1 cells.

Figure 6.9 The effect of D609 on bradykinin-stimulated protein kinase C activity

Serum-starved cells were preincubated for 30 min with 50 µg/mL D609 and then stimulated for 1 min with 1 µM BK at 37°C in the presence or absence of D609. Cells were then processed for Ca^{2+}- and phospholipid-dependent membrane PKC activity. Results are expressed as the number of pmoles of peptide phosphorylated per min per mg of protein. The data presented are the means ± S.D. of 3 experiments performed in duplicate. * $P < 0.001$ vs unstimulated control.

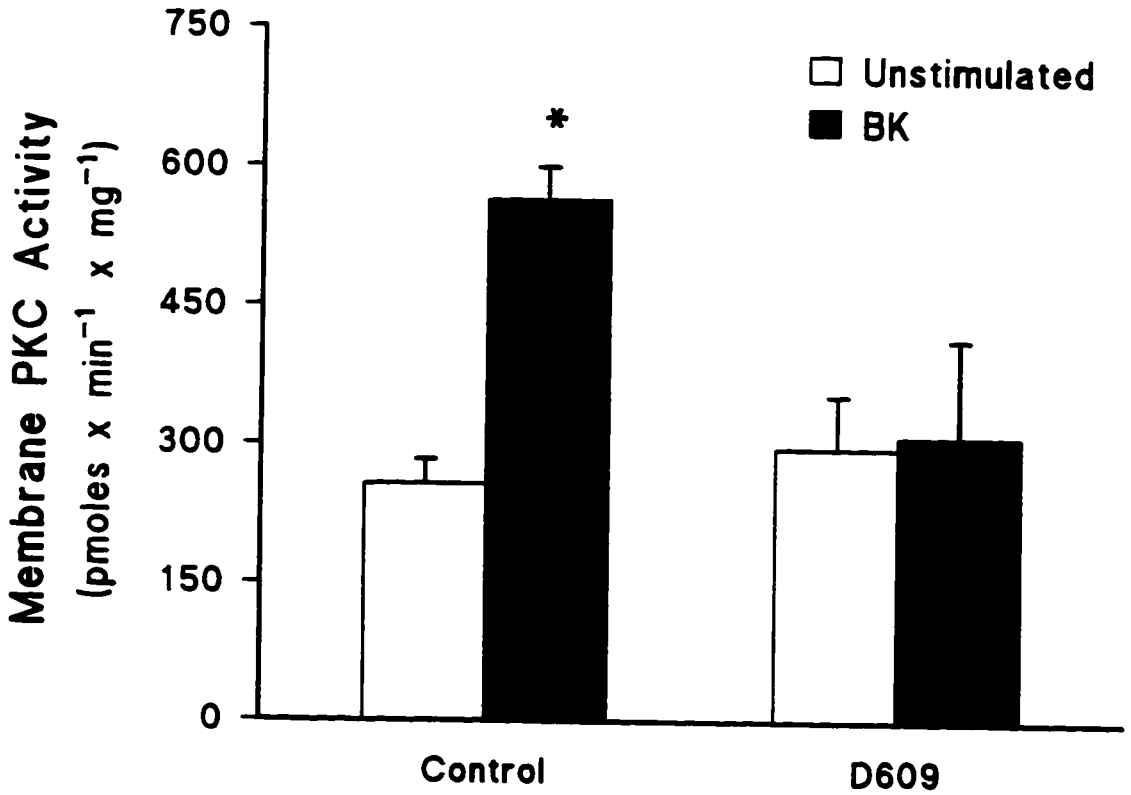

750
600
450
300
150
0
Membrane PKC Activity
(pmoles x min^{-1} x mg^{-1})
*
Unstimulated
BK
Control
D609

Figure 6.10 The effect of D609 and ethanol on bradykinin-mediated phosphorylcholine release

[^{3}H]Choline chloride-labelled cells were preincubated for 30 min with 50 µg/mL D609 and then stimulated for 1 min with 1 µM BK at 37°C in the presence or absence of 2% EtOH or D609. For each sample, the counts of [^{3}H]Pchol released were normalized for total [^{3}H]choline chloride incorporated into the cells and are expressed as the precent of total label incorporated into the cells. The data presented at the means ± S.D. of at least 3 experiments performed in duplicate. * $P < 0.05$ vs basal, unstimulated conditions.

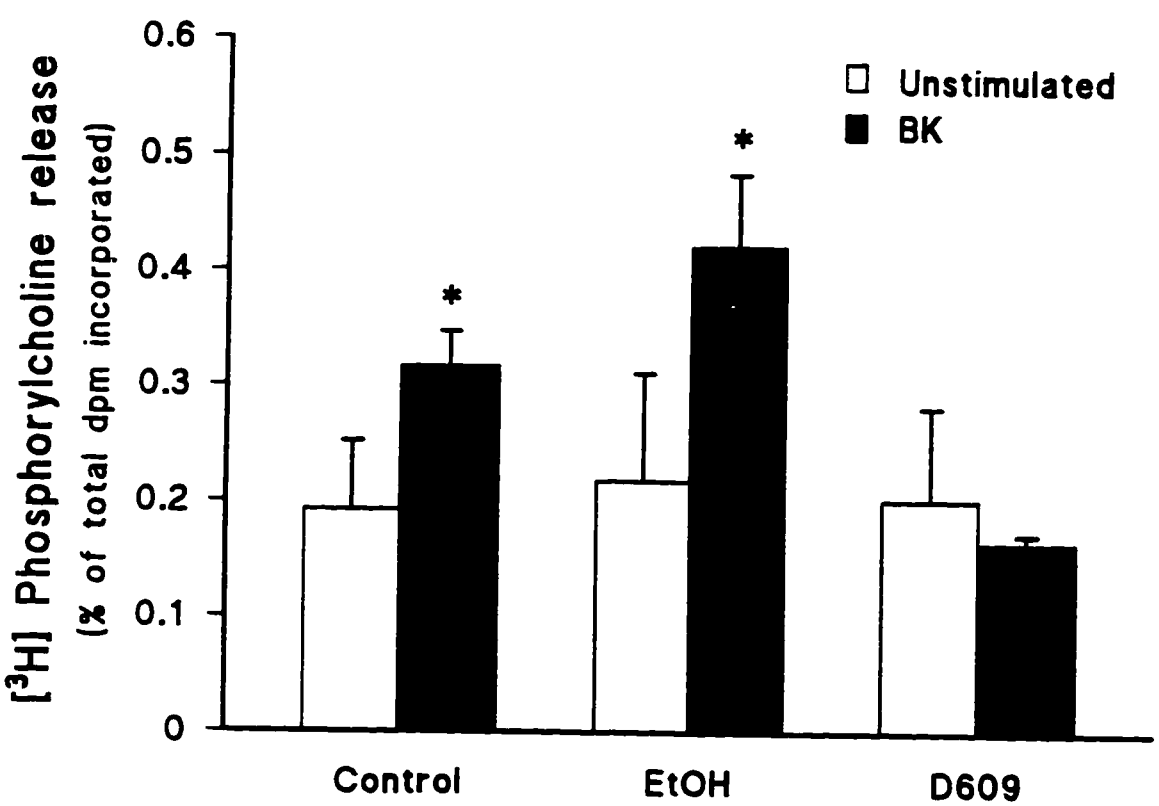

[3H] Phosphorylcholine release
(% of total dpm incorporated)
0.6
0.5
0.4
0.3
0.2
0.1
0
Unstimulated
BK
*
*
Control
EtOH
D609

Figure 6.11 The effect of phosphatidylcholine-specific phospholipase C inhibition and blunting of phosphatidic acid production on bradykinin-mediated arachidonic acid release

MDCK-D1 cells, labelled with [^{3}H]AA, were preincubated for 30 min with 0.3 µg/mL D609 and subsequently stimulated with 1 µM BK at 37°C in the presence or absence of 2% EtOH, D609, or the presence of both for 10 min. All results were normalized for total label incorporated into the cells. The stimulation of AA release by BK in the absence of inhibitors was counted as 100%. Actual counts were: Control release, 0.56 ± 0.05% of total dpm incorporated into cells vs BK, 1.42 ± 0.11% of total dpm incorporated. The data presented are the means ± S.E.M. of at least 4 experiments performed in duplicate. A SNK test was used to determine the significant difference between each group. * $P < 0.05$ vs. any other group.

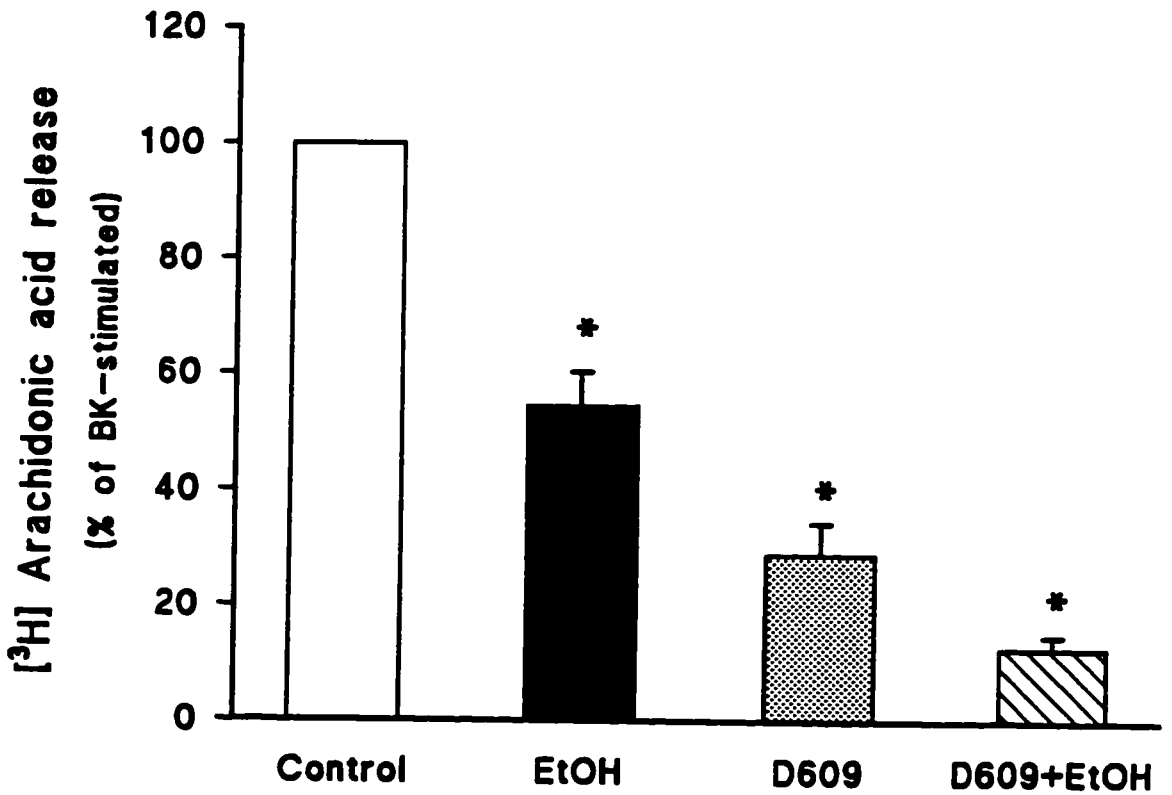

[3H] Arachidonic acid release
(% of BK-stimulated)
120
100
80
60
40
20
0
*
*
*
Control
EtOH
D609
D609+EtOH

Figure 6.12 Bradykinin increases both phosphatidic acid and phosphtidylethanol levels primarily through a phospholipase D-mediated process

Subconfluent cells were serum starved overnight and labelled with 2.0 µCi [^{3}H]palmitic acid. Cultures were then preincubated for 30 min with 50 µg/mL D609 or 300 µM propranolol (Pro) and subsequently stimulated with 1 µM BK for 1 min at 37°C (2% EtOH was added to measure the transphophatidylation reaction). Incubations were terminated with ice-cold methanol containing 1% HCl and the lipids processed for either [^{3}H]PA or [^{3}H]PEt. The results are expressed as the percent of the total labelled [^{3}H]palmitic acid incorporated into the phospholipid pool. The data reported are the means ± S.E.M. of at least 4 separate experiments performed in duplicate.* $P < 0.05$ vs. unstimulated control.

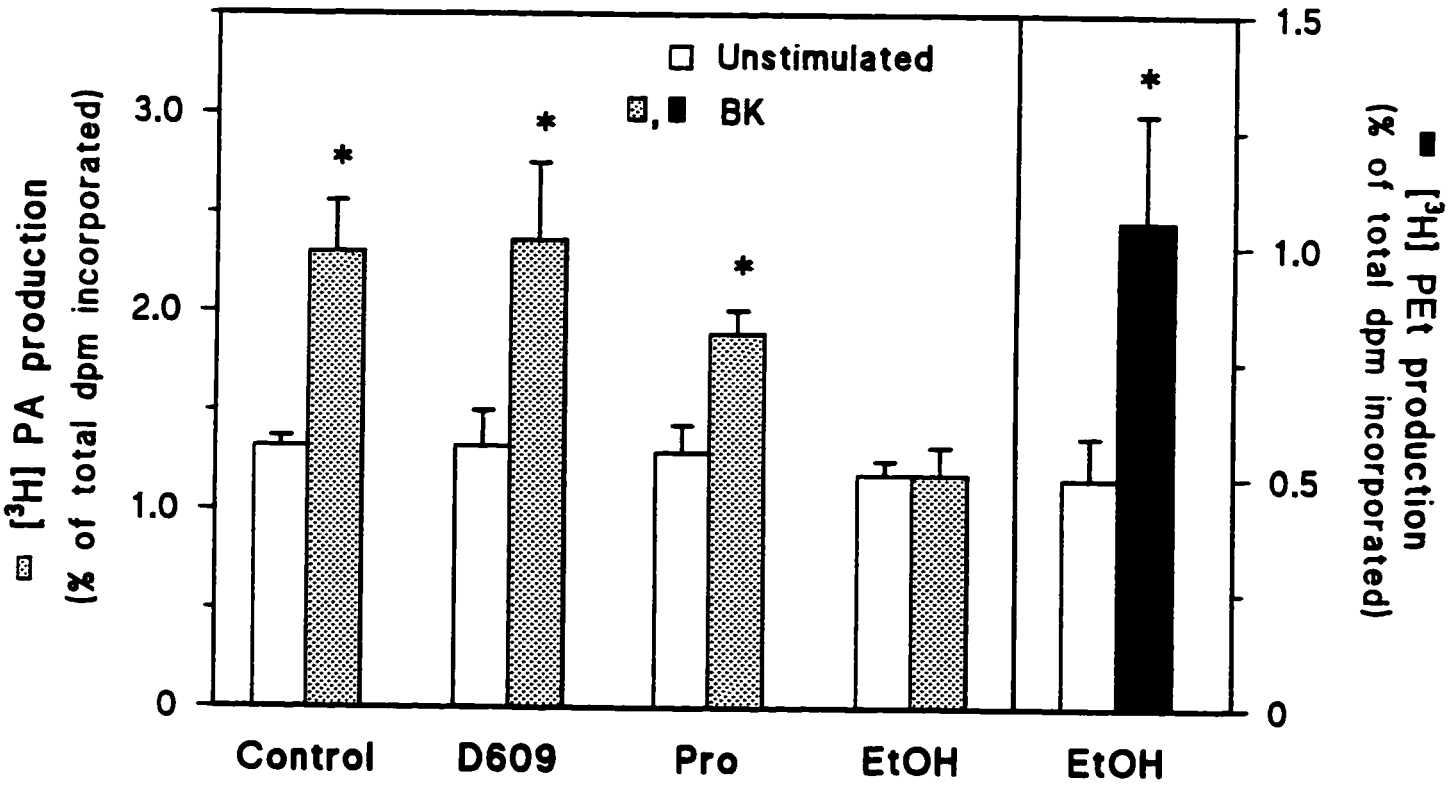
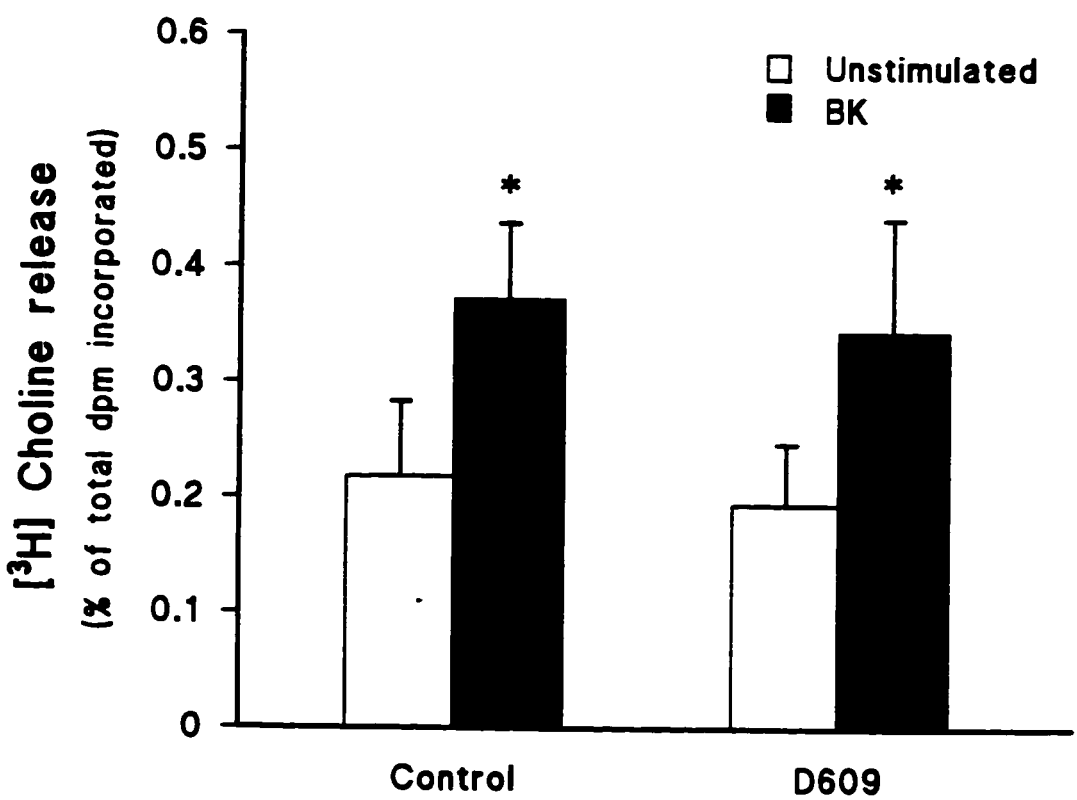

Unstimulated
BK
[3H] PA production
(% of total dpm incorporated)
[3H] PEt production
(% of total dpm incorporated)
3.0
2.0
1.0
0
1.5
1.0
0.5
0
Control
D609
Pro
EtOH
EtOH
*

Figure 6.13 The effect of D609 on bradykinin-mediated choline release

[^{3}H]choline chloride-labelled cells were preincubated for 30 min with 50 µg/mL D609 and then stimulated for 1 min with 1 µM BK at 37°C in the presence or absence of D609. The choline hydrolysis products were then extracted from the media. The release was expressed as the percent of the total labelled choline incorporated into the cells. The data presented are the means ± S.D. of at least 3 experiments performed in duplicate. * $P < 0.05$ vs basal unstimulated.


0.6
0.5
0.4
0.3
0.2
0.1
0
[3H] Choline release
(% of total dpm incorporated)
Unstimulated
BK
*
*
Control
D609

Accordingly, the present study confirmed that inhibition of this latter enzyme with neomycin does not affect BK-induced signalling and the release of AA (Slivka and Insel, 1988).

Phosphatidic acid can be produced directly from the activation of PLD or by the combined activation of PLC and DAG kinase. Results illustrated in **Figure 6.12** indicate that in the presence of EtOH, a condition which promotes the transphosphatidylation activity of PLD and has no inhibitory effect on PC-PLC (**Figure 6.10**), all of the BK-enhanced production of labelled PA is lost and replaced entirely by labelled PEt formation. Indeed, the degree of stimulation of PEt formation by BK was comparable to that of BK-enhanced Chol and PA formation. As mentioned earlier, inhibition of PC-PLC activity with D609 had no effect on PA production. This evidence precludes a pathway involving, to more than a minor extent, the combined action of PLC and DAG kinase for the stimulated production of PA. Ethanol also inhibited the BK-stimulated AA release by some 50% whereas EtOH and D609 together almost completely blunted this effect (**Figure 6.11**). In addition, one must consider, however, the possibility that this newly formed PEt could act as a substrate for PLA_2 thereby causing the observed inhibitory effect of EtOH upon AA release to be an underestimation of the role played by PLD in this transduction pathway. It is also appropriate to mention however, that 2% EtOH added to cytosol extracts had a small inhibitory effect on PLA_2 activity assayed *in vitro* (< 15%). It is doubtful however that this concentration of free EtOH actually reaches the interior of the cell given the short period of time involved and its possibility for interaction with membrane lipids and the PLD-catalysed transphosphatidylation process . Thus it can be

concluded from the evidence as a whole that BK-stimulated AA release depends heavily upon the activation of both PC-PLC and PLD.

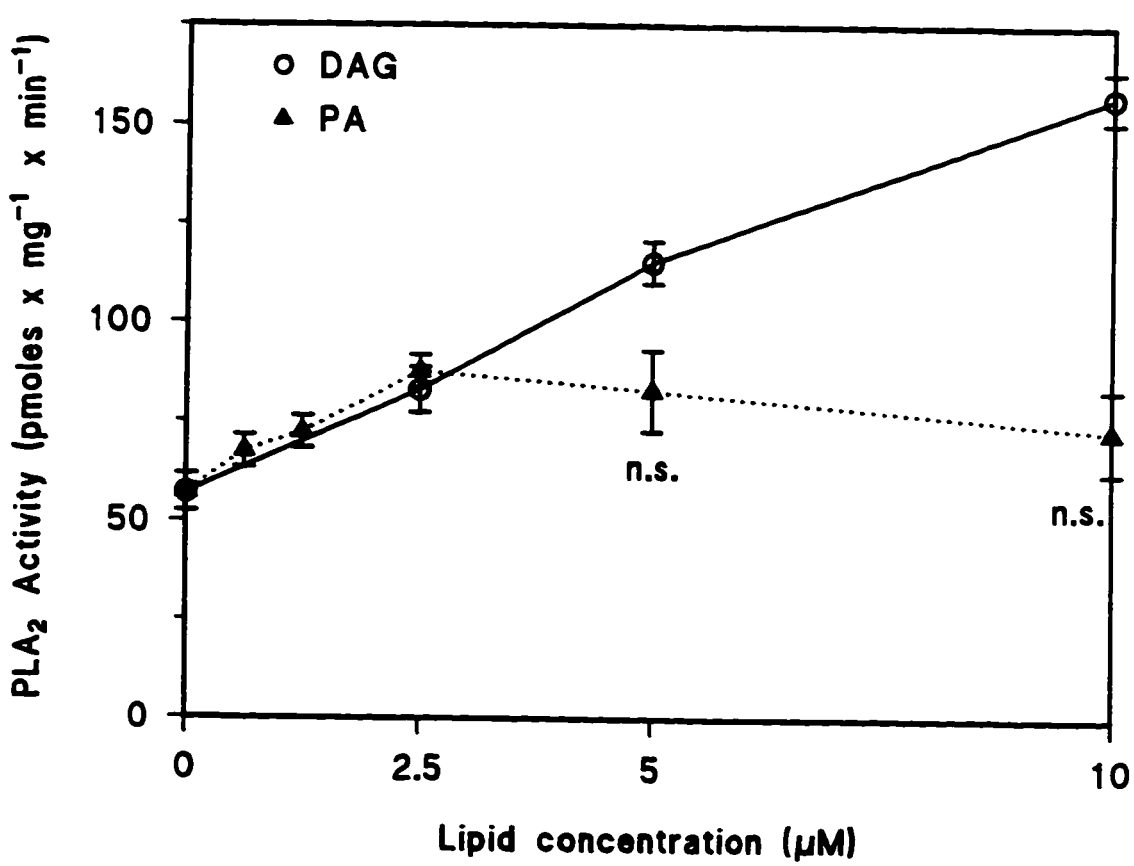
6.2.3 *Effect of phosphatidic acid and diacylglycerol on phospholipase A_2 activity*

The question arose as to whether the effect of PA and DAG on PLA_2 was mediated through activation of effectors such as PKC or resulted from alternative mechanisms seen in other cell types (Dawson et al., 1983; Dawson et al., 1984; Kramer et al., 1987; Leslie and Channon, 1990; Nosal, 1994). Accordingly, a direct action on the enzyme or some lipid perturbating effect on the lyotropic mesomorphism of the substrate to improve accessiblity of the enzyme could be implicated. To test these possiblities, increasing concentrations of added 1-stearoyl-2-arachidonyl-*sn*-glycerol and 1-stearoyl-2-arachidonyl-*sn*-3-glycero-3-phosphate were incubated in the presence of 100 nM Ca^{2+} and cytosolic fraction. As shown in **Figure 6.14**, 10 μM DAG very markedly stimulated PLA_2 activity to nearly 3-fold the basal value (control, 57.1 ± 4.7 pmoles /mg/min as compared to DAG, 157.4 ± 6.3 pmoles /mg/min).

Phosphatidic acid was also able to induce a significant increase in PLA_2 activity, though to a lesser extent than DAG. The most effective concentration of PA was found to be 2.5 μM, under which condition the activity of the enzyme increased from a basal value of 57.1 ± 4.7 pmoles/mg/min to 87.7 ± 4.1 pmoles/mg/min. Above 2.5μM PA, the activity appeared to drop: this could be explained by a possible competition between PC and PA as substrates for PLA_2 not unlike that seen in platelets (Billah et al., 1981). Indeed PLA_2 activity against PA was

Figure 6.14 The effect of phosphatidic acid and diacylglycerol on *in vitro* phospholipase A_2 activity

Cytosolic extracts, 22.5 µg of protein, were assayed for PLA_2 activity for 1 hr at 37°C. The labelled substrate, 1-stearoyl 2-[^{3}H]arachidonyl PC, was employed at a concentration of 30 µM in a volume of 100 µL in the presence or absence of various concentrations of 1-stearoyl 2-arachidonyl PA or 1-stearoyl 2-arachidonyl glycerol. Free calcium concentrations were maintained at 100 nM. The results are expressed as the number of pmoles of AA liberated per min per mg of cytosolic protein. Values are the means ± S.E.M. of at least 4 separate experiments performed in duplicate. Results for experimental conditions are statistically significant vs control conditions as analysed by ANOVA (i.e., no PA or DAG added), $P<0.01$, unless otherwise indicated by n.s. (i.e., not significantly different from control).


DAG
PA
150
100
50
0
0
2.5
5
10
n.s.
n.s.
PLA2 Activity (pmoles x mg-1 x min-1)
Lipid concentration (μM)

observed when tested in cytosolic fractions. The activity obtained with 30 μM PA (same concentration as for labelled PC) was about 25 % (12 pmoles /mg/min) of that obtained with the PC analogue at the same concentration.

Part II Protein kinases and bradykinin-stimulated release of arachidonic acid

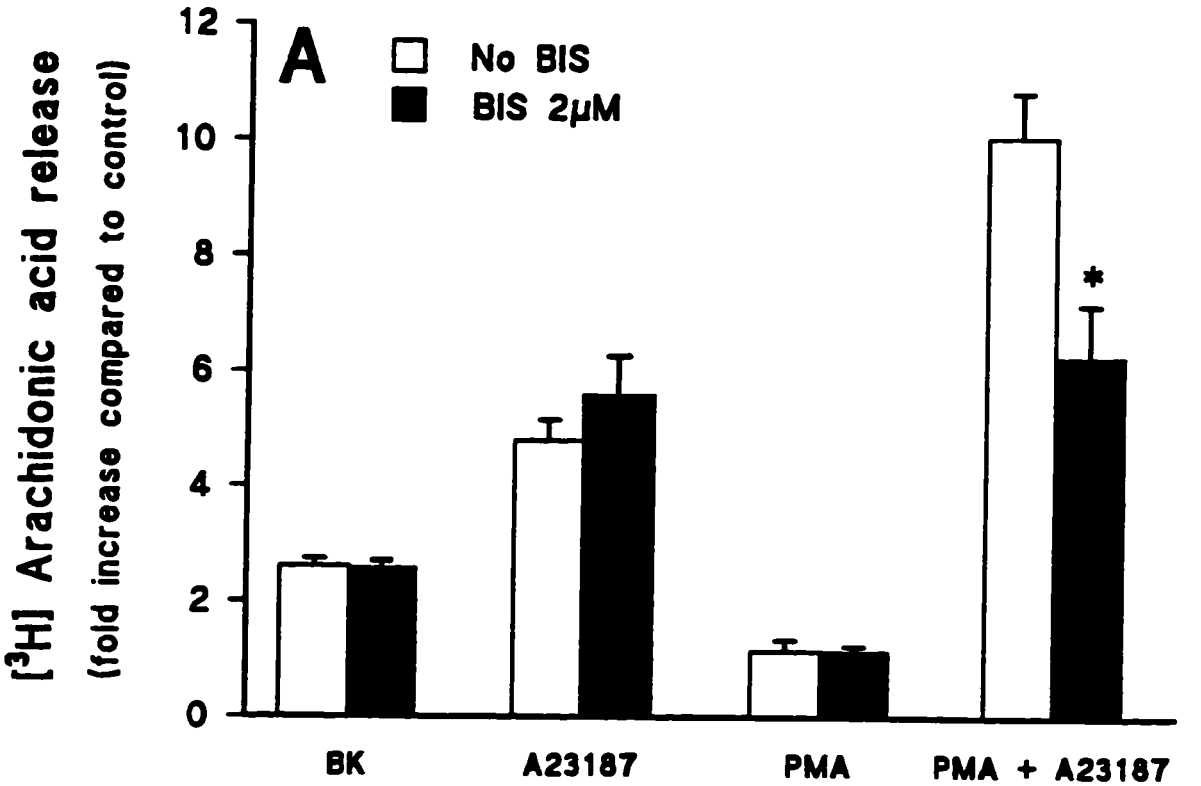
6.3 The role of protein kinase C defined for bradykinin-stimulated arachidonic acid release

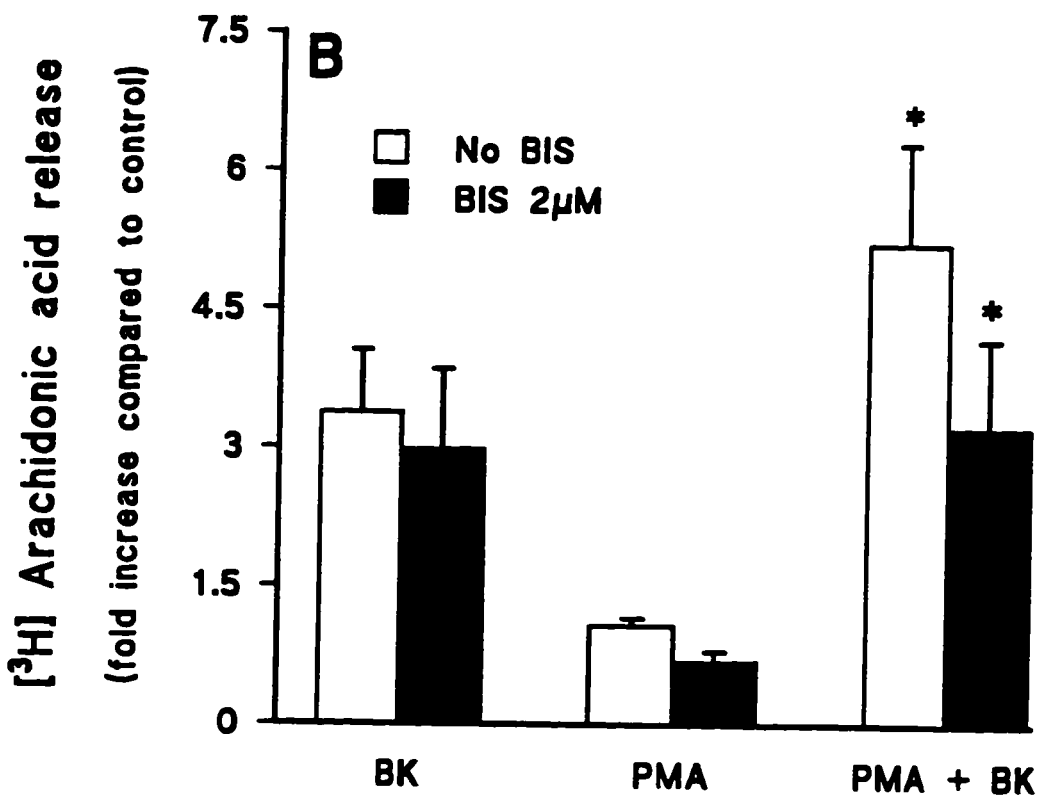
6.3.1 *The effects of the protein kinase C inhibitor, bisindolylmaleimide I, upon arachidonic acid release in MDCK-D1 cells*

Experiments were performed to determine whether chemical inhibition of PKC would cause a concomitant decrease in agonist-stimulated AA release in MDCK-D1 cells. For this purpose, sub-confluent cells were incubated in DMEM containing 0.05 % BSA (w/v). Thirty min prior to stimulation with either 1 μM BK, 10 μM A23187, 100 nM PMA, both PMA and BK, or both PMA and A23187, cells were preincubated with or without 2 μM BIS, a potent and very specific inhibitor of PKC (Martiny-Baron et al., 1993; Toullec et al., 1991). After stimulation for 10 min with the above-mentioned agents, sample media were collected and processed for [^{3}H]AA released. As shown in **Figure 6.15A**, calcium ionophore was able to induce a 4.8 ± 0.4-fold increase in AA released, which was not significantly altered by PKC inhibition in the presence of 2μM BIS (5.6 ± 0.7-fold increase). This result agrees with those observed in other cell types (Börsch-Haubold, 1995; Qiu and Leslie, 1994; Ambs et al., 1995) and indicates that the ability of ionophore to cause a release of AA does not depend upon PKC but predominantly upon the elevation of intracellular Ca^{2-} levels which enables $cPLA_2$ to translocate from the cytosol to membranes where it can access its substrate (Nalefski et al., 1994). On the other hand, activation of PKC by phorbol ester has been shown to lead to increased $cPLA_2$ activity (Xing and Insel, 1996) and AA release in MDCK wildtype cells following incubations of 30 min to 1 hr in length (Robinson et al., 1995; Parker et al., 1987).

Figure 6.15 Bisindolmaleimide inhibits phorbol ester-induced synergistic effect on arachidonic acid release but blocks neither A23187- nor bradykinin-stimulated arachidonic acid release

[^{3}H]AA-labelled cells were first preincubated with or without 2μM BIS for 30 min followed by a 10 min stimulation with or without 100nM PMA. Cells were then treated at 37°C with or without 1μM BK, 10μM A23187, 100nM PMA, both PMA and A23187 together (**A**), or both PMA and BK together (**B**) for 10 min. Media were removed and counted for [^{3}H] AA released by the cells. The amount of AA released is normalized for the total label incorporated into the cells and expressed as fold increase in release compared to control. The values represent the mean ± S.E.M. of 4 experiments performed in duplicate *, $P < 0.05$ vs no BIS pretreatment + PMA + A23187, or + PMA + BK.


A
No BIS
BIS 2μM
[3H] Arachidonic acid release
(fold increase compared to control)
12
10
8
6
4
2
0
*
BK
A23187
PMA
PMA + A23187


B
No BIS
BIS 2μM
[3H] Arachidonic acid release
(fold increase compared to control)
7.5
6
4.5
3
1.5
0
*
*
BK
PMA
PMA + BK

However, after 10 min of incubation with PMA alone, only a minor enhancement of AA release was presently found (1.1 ± 0.2-fold above control). This lack of effect could be explained on the basis that PMA fails to elicit any Ca^{2+} mobilization (Aboolian et al., 1989; Kast et al., 1993) and therefore would not provoke translocation of $cPLA_2$ to the membranes. One could picture that even in resting cells there is a limited pool of membrane $cPLA_2$ which turns over and is replaced by new cytosolic enzyme at the low calcium concentration prevailing under resting conditions. If the cytosolic enzyme becomes phosphorylated by a PKC-dependent mechanism, this would result in a slow increase in membrane activity and AA release. Therefore incubation periods, greater than 10 min are required for a sufficient proportion of the PMA-induced phosphorylated $cPLA_2$ pool to become associated with the cellular membranes under resting Ca^{2+} levels.

The idea that rapid release of AA requires Ca^{2+} influx is demonstrated when both PMA and A23187 are incubated together. The combination of these two agents causes a synergistic release of AA over and above that seen with ionophore alone, increasing from 4.8 ± 0.4-fold to 10.0 ± 0.8-fold upon addition of 100 nM PMA. The PMA-stimulated portion of the AA release was virtually eliminated by BIS, as seen by a return to levels similar to those obtained with ionophore alone (compare 5.6 ± 0.7-fold for A23187 + BIS, to 6.3 ± 0.9-fold for BIS + ionophore + PMA) thereby demonstrating the efficacy of this particular PKC inhibitor. In direct contrast, BIS was unable to exert any effect upon the AA liberated by BK alone as shown in **Figure 6.15B** (i.e., 2.6 ± 0.1 with BK versus 2.6 ± 0.1-fold with BK + BIS). In

addition, the synergistic effect observed when the combination of PMA and BK was employed was eliminated by pretreatment with BIS (compare 5.2 ± 1.1-fold for BK + PMA, to 3.2 ± 0.9-fold for BIS + BK + PMA). Therefore, in spite of being able to eliminate the PMA-induced synergistic release of AA in combination with ionophore or BK, BIS was incapable of blocking that observed with either BK or A23187 alone.

6.3.2 *Determination of the effects of long-term down regulation of protein kinase C by phorbol ester upon both bradykinin- and A23187-induced arachidonic acid release*

In contrast to the lack of effect by chemical inhibitors upon BK-mediated AA release, down regulation of PKC via long-term exposure to phorbol ester has consistently yielded results which argue for the involvement of this serine/threonine kinase in the signalling mechanism used by BK to liberate AA. However, this protocol may have undesirable secondary effects upon other signalling pathways and the various enzymes required therein (Ricanati and Horwitz, 1992). In order to determine whether long-term pretreatment of cells with 100 nM PMA could exert negative effects upon AA release, both BK and A23187 were used. It should be noted that preliminary experiments were carried out in which cells were either down regulated with PMA for 24 hrs, followed by a 12 to 16 hr labelling period, or were labelled with [^{3}H]AA prior to the addition of PMA, or alternatively labelled in the presence of PMA for 24 hr. Regardless of the protocol employed, the qualitative nature of the effects were not significantly different from one another. Nevertheless, the protocol of down regulation followed by overnight labelling with [^{3}H]AA was chosen as it gave the most consistent results. The action of calcium ionophore and the onset responses of BK, as demonstrated earlier, do

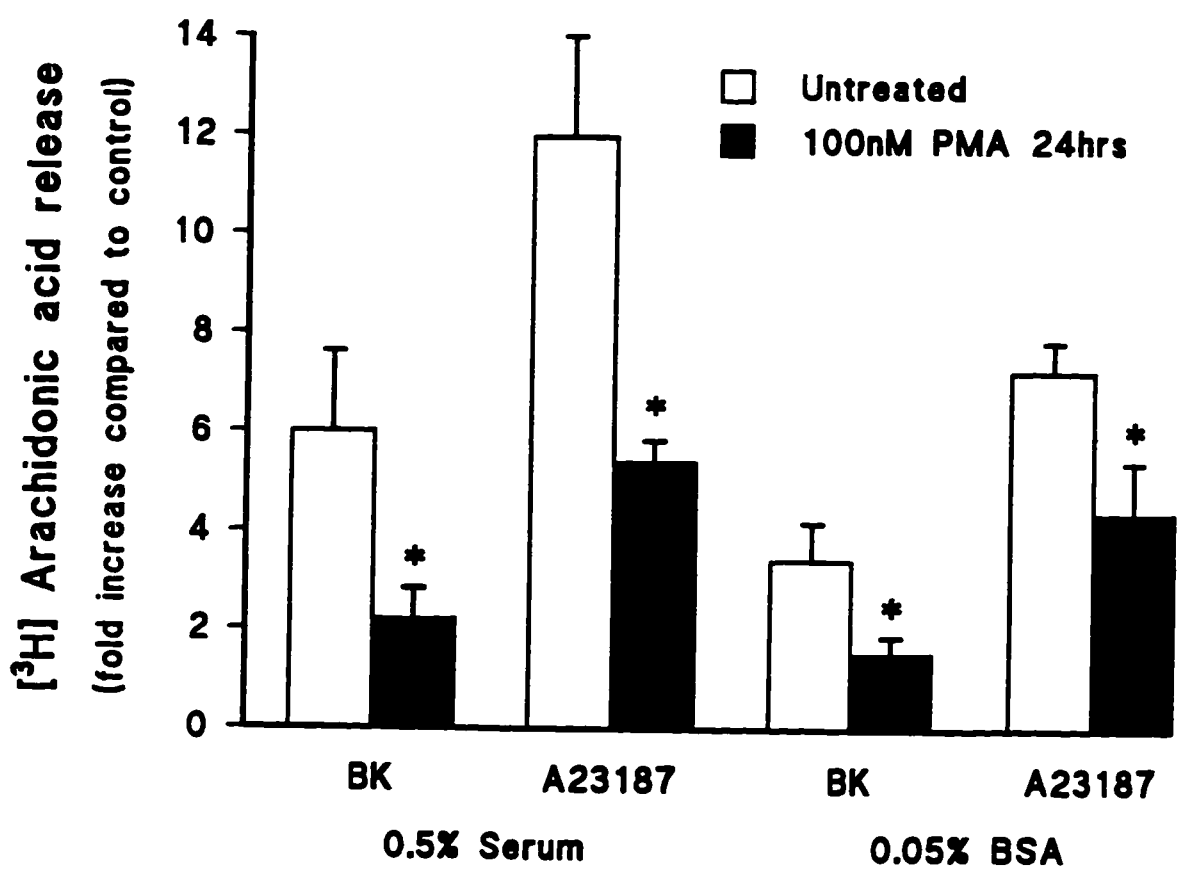
not require PKC mediation but rely heavily instead, upon their ability to increase intracellular Ca^{2+}. Therefore, one would expect that long-term down regulation of PKC levels should not alter A23187-stimulated AA release. However, as depicted in **Figure 6.16**, ionophore-induced release of AA was significantly inhibited in a manner not unlike that observed for BK (i.e., from 3.4 ± 0.8-fold above control for BK, to 1.6 ± 0.3-fold for PMA+ BK; from 7.2 ± 0.6-fold for A23187 alone, to 4.4 ± 1.0-fold for PMA + A23187). Qualitatively similar results were obtained when the cells were preincubated for 24 hrs with 100 nM PMA in DMEM-containing 0.5 % FCS (**Figure 6.16**). From these results, it would appear that a secondary, non-specific effect of long-term down regulation of PKC might account for the observed inhibition of both A23187- and BK-mediated AA release. It was therefore hypothesized that one target might be $cPLA_2$ itself. It will be noticed also from **Figure 6.16** that the presence of serum greatly enhanced the effects of BK on AA release and that this enhancement was abolished by down regulation of PKC. One could suggest from this that serum contains agonists which cause responses at the level of $cPLA_2$ mediated by PKC. This phenomenon is further described in the section that follows.

6.3.3 *Examination of the effects of long-term down regulation of protein kinase C by phorbol ester upon basal in vitro $cPLA_2$ activity*

In order to determine whether $cPLA_2$ itself was being affected by phorbol ester-mediated down regulation of PKC, the $cPLA_2$ activity was measured in cell lysates obtained from cells grown in the presence and absence of serum but not stimulated by BK. Serum has growth factors and other agonists which, by activating signalling systems, would be expected

Figure 6.16 Long-term down regulation of protein kinase C by phorbol ester inhibits both bradykinin and A23187-induced arachidonic acid release

MDCK-D1 cells were incubated for 24 hrs with or without 100nM PMA and then labelled overnight with [^{3}H]AA in DMEM containing 0.05% BSA (w/v) or 0.5% FCS. Cells were washed with HBSS + 0.05% BSA to remove unincorporated label and then preincubated for 10 min in wash HBSS to equilibrate the cells. Stimulations were carried out for 10 min at 37°C using 1μM BK or 10μM A23187. The amount of label released is divided by the total incorporation to normalize for differences in cell number and is then expressed as fold increase in release above control and represents the mean ± S.E.M. of 4 independent experiments performed in duplicate. *, $P < 0.01$ vs respective untreated controls.


[3H] Arachidonic acid release
(fold increase compared to control)
14
12
10
8
6
4
2
0
Untreated
100nM PMA 24hrs
*
*
*
*
BK
A23187
BK
A23187
0.5% Serum
0.05% BSA

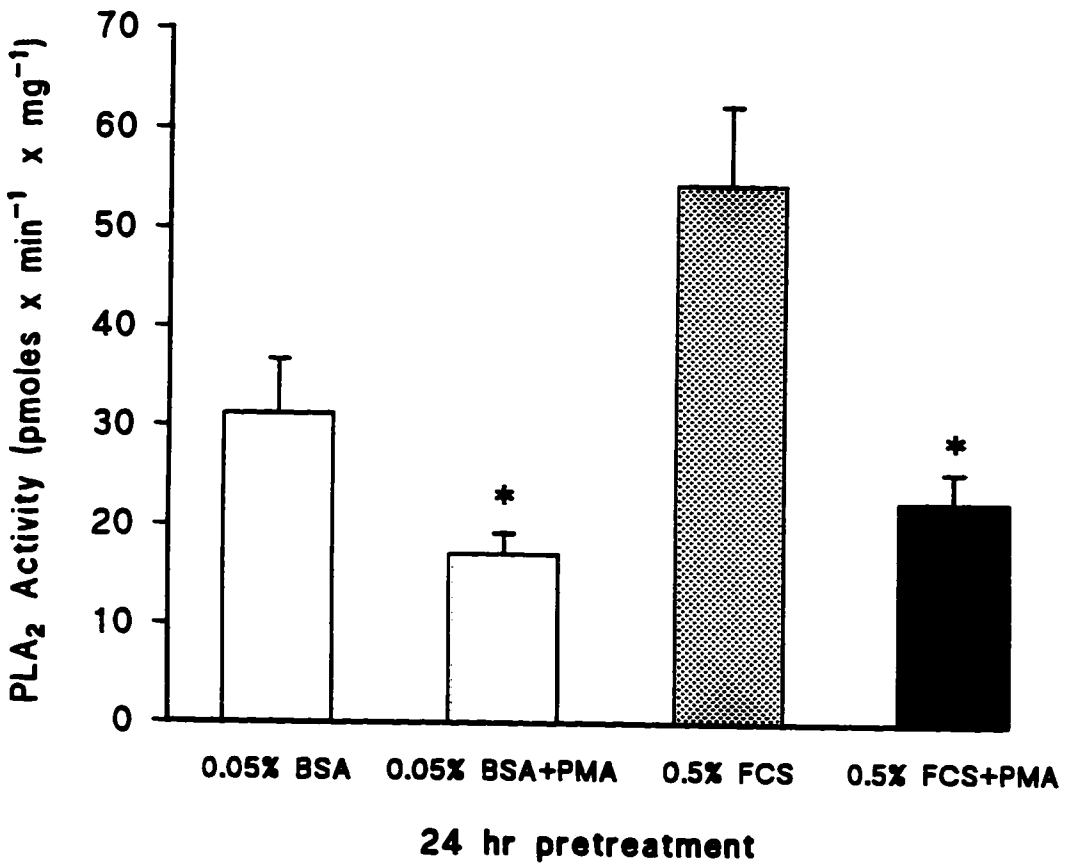
to keep phosphorylation levels of enzymes including PLA_2 at a certain steady state. As shown in **Figure 6.17**, preparations from cells which had been serum-starved (i.e., incubated in DMEM containing 0.05 % BSA) in order to minimize basal $cPLA_2$ phosphorylation, displayed a much lower degree of activity when compared to lysates derived from cells exposed overnight to DMEM containing 0.5 % FCS (i.e., 54.4 ± 7.9 pmoles x min^{-1} x mg^{-1} for 0.5 % FCS, compared to 31.2 ± 5.5 pmoles x min^{-1} x mg^{-1} for 0.05 % BSA). This result agrees with that observed in the arachidonate release studies in which an enhancing effect of serum on ionophore- and BK-induced release could be observed (**Figure 6.16**). Again, pretreatment for 24 hrs with 100 nM PMA caused a significant decrease in PLA_2 activity of cell lysates, reaching levels which were very comparable in serum-starved and serum-treated cells (i.e., 22.5 ± 2.9 pmoles x min^{-1} x mg^{-1} for PMA + 0.5 % FCS versus 17.1 ± 2.1 pmoles x min^{-1} x mg^{-1} for PMA + 0.05 % BSA). These results suggest that serum is able, in this particular case, to maintain a higher state of either phosphorylation, or level of expression of $cPLA_2$, over and above that seen following serum-starvation, and that long-term exposure to PMA all but abolishes this effect. This reduction in activity of lysates by PMA is comparable to that seen for BK-induced AA release from cells preincubated overnight in the presence of PMA, with or without serum.

6.3.4 *Examination of the levels of $cPLA_2$ following long-term down regulation of protein kinase C by phorbol ester as measured by Western blotting*

To determine whether long-term incubation of MDCK-D1 cells with PMA causes a

Figure 6.17 Long-term down regulation of protein kinase C levels reduces basal *in vitro* cytosolic phospholipase A_2 activity derived from cell lysates

Cells grown on 100mm dishes were incubated overnight in DMEM containing either 0.05% BSA or 0.5% FCS with or without 100nM PMA. Cells were then rinsed 2 times with ice-cold Tris buffer (50mM Tris, pH 7.4, 250 mM sucrose, 1mM EGTA) and scraped off the plate. Cells were resuspended and sonicated in 50mM Tris, pH 7.4, containing, 250mM sucrose, 1mM EGTA, 1mM PMSF, 10 µg/ml leupeptin, 50mM NaF, 1mM $NaVO_3$, 10mM sodium pyrophosphate. Cells lysates were then normalized for protein content and assayed for PLA_2 activity. Results are the means ± S.E.M. of 4 independent experiments performed in duplicate. *, $P < 0.01$ vs samples without PMA pretreatment.


70
60
50
40
30
20
10
0
PLA2 Activity (pmoles x min-1 x mg-1)
*
*
0.05% BSA
0.05% BSA+PMA
0.5% FCS
0.5% FCS+PMA
24 hr pretreatment

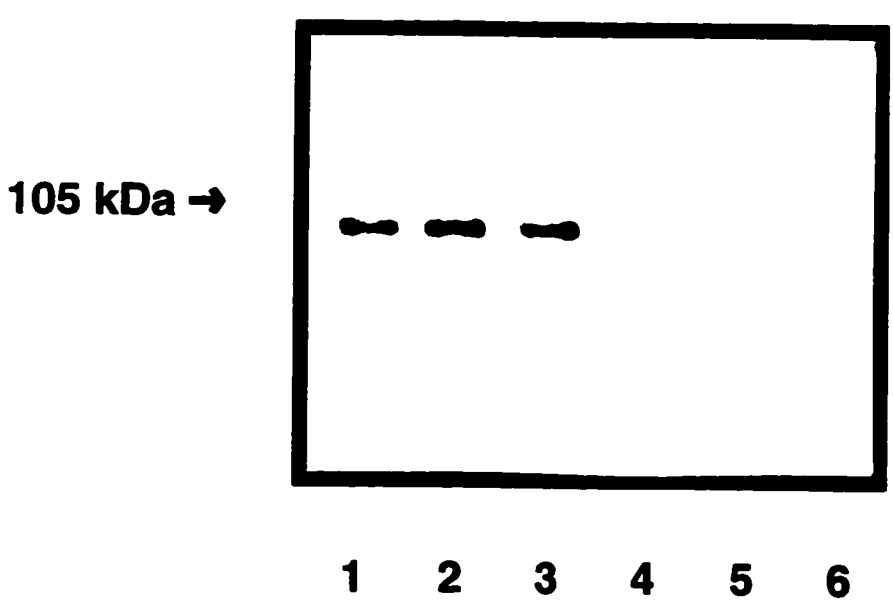
reduction in the expression level of $cPLA_2$, Western blots were performed on cell lysates by means of a rabbit polyclonal antibody directed against the 85 kDa form of phospholipase A_2. As shown in **Figure 6.18**, down regulation with 100 nM PMA did not alter $cPLA_2$ expression in MDCK-D1 cells. Furthermore, cells that were serum-starved failed to display any significant differences in $cPLA_2$ levels compared to those in serum-treated cells. These results demonstrate unequivocally that the decrease of basal $cPLA_2$ activity is not due to a reduction in the expression level of this phospholipase.

6.3.5 *Determination of the level of basal phosphoserine phosphorylation of $cPLA_2$ following long-term phorbol ester pretreatment*

A likely explanation for the observed decrease of $cPLA_2$ activity brought about through long-term PMA exposure is that of a reduction in the phosphorylation state of the phospholipase. It is well established that the activity of $cPLA_2$ can be enhanced by phosphorylation of certain serine residues such as Ser505 (Lin et al., 1992; Lin et al., 1993). Indeed, it was demonstrated *in vitro* that MAPK can carry out this phosphorylation of Ser505, an event which increases the enzyme's ability to hydrolyse substrate (Lin et al., 1993). One could hypothesize then, that basal PKC activity, being enhanced through the signalling induced by the presence of serum, might serve tomaintain a variable portion of the $cPLA_2$ in a phosphorylated state through its positive effects on the MAPK cascade (Wang et al., 1996). Alternatively, MAPK could be activated by PKC-independent signalling. In order to determine whether long-term down regulation of PKC levels could reduce the basal phosphorylation state of $cPLA_2$, Western blotting of $cPLA_2$ immunoprecipitates was performed with a rabbit

Figure 6.18 **Long-term down regulation of protein kinase C levels does not alter cytosolic phospholipase A_2 expression**

Cells grown on 100mm dishes were incubated overnight in DMEM containing either 0.05% BSA (w/v) or 0.5% FCS with or without 100nM PMA. Cells lysates were then normalized for protein content and 2x Laemmli buffer was added. Western blots were prepared with a specific rabbit polyclonal $cPLA_2$ antiserum developed with the Amersham ECL system. Lanes 1-3 were loaded with 10 µg protein while lanes 4-6 were loaded with 5 µg protein. Lanes 1 and 4, DMEM + 0.05% BSA; lanes 2 and 5, DMEM + 0.5% FCS; lanes 3 and 6, DMEM + 0.5% FCS + 100nM PMA.


105 kDa
1
2
3
4
5
6

polyclonal antibody directed specifically towards phosphoserine residues. As depicted in **Figure 6.19**, down regulation of PKC levels by 100 nM PMA significantly reduced the phosphoserine content of $cPLA_2$.

6.3.6 *The effect of inhibiting mitogen-activated protein kinase kinase on bradykinin-stimulated arachidonic acid release*

The requirement of phosphorylation of $cPLA_2$ in mediating the enhanced AA release by certain agonists has been reported for a number of cell types and signalling agents. Typically, an agonist which depends upon PKC for AA release will likewise require MAPK-mediated phosphorylation of $cPLA_2$, effectively receiving the signal through the sequential action of RAF-1, mitogen-activated protein kinase kinase (MAPKK) and finally, MAPK. Despite the conclusion from the present study that onset responses to BK stimulation in regards to AA release are independent of PKC activity, one cannot exclude the involvement of MAPK since PKC-independent signalling routes have been recently reported in other cell types which link G-proteins with MAPK (Araki et al., 1995; Kasuya et al., 1994; Kruger et at., 1995). In order to determine whether BK-stimulated AA release was dependent upon MAPK activation, a potent and specific MAPKK inhibitor, PD 98059 was employed. As demonstrated in **Figure 6.20**, this agent significantly reduced the ability of BK to elicit AA release from MDCK-D1 cells. This observation is similar to that obtained with epinephrine as agonist by Xing and Insel (1996). However, in the case of epinephrine, AA release was shown to require PKC activation at a step proximal to MAPK recruitment. Moreover, since BK-mediated AA release occurs

Figure 6.19 Long-term down regulation of protein kinase C levels reduces phosphoserine content of cytosolic phospholipase A_2

Cells grown on 100mm dishes were incubated overnight in DMEM containing 0.5% FCS with or without 100nM PMA. Cells lysates were then normalized for protein content and immunoprecipitated overnight with a rabbit polyclonal $cPLA_2$ antiserum coupled to protein A agarose. Immunoprecipitate complexes were washed 5x with lysis buffer and dissociated in 2x Laemmli buffer at 100°C for 5 min. Western blotting was carried out with 100 µg of protein and $cPLA_2$ which had been phosphorylated on serine residues was detected using a specific rabbit polyclonal antibody directed against phosphoserine residues. Blots were developed with the Amersham ECL system. The sample in lane 1 is derived from cell lysate that was treated overnight with 0.5% FCS, in lane 2 with 100 nM PMA in the presence of 0.5% FCS while that in lane 3 was treated with 0.05% BSA and lane 4 with 0.05% BSA in the presence of 100 nM PMA

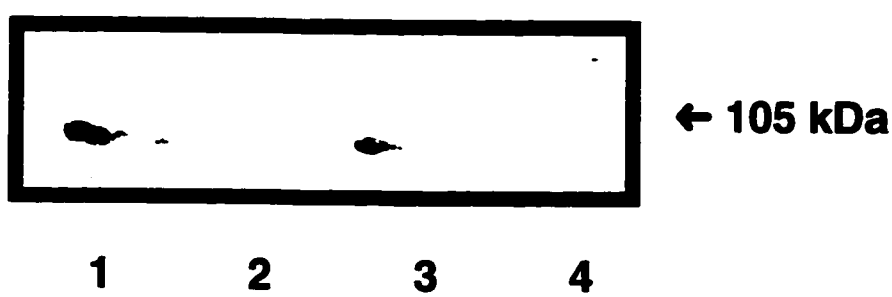
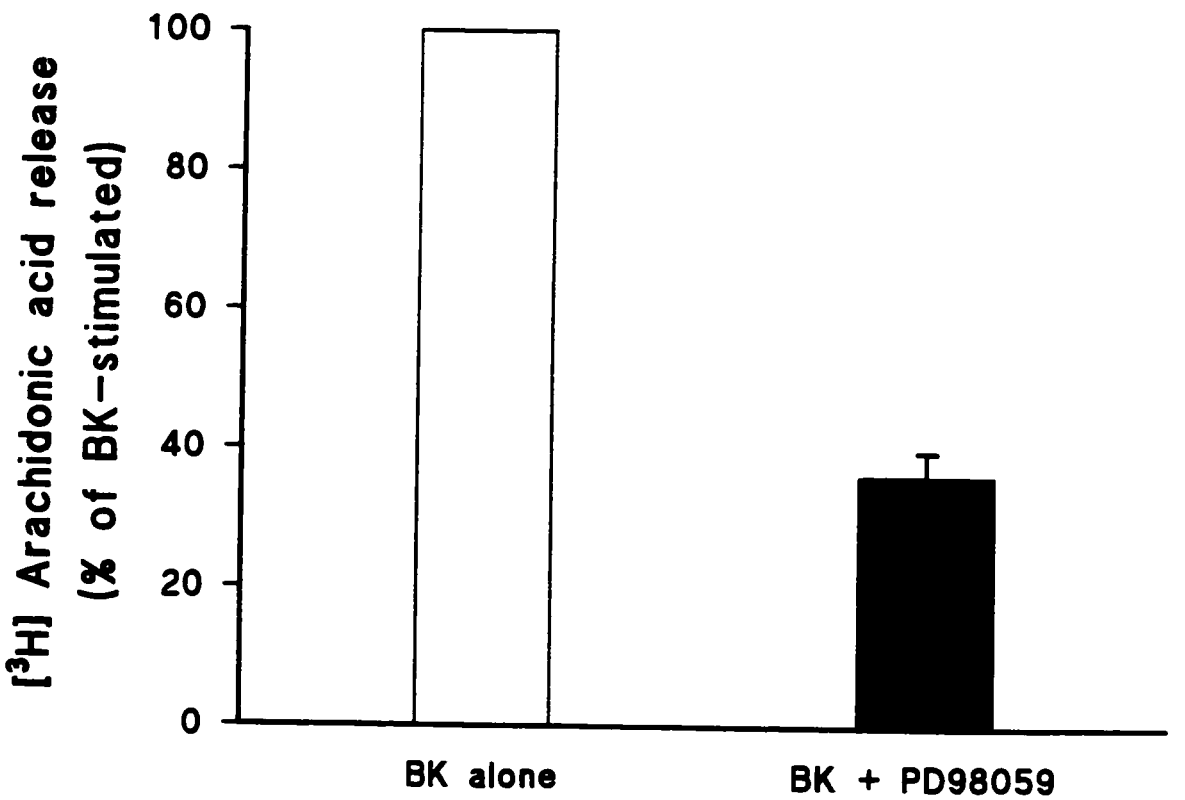

← 105 kDa
1
2
3
4

Figure 6.20 The effect of PD 98059 on bradykinin-mediated arachidonic acid release from MDCK-D1 cells

[^{3}H]AA-labelled cells were preincubated with or without 30μM PD 98059 for 10 min followed by 10 min treatment with or without 1μM BK at 37°C. The amount of AA released is normalized for the total label incorporated into the cells and expressed as fold increase in release compared to control and represents the mean ± S.E.M. of 4 independent experiments performed in duplicate. $P < 0.001$.


[3H] Arachidonic acid release
(% of BK−stimulated)
100
80
60
40
20
0
BK alone
BK + PD98059

independent of PKC activation, an alternate signalling route employing MAPK may prevail in this case. This possibility remains unexplored at this moment, but could involve BK-induced G-protein activation of the *ras* pathway.

6.4 Negative regulation of bradykinin-mediated signalling pathways

In addition to factors exerting positive control as described in earlier sections, there is evidence suggesting that certain elements of negative control are at work to influence release of AA. Hassid (1983) demonstrated that ionophore-stimulated PGE_2 production from MDCK wildtype cells could be blunted following elevations of intracellular cAMP. This method of inhibition could represent a physiological mechanism of negative feedback promoted by agents which increase cAMP levels within the cells of the distal tubule and collecting duct. Therefore, experiments described in the following sections were carried out to further elucidate such negative regulatory effects upon the BK signalling pathways.

6.4.1 *Effects of forskolin, arginine vasopressin and bradykinin on intracellular cyclic adenosine monophosphate levels*

Experiments were performed to determine whether cAMP levels could be adequately raised in MDCK-D1 cells under the experimental conditions provided. The levels of intracellular cAMP were examined at two time points following treatment with 10μM FSK (**Table 6.3**) and were found to increase significantly within the first 20 min of incubation and remained elevated even after 60 min. Acting through the V_2-receptor, AVP increased cAMP levels after 20 min of incubation but levels returned to control values after 60 min. At a 1 μM

Table 6.3 **Effects of forskolin, arginine vasopressin, and bradykinin on cyclic adenosine monophosphate production after 20 and 60 minutes of incubation**

MDCK-D1 cells were incubated 20 and 60 min at 25°C in the presence of 0.5mM IBMX along with either 10μM FSK, 1μM AVP or 1μM BK. Assays were determined in duplicate and the results are expresssed as means ± S.E.M. (n=3). a = $P < 0.05$ vs. control; n.s. = non-significant compared to control.

Treatment	cAMP produced (pmoles/well/20min)	cAMP produced (pmoles/well/60min)
control	19.4 ± 4.5	21.1 ± 6.6
BK	12.8 ± 2.0[n.s.]	27.3 ± 4.5[n.s.]
AVP	29.1 ± 1.0[a]	17.1 ± 2.6[n.s.]
FSK	42.4 ± 1.7[a]	45.6 ±2.3[a]

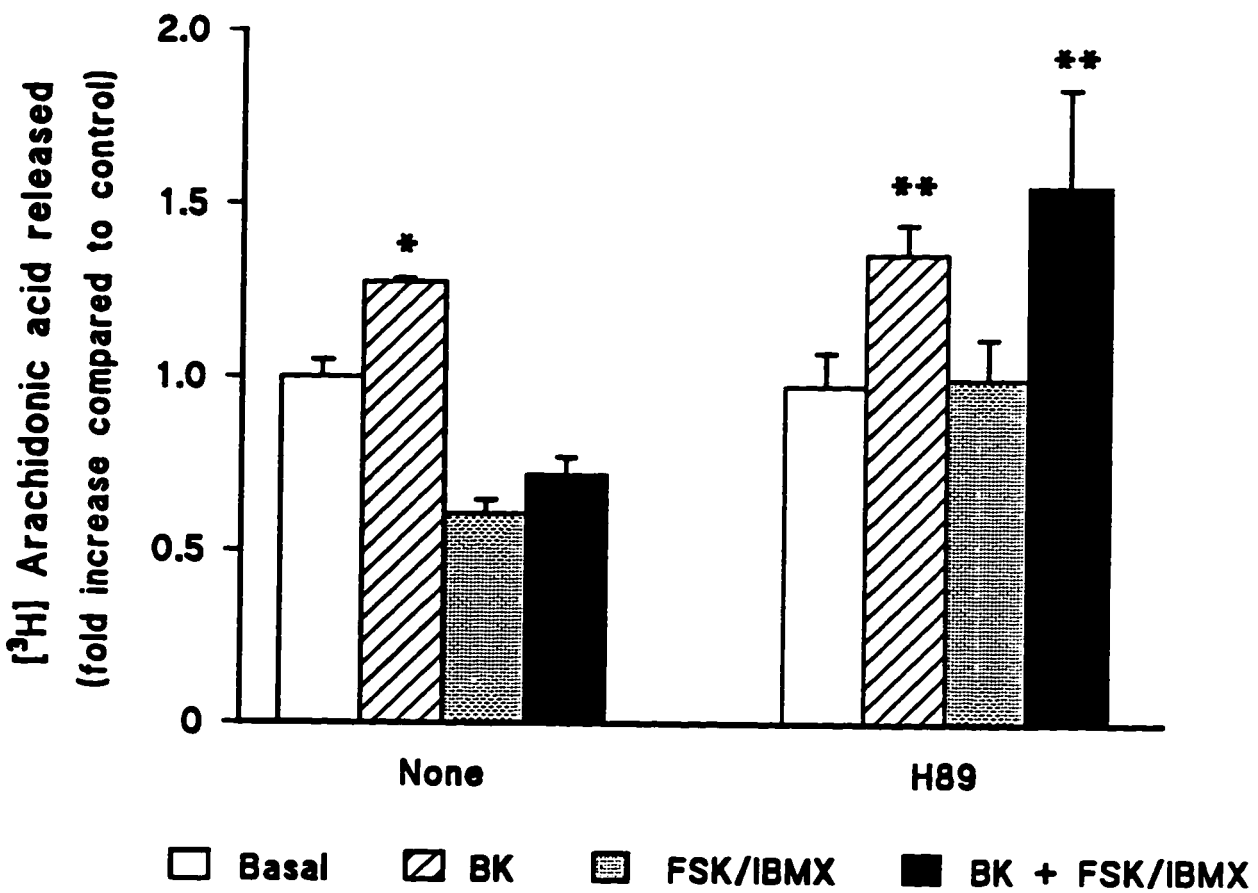
concentration, BK failed to significantly alter cAMP levels at either 20 or 60 min. It was concluded on the basis of these results that a 20 min incubation with either FSK + IBMX, or AVP would be suitable to achieve activation of PKA and that BK in the concentration range to be used in further experiments would not affect the activity of this enzyme.

6.4.2 *The effects of altering cAMP-dependent protein kinase activity on the release of labelled arachidonic acid*

An investigation was made of whether or not PKA could play a regulatory role with respect to AA release in MDCK-D1 cells. In early experiments dealing with AA release, conditions differed slightly from those of later experiments. In the former case, there was the use of DMEM as opposed to HBSS for both preincubations and incubations. Also incubations carried out in DMEM were performed at room temperature instead of 37°C. It was subsequently determined that only the quantitative but not the qualitative aspects of the results were altered by adopting the later conditions. The next experiments described performed with DMEM at room temperature were retained. In these experiments, labelled cells were preincubated for 20 min with FSK + IBMX, and subsequently stimulated with BK for 20 min. As shown in **Figure 6.21**, BK significantly stimulated the release of labelled arachidonate (1.27 ± 0.10-fold above control) while a 20 min preincubation with FSK + IBMX inhibited basal AA release and decreased the BK effect by more than half. The inhibitory effects of cAMP-elevating agents on AA release were completely blunted by the PKA inhibitor, H-89.

Figure 6.21 Effect of protein kinase A activation and inhibition on arachidonic acid release under various conditions

Cells, equilibrium-labelled with [^{3}H]AA were preincubated for 60 min with 30μM H-89. Twenty minutes prior to the completion of the preincubation period, 10μM FSK + 0.25mM IBMX was also added. Upon completion of all pretreatments, the cells were washed with DMEM containing 0.05% BSA to remove unincorporated label and were subsequently stimulated with 1μM BK at 25°C for 20 min. Results are expressed as the ratio of [^{3}H]AA in the media of treated cells compared to that of non-treated cells. The untreated cells constitute the basal conditions and represent the means ± S.E.M. of 3 experiments (n=3). Significant difference from untreated control: * $P < 0.001$. Significant difference from basal + H-89 or FSK + IBMX + H-89: ** $P < 0.05$.


[3H] Arachidonic acid released
(fold increase compared to control)
2.0
1.5
1.0
0.5
0
*
**
**
None
H89
Basal
BK
FSK/IBMX
BK + FSK/IBMX

6.4.3 *Effect of increased cyclic adenosine monophosphate production on resting in vitro phospholipase A_2 activity*

The fact that the preceding results demonstrate a decreased basal release of AA following elevations of intracellular cAMP implies that modifications affecting $cPLA_2$ itself may occur subsequent to PKA activation. Further evidence for this possibility was obtained when cells were incubated for 20 min with FSK + IBMX, lysed and the PLA_2 activity determined. As shown in **Figure 6.22**, elevations of intracellular cAMP caused a significant decrease (i.e., 56 %) of the resting *in vitro* PLA_2 activity. Such short term elevations of cAMP (i.e., 20 min) had no effect on expression levels of $cPLA_2$ (results not shown).

6.4.4 *The effect of stimulating cAMP-dependent protein kinase on phosphatidylethanol production*

In order to determine whether PKA activation could inhibit signalling elements located upstream from $cPLA_2$, the transphosphatidylation product of PLD activity was measured. As shown in **Figure 6.23**, PEt accumulation, in response to BK was significantly reduced subsequent to elevations of intracellular cAMP by preincubations with FSK + IBMX (i.e., BK alone, 1.00 ± 0.12 % of total dpm incorporated, vs. BK + FSK + IBMX, 0.69 ± 0.08 % of total dpm incorporated, $P < 0.01$). Interestingly, FSK + IBMX caused a small but significant increase in the basal production of PEt (i.e., control, 0.32 ± 0.04 % of dpm incorporated, vs. FSK + IBMX, 0.51 ± 0.10 % of dpm incorporated).

Figure 6.22 . The effect of protein kinase A activation *in vivo* on phospholipase A_2 activity

Subconfluent MDCK-D1 cells were incubated for 20 min at 37°C in DMEM with or without 10 μM FSK + 0.5 mM IBMX. Cells were subsequently lysed and assayed for PLA_2 activity for 1 hr at 37°C. The results are expressed as the number of pmoles of AA liberated per minute per mg of protein. Values are the means ± S.E.M. of at least 4 separate experiments (n=4) performed in duplicate. * $P<0.05$, vs. control.

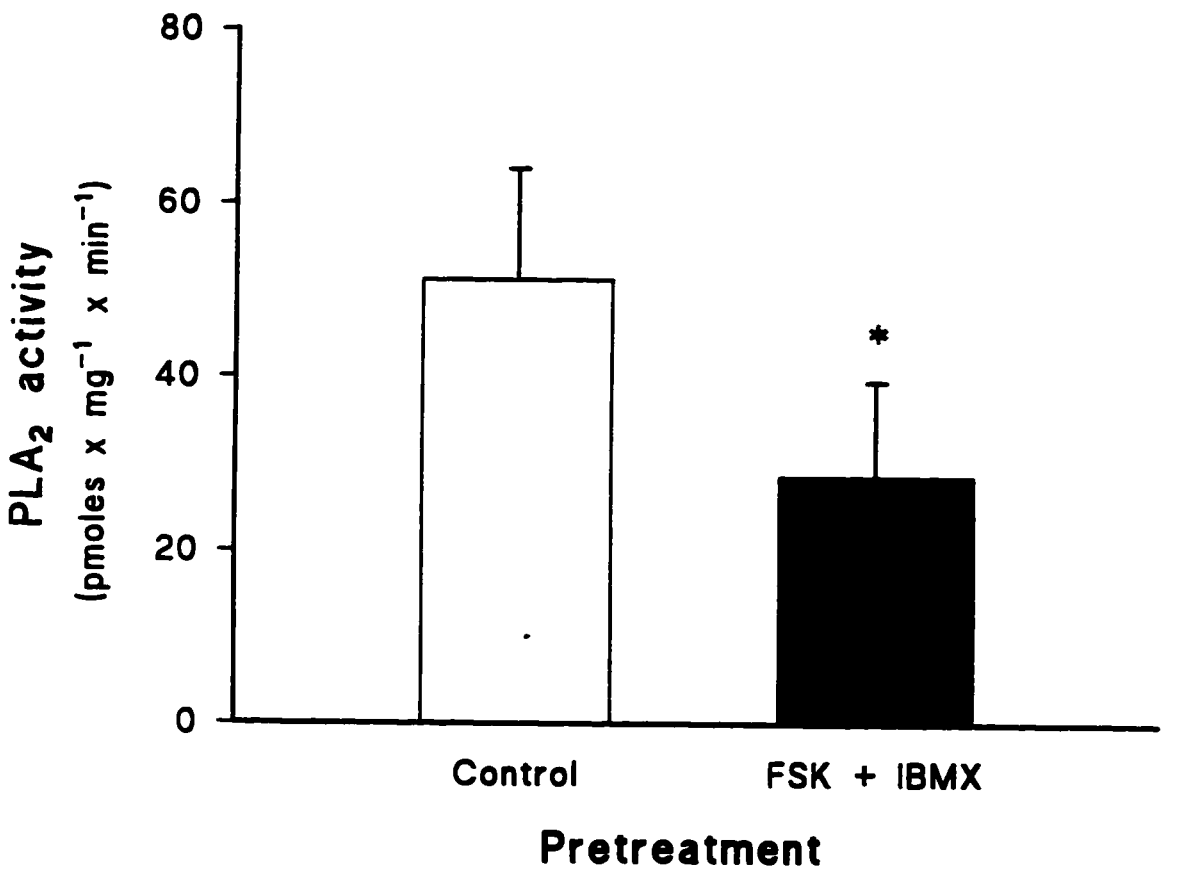

80
60
40
20
0
PLA2 activity
(pmoles x mg-1 x min-1)
*
Control
FSK + IBMX
Pretreatment

Figure 6.23 The effect of protein kinase A activation on bradykinin-stimulated phosphatidylethanol production

Subconfluent cells were labelled overnight with 2.0 μCi [^{3}H]palmitic acid. These cultures were then preincubated for 20 min with 10 μM FSK + 0.5 mM IBMX and subsequently stimulated with 1 μM BK for 1 min at 37°C (2% EtOH was added to measure the transphosphatidylation reaction). The results are expressed as the percent of the total labelled [^{3}H]palmitic acid incorporated into the phospholipid pool. The data reported are the means ± S.E.M. of at least 4 separate experiments performed in duplicate.* $P < 0.05$ vs. unstimulated control.

[3H] PEt production
(% of total dpm incorporated)
1.5
1.0
0.5
0
No BK
BK
*
Control
FSK + IBMX

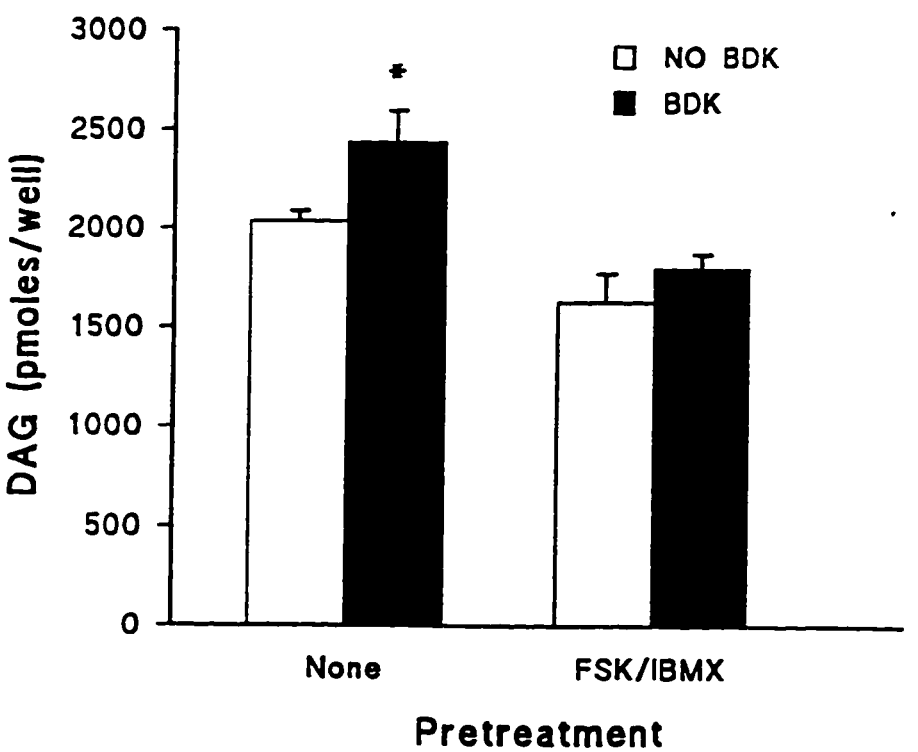
6.4.5 *The effect of stimulating cAMP-dependent protein kinase on diacylglycerol production*

To substantiate the role of PKA in the regulation of PLC, the effect of preincubation with FSK + IBMX on DAG production was examined (**Figure 6.24**). Asmentioned earlier, BK-derived DAG can originate from three different sources: 1) through the action of PI-PLC upon phosphatidylinositol-4,5-bisphosphate; 2) through the sequential action of PLD and PAP; 3) through the recruitment of PC-PLC which uses phosphatidylcholine as its substrate. However, as shown earlier, the predominant enzyme responsible for DAG production as messenger source for early (i.e.,< 1min) activation of PKC in MDCK-D1 cells in response to BK stimulation, is PC-PLC, since D609 was able to significantly blunt BK-induced PKC activity and BK-generated responses are not affected by neomycin.

In the absence of PKA-stimulating conditions, BK significantly enhanced the formation of DAG as was expected from the results of the preceding experiment and this further supported the involvement of PC-PLC. However, when cells were preincubated with FSK + IBMX, the BK effect was almost completely abolished. Therefore, it appears again that conditions which enhance PKA activity elicit a negative regulatory effect on PLC activity in MDCK-D1 cells as could be assessed by examining water-soluble and lipid-soluble products of this enzyme.

Figure 6.24 Effect of activating protein kinase A on diacylglycerol production under various conditions

Serum-starved MDCK-D1 cells were preincubated 20 min with or without 10μM FSK + 0.5mM IBMX. They were then, either stimulated with 1μM BK or treated with vehicle for 40 min at 25°C. Diacylglycerol was determined with DG kinase. Results are the means ± S.E.M. (n=5), * $P < 0.01$.

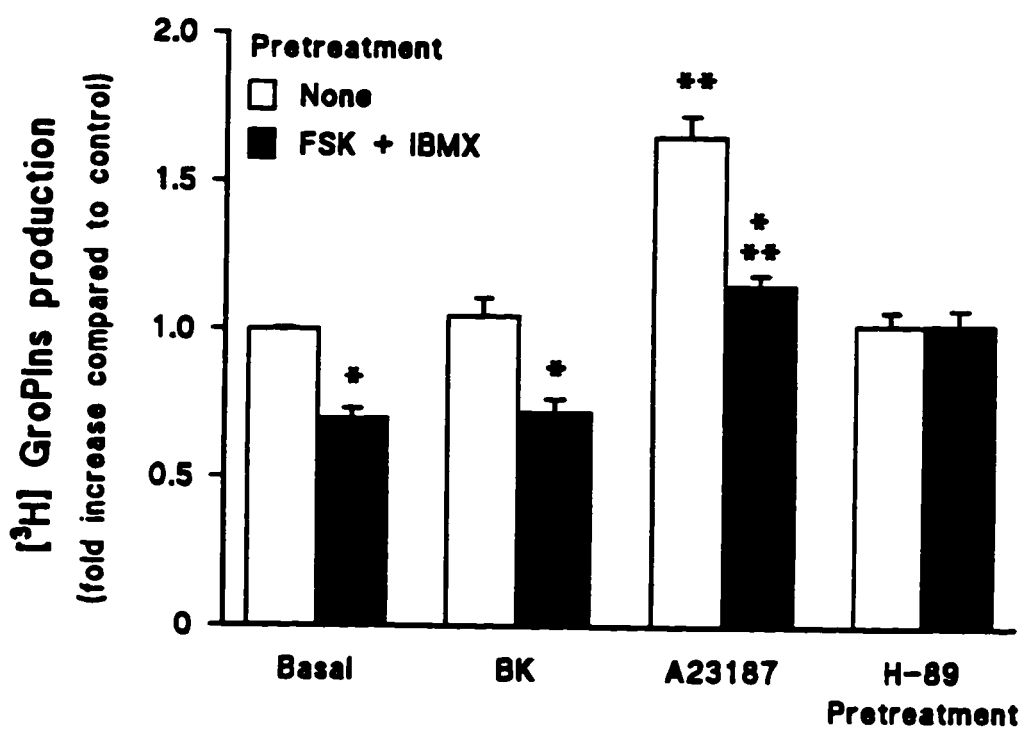

3000
2500
2000
1500
1000
500
0
DAG (pmoles/well)
NO BDK
BDK
*
None
FSK/IBMX
Pretreatment

6.4.6 *Effect of increased cyclic adenosine monophosphate production on glycerophosphoinositol production*

Products characterizing PLA_2 activity, apart from AA are the deacylated glycerophosphodiesters. The latter can arise only from PLA activity as the initial step in the degradation of phospholipids. They do not allow the distinction between PLA_1 and PLA_2 activity, nor do they preclude the involvement of phospholipase B. Accumulation of radioactive glycerophosphoinositol in [^{3}H]inositol-labelled cells would report on only a fraction of the total PLA_2 activity which in MDCK cells is involved with other phospholipids as well (Daniel et al., 1981). This is probably why only a small non-significant enhancement of GroPIns production resulting from BK stimulation was observed (**Figure 6.25**). However, the cAMP-elevating agents decreased the production GroPIns in resting (control) and BK-treated cells. The calcium ionophore, A23187, substantially enhanced GroPIns production by greater than 50 %. Interestingly, preincubation for 20 min with FSK + IBMX significantly reduced the ionophore-stimulated GroPIns production by more than 65 %. As well, H-89 was found to restore GroPIns to normal resting levels in the presence of FSK + IBMX. The ability of increased PKA activity to blunt both AA release and GroPIns production strengthens the argument that it is a PLA activity that is reduced following increased in PKA activity and not the combined activity of other enzymes displaying as a sum, PLA-like activity resulting in AA release. Since most of the AA releasing activity of MDCK-D1 cells was shown to be due to $cPLA_2$, one can surmise that it is this form of the enzyme that ismainly affected by PKA.

Figure 6.25 Effect of protein kinase A activation and inhibition on glycerophosphoinositol production under various conditions

MDCK-D1 cells were equilibrium-labelled with [^{3}H]-myo inositol. Ten micromolar FSK + 0.5 mM IBMX was added for a pretreatment of 20 min. Cells were then stimulated for 40 min with either 1μM BK or 10μM A23187 at 25°C. In some trials, the addition of these latter agents was preceeded by a 60 min incubation with 30μM H-89. Incubations were terminated by addition of ice-cold methanol. The aqueous portion of the Bligh and Dyer extraction was applied to anion exchange columns to separate inositol phosphates. Counts of GroPIns are normalized for total incorporation into the cells while the results are expressed as the ratio of [^{3}H]AA in media of treated cells compared to that of non-treated cells. The untreated cells constitute the basal conditions and the values are expressed as the means ± S.D. (n=3), * $P < 0.01$; ** $P < 0.001$ compared to basal control; *** $P < 0.001$ compared to A23187 alone.


2.0
1.5
1.0
0.5
0
[3H] GroPIns production
(fold increase compared to control)
Pretreatment
None
FSK + IBMX
*
*
**
*
**
Basal
BK
A23187
H-89
Pretreatment

6.4.7 *The effect of stimulating cAMP-dependent protein kinase on accumulation of inositol phosphates*

The question of whether the effect of PKA is specific for the signalling pathway required for AA release was examined. This possibility was addressed by measuring the products of a phospholipase not involved in BK-stimulated AA release, namely, PI-PLC. Accordingly, [^{3}H]inositol-labelled cells were preincubated with cAMP-elevating agents (**Figure 6.26**) and treated with BK for 10 sec at 37°C which resulted in a stimulation of $InsP_3$ production corresponding to 1.45 ± 0.13-fold above control values. This increase was significantly inhibited by a 20 min pretreatment of cells with FSK + IBMX (1.1 ± 0.1-fold above control). Longer incubations of 40 min with BK produced a very similar stimulation of the more readily measurable breakdown product of $InsP_3$, labelled InsP (1.30 ± 0.05-fold above control, cf **Figure 6.27**) which again was inhibited by cAMP-elevating conditions. The effects of cAMP-elevating agents were blunted by H-89, indicating the involvement of PKA. Under these prolonged incubation conditions, $InsP_3$ production could not be reliably measured but InsP formation, being more extensive, was readily measurable with less variation than $InsP_3$ levels after 10 sec incubations. Since the more easily measured changes in InsP reflected those of $InsP_3$, the metabolism of which could be affected by cAMP levels, changes in PI-PLC activity in subsequent experiments were based on measurement of InsP production. To test whether this cAMP-dependent effect could be mediated also through stimulation of an adenylate cyclase-coupled receptor, InsP production was measured after preincubation with AVP. As for that of the other agents, the hormonal effect was to decrease BK-stimulated InsP accumulation (i.e., AVP + BK, 0.93 ± 0.04-fold of control, $P < 0.0001$). The basal inositol

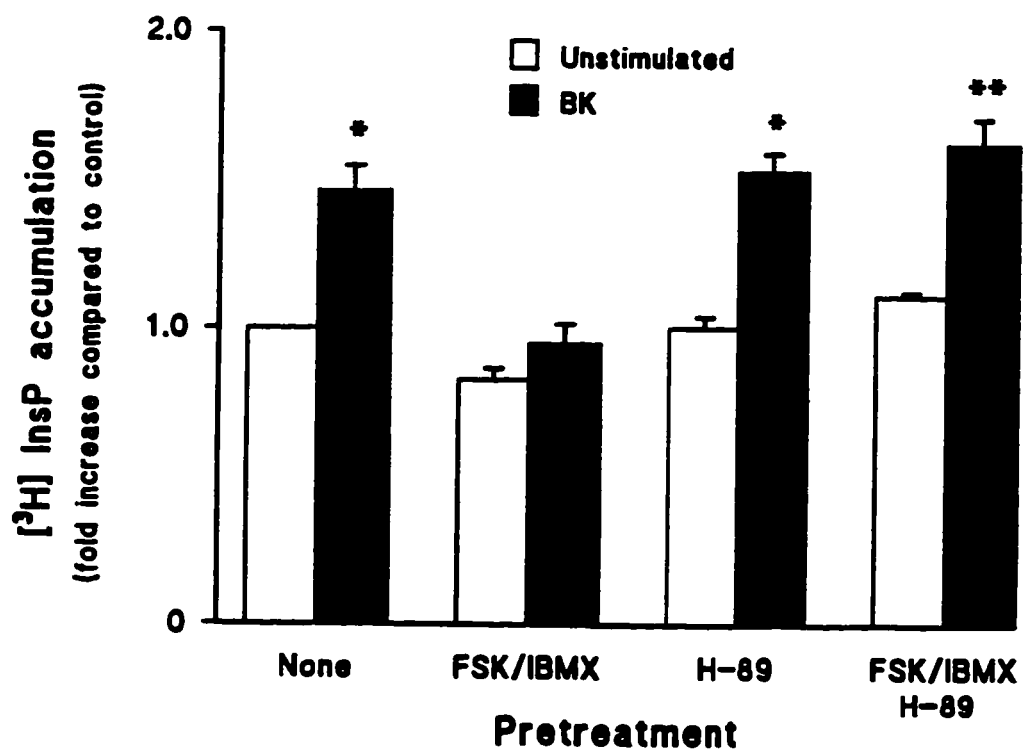
Figure 6.26 Effects of protein kinase A activation on bradykinin-stimulated inositol trisphosphate production

MDCK-D1 cells equilibrium-labelled with [^{3}H]myo-inositol were preincubated for 20 min in DMEM containing 10μM FSK + 0.5mM IBMX. Cells were stimulated with 1μM BK for 10 sec at 37°C and the medium was then quickly removed, replaced with ice-cold TCA (10%) and allowed to stand 30 min at 4°C. The TCA was removed by 4 extractions with 4 volumes of water-saturated ether and the sample, brought to pH 7.4 with Tris base. They were then frozen at -85°C, lyophilized and dissolved in 20μl of 10mM ammonium phosphate. Accumulated $InsP_3$ was separated by HPLC. Results are expressed as fold increase above the unstimulated control and are the means ± S.D. (n=3). Control counts were 1635 ± 95 dpm. * $P < 0.001$ compared to FSK + IBMX + BK.

[3H] Inositol trisphosphate
(fold increase compared to control)
2.0
1.5
1.0
0.5
0
Pretreatment
None
FSK + IBMX
*
Control
Bradykinin

Figure 6.27 Effect of protein kinase A activation and inhibition on inositol monophosphate accumulation under various conditions

Cells, equilibrium-labelled with [^{3}H]myo inositol, were preincubated 60 min with or without 30μM H-89. Following this, the cells were preincubated another 20 min with or without 1μM FSK + 0.5mM IBMX and subsequently treated 40 min at 25°C with either vehicle or 1μM BK in DMEM-containing 50mM LiCl. Incubations were terminated by addition of ice-cold methanol. The aqueous portion of the Bligh and Dyer extraction was applied to anion exchange columns to separate inositol phosphates. Counts of InsP are normalized for total incorporation into the cells while the results are expressed as the fold increase above control and are the means ± S.E.M. (n=5). * $P < 0.01$, ** $P < 0.0001$ compared to respective non-BK treated.


2.0
1.0
0
[3H] InsP accumulation
(fold increase compared to control)
Unstimulated
BK
*
*
**
None
FSK/IBMX
H-89
FSK/IBMX
H-89
Pretreatment

phosphate production was also significantly inhibited (5-20 %) by these various cAMP elevating agents. The results as a whole demonstrate the inhibitory effects of elevating cAMP and suggest a role for PKA in the regulation of the products of PI-PLC activity in MDCK-D1 cells.

7 DISCUSSION

Part I Phospholipases and bradykinin-stimulated arachidonic acid release

7.1 Determination of the enzyme required for arachidonic acid release in MDCK-D1 cells

7.1.1 *Cytosolic phospholipase A_2 is present and active in MDCK-D1 cells*

The release of AA and subsequent production of eicosanoids has long been recognized as an integral component of many signalling processes. Indeed, within the renal setting of the collecting duct, the synthesis and release of PGE_2 plays an important role in modulating AVP-stimulated water and salt transport. Since prostaglandins are not stored within the cellular milieu, the synthesis of such eicosanoids is always contingent upon the availability of arachidonate. A key enzyme thought to be responsible for release of AA from membrane phospholipids, termed $cPLA_2$, was recently cloned (Clark et al., 1991). This particular phospholipase is favoured as the key mediator of intracellular release of arachidonic acid since its properties fulfill many of the requirements for a signal transducer. For example, $cPLA_2$, unlike $sPLA_2$ counterparts, does not contain any disulfide bridges and is therefore ideally suited for the reducing environment of the cytosol. Furthermore, this 85 kDa PLA_2 is significantly active at sub-micromolar concentrations of Ca^{2+}, not unlike those attained within the cytosol following agonist-stimulation of cells. Such small Ca^{2-} increases are sufficient to cause translocation of $cPLA_2$ to the membrane, at the site of its substrates. Mutant constructs

of $cPLA_2$ lacking the CaLB domain, when overexpressed in CHO cells, fail to associate with added phospholipid membranes (Nalefski et al., 1994). Thus, for onset responses the requirement for calcium mobilization within the cytosol and therefore a signal would seem imperative for increased $cPLA_2$ translocation to sites where the phospholipid substrate is located. Lastly, this enzyme has been shown to be phosphorylated *in vitro*, thereby enhancing its ability to hydrolyze substrate. This post-translational modification can be carried out by MAPK (Lin et al., 1993), a key component of many agonist-induced signalling pathways.

Making use of a cell lysis procedure which omits Ca^{2+}-chelators as described by Paglin et al (1993), the present study demonstrated a very significant increase in membrane PLA_2 activity following stimulation of MDCK-D1 cells by a concentration of BK (1μM) (**Table 6.1**) that maximally stimulated AA release. Indeed, BK is known to cause a Ca^{2+} signal in MDCK cells (Aboolian et al., 1989) and others have observed that elevations of Ca^{2+} within cytosolic extracts or artificial media can lead to increased association of $cPLA_2$ with either natural membranes (Ambs et al., 1995; Nalefski et al., 1994) or synthetic liposomes (Nalefski et al., 1994). The present results demonstrate an increased activity within the membrane fraction which represents less than 10% of the total PLA_2 activity found in the cell lysate. Although the proportion of enzyme translocated appears relatively small it can be added that the minimum quantities of this enzyme required to produce AA in any given signalling pathway are unknown. In fact, only minor proportions of the total $cPLA_2$ pool may be required to generate the AA release observed following agonist stimulation of cells. This possibility gains credence

upon examination of the results of Goldberg et al (1994) who demonstrated, using an antisense technique in NIH 3T3 cells, that a 60% reduction in $cPLA_2$ expression levels failed to decrease agonist-stimulated AA release.

A polyclonal antibody raised against an epitope of amino acids 42-58 located within the CaLB domain of $cPLA_2$ (Lin et al., 1992), was used to selectively immunodeplete the cell lysate, through its ability to preferentially bind and thereby sequester $cPLA_2$ exclusively. Anti-$cPLA_2$ abolished both the basal and BK-enhanced activities of the acylhydrolase enzyme extracted from the membrane fraction (**Table 6.1**), indicating that the bulk of the activity within the membrane can be attributed to the increased presence of $cPLA_2$, as determined by Western blotting (**Figure 6.5**). It remains to be determined whether this increase in $cPLA_2$ activity occurs solely as a result of translocation of $cPLA_2$ to the membranes or whether enhanced phosphorylation accompanies its translocation.

The nature of the membranes involved in the translocation (plasma, mitochondrial, nuclear etc.) remains uncertain. For a better understanding of the signalling process, future work should be directed towards determining the target of $cPLA_2$ translocation in epithelial cells. Others have recently noted, using immunohistochemical techniques, a surprising absence of this phospholipase in the plasma membrane of rat basophilic leukemia cells and CHO cells stably overexpressing $cPLA_2$, while an association with the nuclear envelope and endoplasmic reticulum could be clearly seen (Schievella et al., 1995; Glover et al., 1995). More recently,

however, Pouliot et al (1996) showed that $cPLA_2$ can translocate from the cytosol to the nuclear membrane and to a lesser extent, the plasma membrane of A23187-stimulated human neutrophils. Furthermore, since PGE_2 transport across epithelial cells has been shown to be a vectorial process, beginning at the basolateral membrane and ending with the secretion into the luminal side across the apical membrane (Cortizo et al., 1992), it would be interesting in future studies to investigate the possibililty that $cPLA_2$ is preferentially translocated to, not only nuclear, but also to the basolateral membranes of MDCK-D1 cells.

7.1.2 *The possible presence of other types of PLA_2*

Although the present results indicate that $cPLA_2$ is most likely the sole enzyme involved in AA release within MDCK-D1 cells, it cannot be precluded that other relevant phospholipase A_2 forms might exist in this cell type, not surviving the lysis procedure employed, or perhaps not being detected with the assay conditions chosen.

Ca^{2+} -independent phospholipases A_2 have recently been isolated and characterized from dog myocardium (Wolf and Gross, 1985), representing a 40 kDa form, and from rabbit kidney, a form 28 kDa in size (Portilla and Dai, 1996). The 40 kDa form prefers the plasmalogen form of phosphatidylcholine, is cytosolically located and is activated by anoxic conditions. This enzyme has been shown to be sensitive to and inhibited by HELSS, a halolactone compound. The more recently isolated 28 kDa form prefers arachidonate at position sn-2 from diradylglycerophospholipids and is optimally active at neutral pH. This

group of investigators noted that most of the PLA_2 activity within the cytosol of rabbit proximal tubule cells was Ca^{2+} -independent, was recruited during hypoxia preceding cell death, and was accompanied by hydrolysis of endogenous plasmalogen substrates, leading to the generation of AA and accompanying phospholipid catabolism (Portilla et al., 1994; Portilla and Creer, 1995). It would therefore seem that these forms of PLA_2 participate in stressful or abnormal catabolic circumstances, and not those which affect normal prostanoid production. The involvement of these types of PLA_2 in the ability of BK to elicit a release of AA is doubtful in light of the results presently obtained namely that in MDCK-D1 cells, most of the PLA_2 activity from cell lysates was found to be dependent upon Ca^{2+} concentrations < 1 mM (**Figure 6.4**). Additionally, PLA_2 activity of cell lysates assayed with endogenous membranes derived from [^{3}H]arachidonate-labelled cells was found to be exclusively Ca^{2+}-dependent (results not shown).

Further evidence favouring the involvement of $cPLA_2$ was provided through the use *in vivo* of an inhibitor of $cPLA_2$, $AACOCF_3$, which was able to eliminate BK-stimulated AA release (**Figure 6.3**). However, although this inhibitor is reportedly some ten-fold more specific for the 85 kDa enzyme than for the secreted forms, it was found recently to also inhibit Ca^{2+}-independent species of PLA_2 (Lio et al., 1996). Countering this drawback, is the fact that the inhibitor to the 40 kDa form of PLA_2, HELSS, inactive towards Ca^{2+}-dependent PLA_2 activity, failed to block BK-induced AA release from MDCK-D1 cells (**Figure 6.3**). However, it is not certain whether all forms of Ca^{2+}-independent PLA_2 would be sensitive to

HELSS. Lastly, in addition to calcium ionophore's ability to release AA from this cell line, the requirement for extracellular calcium influx in BK's stimulation of AA release was unequivocally demonstrated by the fact that blockage of receptor-mediated Ca^{2+} entry into the cell very sizeably reduced BK-stimulated AA release (**Figure 6.6**).

Recently, evidence was presented showing that group I $sPLA_2$ is found in the cytosolic fraction of rat kidney homogenates (Aarsman et al., 1996). Additionally, such $sPLA_2$-like activity was demonstrated in MDCK cell supernatants which had been treated for 20 hrs with PMA (Schaefers et al., 1996). Approximately 50 % of PGE_2 produced following this extended incubation with the phorbol ester could be accounted for by this $sPLA_2$ activity while the remaining quantity was most likely supplied through $cPLA_2$ activation. However inhibitors of protein synthesis and mRNA translation completely eliminated the appearance of $sPLA_2$ activity observed following PMA pretreatment. This report demonstrates that synthesis and secretion of a $sPLA_2$ subtype can take place exceptionally following prolonged periods of PMA stimulation. With MDCK-D1 cells not treated in this manner, as in the present study, Western blotting failed to reveal the presence of group I $sPLA_2$ (**Figure 6.1**). Furthermore, *in vitro* PLA_2 activity of MDCK-D1 cells was insensitive to reducing agents such as DTT and was maximally active in micromolar amounts of Ca^{2+} (**Figure 6.4**). The mechanism whereby BK induces AA release within seconds is therefore unlikely to also induce production of $sPLA_2$ which is a slow process. Consequently, the initial release of AA and subsequent PGE_2 synthesis, can be explained by the activation of $cPLA_2$ while later sources of arachidonate could

be provided by the action of a yet unidentified $sPLA_2$, if situations mimicking prolonged stimulation with PMA would arise.

Taken together, the evidence at hand strongly supports the conclusion that the onset of BK-stimulated AA release is mediated by the 85 kDa form of PLA_2, while denying a role for other forms of PLA_2. Accordingly, BK is capable of inducing intracellular changes which favour the association of the 85 kDa $cPLA_2$ with cellular membranes and concomitant release of AA from cellular phospholipids.

7.2 The extracellular Ca^{2+}-requirement of bradykinin-mediated arachidonic acid release

The source of Ca^{2-} required for AA release from MDCK cells remained unresolved. Prior to the present study, evidence supported the requirement for a basal level of intracellular Ca^{2-} in sustaining the signalling pathways which led to AA release. Indeed, using MDCK-D1 cells, Weiss and Insel (1991) demonstrated that immobilization of intracellular calcium with BAPTA, a chelator of $[Ca^{2-}]_i$ which would also be expected to chelate influxes of extracellular Ca^{2+}, significantly reduced BK-mediated AA release. Similarly, Slivka and Insel (1988) demonstrated that BK-stimulated AA release from these same cells can be reduced when incubations are carried out using Ca^{2+}-free media. However, studies by Borle et al (1990), demonstrated that removal of extracellular Ca^{2-} from the media causes a rapid efflux of calcium followed by a concomitant reduction of resting $[Ca^{2+}]_i$ in MDCK wildtype cells. Consequently, such strategies make it difficult to identify the source of Ca^{2-} required for AA release and conclusions drawn from procedures which cause significant reductions in the resting $[Ca^{2-}]_i$, such as those involving either removal of extracellular Ca^{2-} or chelation of intracellular Ca^{2+} with BAPTA, may falsely implicate the necessity for extracellular calcium.

In order to determine whether release of AA in response to BK is dependent upon Ca^{2+} originating from either intra- or extracellular sources the present study employed the inhibitors, neomycin sulfate and SK&F 96365. Neomycin is a compound which selectively binds to phosphatidylinositol-containing phospholipids thereby preventing PI-PLC from accessing and hydrolysing its substrate. The action of this agent leads to an inhibition of

receptor-induced production of $InsP_3$, the second messenger required for the release of Ca^{2+} from intracellular stores. In contrast, SK&F 96365 is a synthetic inhibitor of receptor-mediated extracellular Ca^{2+} entry and its use was successful in blocking thrombin-stimulated Ca^{2+} entry in human platelets (Merritt et al., 1990).

In the present study, neomycin, even at a concentration of 100 μM, failed to block BK-mediated AA release while 10 μM SK&F 96365 significantly reduced the nonapeptide's effect (**Figure 6.6**). When combined together, neomycin and SK&F 96365 did not achieve any greater inhibition than that obtained using SK&F alone. These results strongly suggest that it is the extracellularly-derived Ca^{2+} which is required for BK-mediated AA release in MDCK-D1 and not that which is mobilized from the endoplasmic reticulum through an $InsP_3$-dependent process. This conclusion is further supported by the observation that BK was able to elicit a significant increase in $[Ca^{2+}]_i$ which was completely eliminated in the presence of 10 μM SK&F 96365 (**Figure 6.7**). Additionally, BK-stimulated inositol phosphate production in MDCK-D1 cells is very weak, achieving increases (above control) no greater than 1.5-fold the control values (**Figures 6.26 and 6.27**). Therefore, coupling of B_2-receptors to PI-PLC in MDCK-D1 cells is relatively low, and may reflect minute expression levels of this enzyme in this particular clone of MDCK, a possibility that requires further investigation.

7.3 The role of other phospholipases in bradykinin-mediated arachidonic acid release

7.3.1 *Phosphatidylinositol-specific phospholipase C is not involved in arachidonic acid release by bradykinin*

Previous studies with MDCK wildtype cells revealed that stimulation with BK, activates PI-PLC, as demonstrated by increased inositol phosphate production. However, for MDCK-D1 cells, it was reported that the use of neomycin (Slivka and Insel, 1988), had no effect upon epinephrine-stimulated PKC activity and failed to block BK-stimulated AA release as the results of the present study also show (**Figure 6.6**). A role for bisphosphoinositide hydrolysis in mediating an action on PLA_2 is questionable on this basis. The function of PI-PLC in MDCK-D1 cells and the reason for its regulation by BK remain presently unknown. However, roles for other phospholipases in the release of AA have been determined in many other cell types, including rabbit platelets (Sato et al., 1992), and rat peritoneal mast cells (Ishomoto et al., 1994).

7.3.2 *Involvement of phosphatidylcholine-specific phospholipase C and phospholipase D in the release of arachidonic acid by bradykinin*

The present study demonstrated an involvement of PC-PLC and PLD in response to stimulation of the cells by BK. The activities of these enzymes were characterized on the basis of the accumulation of their characteristic products *in vivo* and a demonstration, as far as was possible, that these products did not arise indirectly by the activation of other enzymes. The effect of BK on PC-PLC activity was very rapid and transient not unlike that seen for NIH 3T3 cells (Fu et al., 1992). Its time course correlated well with that of BK-stimulated AA release

which could be blunted by a specific PC-PLC inhibitor, D609 (**Figure 6.11**). The specificity of this agent was verified against PLD and PLA_2, for which enzymes it had no inhibitory effect. It can be concluded from such evidence that there is a mediatory involvement of PC-PLC in the BK enhancement of PLA_2 activity. Interestingly, the activation of PC-PLC has also been reported for MDCK-D1 cells stimulated with epinephrine (Slivka and Insel, 1988).

Activation of PLD in reponse to purinergic agonists and PMA is an event occurring after PKC activation in MDCK-D1 cells (Balboa et al., 1994). On the other hand, stimulation of such cells with BK in the presence of EtOH was shown to cause a rapid transient increase in PEt formation, which is possibly due to a G-protein-mediated event, followed by a slower, sustained increment in PEt formation (Huang et al., 1992), likely involving the participation of PKC as an activator of some more latent form of PLD. The results of the present study confirm such a biphasic activation profile for PLD and demonstrate kinetics of Chol release which are compatible with its mediatory role in BK-enhanced release of AA (**Figure 6.8**). The production of free Chol was very likely due to PLD rather than to enhanced degradation of PChol since the product of transphosphatidylation, PEt, was likewise increased twofold in response to BK after 1 min of stimulation (**Figure 6.12**). The early PLD activation phase could serve together with PC-PLC, at the onset of stimulated AA release whereas the later phase would not likely participate since Ca^{2-} levels return to resting levels within the first min of stimulation, likely causing $cPLA_2$ to dissociate from the substrate-rich membranes. Therefore the rate of AA release no longer increases as shown in **Figure 6.8**. The mediation by PLD is further

supported by the fact that EtOH, which causes a loss of PA production in favor of PEt, very significantly inhibited the BK-enhanced release of AA (**Figure 6.11**). The combined inhibition of PC-PLC (with D609) and PA production (with EtOH) blunted the enhancement of AA release by some 90% attesting to the important role played by these enzymes in the process.

7.3.3 *Phosphatidic acid and diacylglycerol contribute to the bradykinin response by enhancing cytosolic phospholipase A_2 activity*

In rat peritoneal mast cells, the PLD/PAP pathway contributes quite substantially to AA release (Ishomoto et al., 1994) and the DAG produced is further degraded by DAG lipase. In MDCK-D1 cells, the operation of this signalling pathway could not be precluded completely but did not seem likely from the evidence at hand. Propranolol, an inhibitor of PAP failed to elevate PA recovery above that seen with BK alone (**Figure 6.12**). If PAP had been active following BK stimulation, a significant increase in PA levels should have occurred in the presence of propranolol. These results are compatible with those of others who reported that the conversion of PA to DAG was absent in rabbit platelets (Sato et al., 1992). Conversely, the PC-PLC inhibitor, D609, did not alter PA production after 1 min of BK stimulation, making the involvement of DAG kinase unlikely in this process (**Figure 6.12**). In agreement with previous findings (Weiss and Insel, 1991), the action of DAG lipase beyond the PAP step appears not to be involved since the DAG lipase inhibitor, RHC80267 at concentrations which, in the present studies, were reverified to completely inhibit the enzyme, had no effect on BK-stimulated release of AA (**Figure 6.3**).

The question arose as to how DAG, and PA could promote AA release. Earlier reports of activation of PLA_2 by these lipids (Dawson et al., 1983; Dawson et al., 1984; Hashizume et al., 1994; Kramer et al., 1987; Leslie and Channon, 1990; Sato et al., 1992) prompted the present investigation of their action on the cytosolic PLA_2 of MDCK-D1 cells. As illustrated in **Figure 6.14**, both DAG and PA significantly enhanced PLA_2 activity. Since DAG lipase and DAG kinase are membrane enzymes (Bell et al., 1979; MacDonald et al., 1988), it is unlikely that a metabolite of DAG might have been responsible for stimulating a PLA_2 analyzed in the cytosol fraction. Also the cytosol fraction used was extensively dialysed and neither ATP nor phospholipid such as phosphatidylserine were added to the incubation mixture. Thus one can exclude the possibility that a PKC type of activity was able to phosphorylate the $cPLA_2$ and increase its activity in the cytosol. It appeared therefore that the action of DAG was either a direct effect upon the enzyme itself or perhaps following intercalation within the bilayer, an effect due to favorable changes in the order of the lipid substrate increasing its accessibility to the enzyme. The latter mechanism appears the most plausible on the basis that DAG had a stimulatory effect only when it was sonicated together with the substrate. If DAG was sonicated in a separate step and then added to the assay mixture, it had no stimulatory effect. It has been suggested that since DAG is a wedge-shaped molecule, it is able to cause membrane spreading when embedded in lipid bilayers (Leslie and Channon, 1990). This spacing of the phospholipids would then allow $cPLA_2$ improved access to its substrate, resulting in increased activity. Also stimulating PLA_2 activity, PA would not

likely exert its effects by degradation to DAG since the PAP subtype present in cytosol, displays almost negligible activity towards PA suspended in aqueous solution (Cassola and Possmayer, 1981). Its action would be similar to DAG, affecting the order of the lipid substrate since mutually repulsing negative charges give PA molecules a shape equivalent to that of a wedge-shaped molecule.

Whether or not these effects on lipid lyotropic mesomorphism reflect actual *in vivo* mechanisms or not is rather difficult to prove. The molar percentage of DAG and PA at which statistically significant increases in activity were observed *in vitro* were less than 5 % of the substrate concentration. BK-mediated stimulation of MDCK-D1 cells may not produce DAG and PA levels of that magnitude. However, one cannot exclude the possibility that local concentrations of such mediators within the lipid bilayers may approach or perhaps exceed the required levels for increased $cPLA_2$ activity. Determination of the exact location and concentration of DAG and PA within the specific domains of membrane where $cPLA_2$ acts, is experimentally unfeasable at the present time.

Part II Protein kinases and bradykinin-stimulated release of arachidonic acid

7.4 Determination of the role protein kinase C plays in bradykinin-stimulated arachidonic acid release in MDCK-D1 cells

The mechanisms coupling signalling induced by BK and other agents to $cPLA_2$-catalysed AA release are not yet completely resolved. It is known that certain growth factors such as EGF cause the activation of MAPK, an enzyme which can phosphorylate $cPLA_2$ on ser 505 leading to increased $cPLA_2$ activity (Lin et al., 1993). Although PKC was reported to phosphorylate Ser/Thr residues other than Ser505 on $cPLA_2$, thereby increasing its activity (Nemenoff et al., 1993), the role of this protein kinase with regards to AA release in MDCK-D1 cells remained uncertain (Slivka and Insel, 1988). One of the goals of the present study was to further investigate the possible requirement for PKC as a mediator of BK-stimulated activation of PLA_2 and the increased release of AA in MDCK-D1 cells.

One approach of the present study was an attempt to reconcile opposing conclusions drawn from experiments designed to establish whether or not PKC activation is a prerequisite for BK-mediated AA release in MDCK-D1 cells. The predominant conclusion obtained with chemical inhibitors of PKC has been one which denies such a role for this serine/threonine kinase (Weiss et al., 1989). On the other hand, studies involving phorbol ester-mediated down regulation of PKC levels have provided results in support of this role (Weiss and Insel, 1991).

7.4.1 *Inhibition of protein kinase C: Its effect upon bradykinin-induced arachidonic acid release*

The results of the present study demonstrate that the inhibitor of PKC, BIS, while fully capable of blocking the synergistic effect observed with the combination of either PMA and A23187, or PMA and BK, is unable to alter the release of AA due to BK alone (**Figure 6.15**). One possibility for the lack of success with PKC-specific inhibitors is that perhaps a subclass of protein kinase C activity may be insensitive to these agents. However reports indicate that BIS is capable of inhibiting the activities of PKC-α, β_I, δ, ε,and ζ with IC_{50} values of 8.4, 18, 210, 132, and 5800 nM, respectively (Martiny-Baron et al., 1993). The isozymes found in MDCK-D1 cells are indeed comprised in the list just mentioned (Balboa et al., 1994). Furthermore, a wide variety of PKC inhibitors including, sphingosine, H7, and staurosporine (Weiss et al., 1989), have proved unsuccessful in blocking BK-mediated AA release while at the same time eliminating the contribution made by acute phorbol ester treatment. Of these, only sphingosine, when used at high doses (> 10 μM) was able to partially reduce BK-stimlated AA release (Robinson et al., 1995). Unfortunately, results obtained with high doses of these compounds can lead to nonspecific effects, including cytotoxicity and must be viewed with caution. For example, in the present study, at concentrations > 16 μM, BIS was able to reduce BK-induced AA release by > 50 % but also lowered that of ionophore (data not shown) which, as argued earlier, does not require PKC activation as an onset response. The other enzymatic events may be subject to non-specific inhibition by large doses of PKC inhibitors. Therefore, despite its ability to activate PKC, the signal induced by BK which results in AA release from MDCK-D1 cells, does not require onset activation of this kinase. It

should be noted that BK is much weaker than PMA as an activator of PKC, a fact that reinforces the idea that concentrations of inhibitor (i.e., BIS) which block phorbol ester-mediated PKC activity, should in fact significantly reduce that induced by BK. Thus, the magnitude of BK-stimulated PKC activity may not be sufficient to influence AA release from MDCK-D1 cells.

This lack of effect by PKC inhibitors on agonist-stimulated AA release is not unique to MDCK-D1 cells. For example, in HL60 granulocytes, the chemotactic peptide, N-formyl-Met-Leu-Phe (FMLP) can augment AA release through PLA_2 activation via a mechanism insensitive to the PKC inhibitor, H7 (Billah and Seigal, 1987). As well, in human polymorph neutrophils, FMLP-induced AA liberation was actually increased by staurosporine-mediated inhibition of PKC (Nigam et al., 1995). Antigen-stimulated rat mast cells were likewise insensitive to Ro31-7549 (an analogue of BIS) with respect to AA release (Hirasawa et al., 1995). Finally, thrombin-stimulated platelets were shown to phosphorylate $cPLA_2$ and release AA in a manner unaffected by PKC inhibitors (Börsch-Haubold et al., 1995). The authors were confident in reaching this conclusion since the PKC inhibitors Ro 31-8220 (another BIS analogue) and staurosporine were unable to block thrombin's ability to release AA, while abrogating that of collagen. It should be added at this point that certain agonists do display in MDCK-D1 cells, a requirement for PKC mediation in their ability to release AA. As mentioned above, Weiss et al (1989) observed that the PKC inhibitors, H7, sphingosine and staurosporine are each capable of blocking the AA released following epinephrine stimulation

despite being ineffective against that following BK stimulation.

In addition to the negative evidence brought forth by the use of synthetic inhibitors, the case for BK-stimulated AA release occurring independent of PKC action has been strengthened by testing certain naturally-occurring inhibitors of this serine/threonine kinase. Alkylglycerol (AG), recently shown to be produced in unstimulated wildtype MDCK (Warne et al., 1995), was found capable of inhibiting phorbol ester-induced AA release. In fact, it was proposed that increased production of AG during the growth of MDCK wildtype cells to a confluent state contributes to an observed parallel decrease in PKC activity (Warne et al., 1995). However, as in the case of the synthetic inhibitors, AG was unable to block AA release brought about by BK. The mechanism whereby AG carries out the observed inhibition of PKC is, at present, not fully understood. Nonetheless, one study showed that addition of AG is effective in blocking the BK-induced translocation of PKCα but not that of PKCε to the membrane fraction in SF 3271 human skin fibroblasts (Clark and Murray, 1995). Additionally, this group demonstrated that BK's ability to activate MAPK was not altered by the effects of AG upon PKCα. Godson et al (1993), using an antisense strategy, revealed that the α isoform of PKC is most likely the enzyme utilized by phorbol ester, leading to release of AA in MDCK-D1. The observations that AG seemingly prefers inhibition of the α over the ε isoform, coupled with its inability to alter BK-stimulated AA release, points to the possible involvement of PKCε. However, several pieces of evidence argue against this, such that the question remains incompletely resolved. First, most inhibitors, including BIS, which are ineffective

against BK-mediated AA release, do in fact block the activity of PKCε *in vitro* (Wilkinson et al., 1993). Second, BK and phorbol ester have each been shown to enhance both Ca^{2-}-dependent and -independent PKC activity in MDCK-D1, most likely due to the fact that both the α and ε isoforms are DAG-dependent. Furthermore, the degree to which BK activates PKC is much less than that brought about by phorbol ester (Godson et al., 1990). Therefore it can be safely assumed that those concentrations of BIS at which phorbol ester-mediated PKC activity is inhibited would also be sufficient to block that stimulated by BK. In spite of this evidence, the *in vitro* situation within which these inhibitors were tested against the various PKC isoforms, may not accurately reflect that of the intact cell. Therefore, further studies which focus on antisense strategies targetted against the different isoforms present may be required in order to unequivocally determine the role, if any, played by PKC in mediating BK-stimulated AA release.

7.4.2 *Long-term phorbol ester-induced down regulation studies*

Evidence obtained in the present study indicates that long-term treatment of cells with PMA, a process which has been shown to down regulate PKC in MDCK-D1 cells (Godson et al., 1993) leads to decreased AA release in response to BK stimulation. Such results are often the basis for implicating PKC in a signalling phenomenon. However, since PKC inhibitors were ineffective in blunting BK-stimulated AA release, it seemed unlikely that this protein kinase was involved at levels of signalling which engender short term responses and thus would not account for events leading to release of AA after BK stimulation for 1 min. Accordingly it was

observed that the main consequence of PKC down regulation was to lower the activity of $cPLA_2$ by a process which affects its phosphorylation rather than the levels of the enzyme (**Figures 6.17 and 6.18**). It should be noted however, that a reasonable control would have been to test for non specific effects using the inactive version of the phorbol ester, although no reports could be found within the literature documenting such an effect. Nonetheless, it could be concluded from this that PKC plays an important role in maintaining levels of phosphorylated, ready-for-use enzyme depending on the steady state conditions prevailing in the cell but is not implicated in onset responses following BK stimulation which lead to AA release. Steady-state conditions could include processes such as continued stimulation of the cell by serum or autocrine factors dependent for signal transduction on PKC activation. The present results suggest that although some physiological ligands such as BK can activate PKC, the increased enzyme activity was, at least apparently, not functionally relevant to AA release.

One could speculate that under steady-state conditions, PKC may be required to maintain a certain level of RAF-1 activity which would, in turn, increase MAPK activity, resulting in the observed phosphorylation and activity of $cPLA_2$. Alternatively, PKC activity may negatively regulate a phosphatase(s) that serves to reduce $cPLA_2$ phosphorylation levels. Therefore, when PKC levels drop following long-term PMA treatment, $cPLA_2$ would be dephosphorylated. Finally, signalling through the PKC pathway may be required for the regulation of expression levels of other enzymes required in the signalling route of agonist-stimulated AA release. For example, long-term treatment with PMA can reportedly lead to

effects on signalling pathways which, at onset, do not couple to PKC activation, including a 50 % reduction of *in vitro* PLD activity in membranes derived from PC12 cells (Ricanati et al., 1992). These researchers observed that PKC-independent membrane PLD activity induced by fluoride, an agent which presumably acts on guanine nucleotide binding proteins linked to the phospholipase, was attenuated by 55 % in phorbol ester-treated cells. This may reflect a down regulation of levels of PLD itself, or alternatively of factors involved in the positive regulation of PLD, following long-term treatment with PMA. Additionally, evidence has also been presented that extended treatment with PMA can reduce EGF-mediated activation of MAPK in SF 3271 cells, a signalling pathway that is not coupled to PKC activation for proper functioning (Clark and Murray, 1995). Finally, maintainence of PKC levels may be required for proper regulation of certain receptors in MDCK-D1 since a reduction in prazosin binding, due perhaps to decreased levels of α_1-adrenergic receptors was observed by Weiss et al (1989) following down regulation of PKC. Thus, future work should be directed toward testing the effects of PKC down regulation on expression levels and binding capacities of B_2-receptors in MDCK-D1 cells.

7.4.3 *The role of mitogen-activated protein kinase in bradykinin-stimulated arachidonic acid release*

As mentioned earlier, certain agonists do display in MDCK-D1 cells, a requirement for PKC mediation in their ability to release AA. More recently, however, Xing and Insel (1996) demonstrated that epinephrine, signalling through the α_1-adrenergic receptor, relies not only upon PKC, but also on MAPK in order to phosphorylate and activate $cPLA_2$ and release

AA from MDCK-D1 cells . The mechanism for this process is most likely a PKC-mediated activation of RAF-1 which enhances MAPKK (MAPK kinase) activity, followed by MAPK recruitment and phosphorylation of $cPLA_2$.

In MDCK-D1 cells however, since onset responses to BK stimulation do not depend on PKC activation, recruitment of the MAPK cascade by BK, through a PKC-independent pathway, is a definite possibility yet to be examined. The results presented herein demonstrate that while PKC inhibitors fail to abrogate BK-induced AA release, the MAPKK inhibitor, PD 98059 significantly blunts this release (**Figure 6.20**). Indeed, recent studies involving agents such as endothelin-1 in both rat astrocytes (Kasuya et al., 1994), and CHO cells transfected with the human ET_A receptor (Kruger et al., 1995), and leukotriene B_4 in guinea pig eosinophils (Araki et al., 1995) have provided evidence for PKC-independent mechanisms of MAPK activation. These signalling pathways seem to rely upon the $\beta\gamma$ subunits of certain G-proteins which increase *ras* activity through an unknown mechanism. More recent evidence seems to implicate a $\beta\gamma$-subunit-dependent association of shc, sos and grb2 along with the activation of phosphatidylinositol-3-kinase, a signalling cascade which precedes the activation of ras (Daub et al., 1996; Hawes et al., 1996; van Biesen et al., 1995) Thus, one could speculate that a G-protein-to-MAPK cascade exists in certain cell types including MDCK-D1 cells, and may contribute to PKC-independent AA release.

It must be maintained that a vital component of BK-mediated AA release is the influx

of Ca^{2+} from the extracellular medium, serving to translocate $cPLA_2$ to membrane. In agreement with this, thromboxane A_2 -enhanced PLA_2 activity in human platelets was shown to be inseparable from both sustained elevation of cytosolic Ca^{2+} and agonist-induced influx of extracellular Ca^{2+} (Murthy et al., 1995).

The present study provides evidence that long-term down regulation of PKC through the use of phorbol esters can lead to a reduction in the steady-state level of phosphorylation of $cPLA_2$. This would account in this case, for the decreased release of AA following stimulation with BK. The degree of inhibition by PKC down regulation was no greater than 50 %. indicating that some steady state phosphorylation by MAPK might persist via PKC-independent mechanisms. No evidence was obtained in support of immediate PKC involvement as an effector for the release of AA following stimulation with BK. This is probably because, under normal circumstances, the resting levels of phosphorylated PLA_2 in MDCK-D1 cells are sufficiently high to favour a BK response. An alternative explanation is that BK-induced phosphorylation of PLA_2 does occur by an activation of the MAPK cascade in a manner independent of PKC, thereby contributing to the observed AA released. It is also apparent from the present discussion that the use of PKC down regulation as a sole means of demonstrating the involvement of this protein kinase in mediating the onset responses of an agonist constitutes an invalid approach.

At the beginning of this project, the role of PKC in mediating BK effects on AA release

remained incompletely resolved. Not discounting the fact that DAG may stimulate PLA_2 via effects on the lyotropic mesomorphism of the substrate, it appears from the present results that DAG produced by PC-PLC in BK-stimulated cells is also sufficient to induce a very marked increase in PKC activity. These data suggest that although some physiological ligands can activate PKC, the increased enzyme activity is not always functionally apparent or relevant. It would therefore appear that the ability of BK to induce a release of arachidonate from MDCK-D1 cells is dependent upon several key factors: included are, the influx of extracellular Ca^{2-} through membrane-operated channels serving to increase the association of $cPLA_2$ with the phospholipid-enriched membranes, the activation of PC-PLC and PLD, which produce the lipid mediators, DAG and PA which likely cause membrane perturbations enhancing the ability of $cPLA_2$ to access its substrate, and the phosphorylation of $cPLA_2$ by a PKC-independent MAPK recruitment.

7.5 Negative regulation of bradykinin-stimulated signalling pathways by cAMP-dependent protein kinase

Hassid (1983) demonstrated that in wildtype MDCK cells, PGE_2 production could be blunted by elevation of cAMP. Several years later, Teitlebaum et al (1986) were able to show that similar decreases in PGE_2 levels in collecting tubule cells resulted, not from inhibitory effects on cyclooxygenase activity, but from negative constraints on the release of AA itself since exogenously added AA could overcome the cAMP effect. These results pointed to the existence of cAMP-dependent regulatory mechanisms acting on lipid hydrolysing enzymes in

renal epithelial cells, including MDCK cells.

To further reveal such control mechanisms, the approach of the present study was to elevate cAMP levels in the cell, and measure effects on the products of various phospholipases stimulated by BK. These included PI-PLC which is not involved with AA release and other phospholipases such as PC-PLC, PLD, and $cPLA_2$ which are involved. This aim was to identify possible targets of inhibition along the AA release pathway and to see if targeted phospholipases were exclusively those related to AA release.

7.5.1 *Elevated cyclic adenosine monophosphate production reduces phosphoinositide-specific phospholipase C and agonist-stimulated inositol phosphate accumulation*

The effects of BK on the production of inositol phosphates and the release of AA were tested in MDCK-D1 cells with induced elevated cAMP levels. The presently reported inhibitory effect of elevated cAMP on basal and/or BK-enhanced PI-PLC activity (**Figure 6.27**) was also observed in several other cell types (Anwer et al., 1990; McAtee and Dawson, 1989; McAtee and Dawson, 1990; Neylon and Summers, 1988; Teitlebaum et al., 1986). For example PGE_2, the receptor of which is coupled to adenylate cyclase, and 8-bromo-cAMP were able to cause a decrease in steady-state levels of $InsP_3$ in v-Ha-*ras*-transformed MDCK cells (Wu and Lin, 1990). Thrombin-induced platelet responses were again reported to be inhibited by agents that increase cAMP and block the formation of both $InsP_3$ and DAG (Imai et al., 1983). In many of these studies cAMP was elevated with agents such as FSK and IBMX but stimulation of PKA in MDCK-D1 by receptor-mediated phenomena (i.e., with

AVP) or by cAMP-elevating agents gave similar results. It is appropriate to recall at this point the evidence that was presented precluding the involvement of PI-PLC in the BK-stimulated release of AA (**Figure 6.6**) and it appears therefore that negative regulation of phospholipases by cAMP is a general phenomenon not exclusive to enzymes concerned with the AA release pathway.

7.5.2 *Elevated cyclic adenosine monophosphate levels reduce cytosolic phospholipase A_2 activity: Involvement of the mitogen-activated protein kinase cascade*

Interestingly, activation of PKA with cAMP-elevating agents also notably inhibited basal and A23187-enhanced PLA_2 activity as could be judged from corresponding decreases in labelled GroPIns production (**Figure 6.25**). These decreases paralleled those in AA release in control and ionophore-treated cells (results not shown). Such results were again taken as further proof that changes in AA release reflect directly changes in $cPLA_2$ activity and do not involve some indirect mechanism comprising DAG formation and hydrolysis. Because of evidence discussed in **Section 7.1**, it is assumed that the PLA_2 involved is in fact $cPLA_2$. Supporting the activation of PKA and not some possible alternative action of cAMP is the fact that all inhibitions due to FSK + IBMX were reversed by addition of H-89, a specific inhibitor of the cAMP-dependent protein kinase (Chihiwa et al., 1990).

The observation that $cPLA_2$ activity is significantly inhibited by PKA activation accurately reflects the ability of this kinase to reduce release of AA from MDCK-D1 cells. However, this inhibitory effect does raise several questions regarding the maintainence of PLA_2

activity in this cell line. First, what factors contribute to regulate phosphorylation of $cPLA_2$ in MDCK-D1 cells? Second, are these factors kinases or phosphatases, or both? As discussed previously, MAPK is known to phosphorylate and to enhance the activity of $cPLA_2$ and thus could be involved in regulating the phosphorylation of $cPLA_2$ in resting and stimulated cells. By resting in this case is meant, cells that are not being stimulated by BK but that are probably being stimulated to some extent by other agents such as autocrine factors or serum factors. One possible serum factor, EGF, has recently been shown to activate MAPK in MDCK wildtype cells (Yamada et al., 1995). This group demonstrated that AVP-stimulated PKA activity decreased EGF-induced RAF-1, MAPKK, and MAPK activities in MDCK cells. One can then envision the possibility that inhibition of various targets by PKA, such as RAF-1 which lies upstream of MAPK in this signalling cascade, might block activation of $cPLA_2$, resulting in a reduced release of AA. Indeed, RAF-1 was shown to be phosphorylated and its ability to associate with its activator, p21ras, blocked by PKA (Simon and Frank, 1993). This resulted in an inhibition of EGF-stimulated MAPK activity. One corollory from these observations is that since FSK + IBMX-induced inhibition of basal PLA_2 activity occurred following only 20 min of incubation, the turnover rate of phosphorylated $cPLA_2$ in resting cells would be predicted to be rather high. However, the precise regulatory mechanisms involved remain unresolved at this point. The present study also indicates that inclusion of serum (i.e., 0.5% FCS) in the medium overnight prior to stimulation with either A23187 or BK, results in a significantly greater release of AA compared to cells which are serum-starved (i.e., 0.05% BSA) (**Figure 6.16**). Thus, the presence of factors within the serum, signalling through

tyrosine kinase pathways would lead to elevated activity of MAPK and subsequently, increased phosphorylation of $cPLA_2$.

7.5.3 *Increased cyclic adenosine monophosphate levels block bradykinin-mediated phospholipase D activity*

Further inhibitory effects of PKA were observed upon measurement of the product of PLD-catalyzed phosphatidylation. An elevation of intracellular cAMP levels significantly diminished ability of BK to stimulate the production of [^{3}H]PEt (**Figure 6.23**). This indicates that PKA activation can also negatively regulate PLD and one could surmise that, because of the favourable disruptive effects of this acidic lipid on bilayer structure, a reduction in the quantity of PA produced might contribute to the observed loss of AA release. This inhibitory effect of PKA upon PLD activation has been previously demonstrated in human neutrophils where FMLP-induced, but not PMA-induced PA production was diminished by increased cAMP levels (Agwu et al., 1991).

7.5.4 *Increased cyclic adenosine monophosphate levels reduce phosphatidylcholine-specific phospholipase C activity*

As demonstrated earlier, BK-stimulated calcium-dependent membrane PKC activity was abolished by preincubating the cells with D609, an inhibitor of PC-PLC (**Figure 6.9**). This result and the fact that PI-PLC could be shown not to be involved in promoting AA release, implies that the ability of BK to increase DAG production depends predominantly upon PC-PLC activity. The observation that activation of PKA also caused inhibition of BK-

stimulated DAG in MDCK-D1 cells (**Figure 6.24**), suggests that PC-PLC activity was also diminished. As in the case of PA reduction, because of the effects of this lipid on bilayer structure, this reduction in DAG levels would explain the oberved decrease in BK-stimulated AA release.

7.5.5 *Other possible targets of cyclic adenosine monophosphate-dependent protein kinase induced inhibition of the bradykinin signalling pathway*

That PKA decreases the activity of these enzymes *in vivo* but does not cause their complete inhibition cannot be presently explained. The enzymes might exist as PKA-sensitive and -insensitive species perhaps serving different functions in the cell. Alternatively, the PKA-phosphorylated principle involved may apply to all of the enzyme but may just reduce its function rather than abolish it completely. These points require further investigation. However, the fact that the activity of a variety of lipid hydrolases including PI-PLC, PC-PLC, and PLD were reduced following elevations of intracellular cAMP, raises several possibilities regarding the identity of the targets of PKA.

First, each phospholipase might contain PKA-sensitive phosphorylation sites which, when covalently modified by this kinase, would render the phospholipase either inactive, less active or unable to translocate to its substrate's location. However, thus far only phospholipase Cγ has been shown to be directly phosphorylated by PKA (Kim et al. 1989). Despite being unable to change the activity of PLCγ, as assayed *in vitro*, PKA activation with FSK prevented agonist-induced tyrosine phosphorylation of this phospholipase as well as PIP_2

hydrolysis in living Jurkat cells. However, agents such as BK, signalling through STMRs, promote PIP_2 hydrolysis through the action of PLCβ, a subtype for which there has been so far no demonstration of phosphorylation by PKA. Furthermore, no evidence exists at this time demonstrating phosphorylation of PC-PLC and PLD by PKA.

Second, the G-protein(s) which couple the B_2-receptor to the various phospholipases might be subject to PKA-mediated phosphorylation. In NCB-20 cell extracts, 20 kDa and 24 kDa GTP-binding proteins have been observed to be substrates of PKA. Phosphorylation of these *ras*-related proteins correlated with inhibition of phosphoinositide turnover (McAtee and Dawson, 1990). It can be added that two G-protein subtypes, $G\alpha_q$ and $G\alpha_{11}$, which can couple to the B_2-receptor, contain consensus phosphorylation sites. Furthermore, G_{12}, but not G_{13} has been shown to be phosphorylated by PKA (Bushfield et al., 1990). In rat myometrial plasma membranes, PKA was found to phosphorylate several membrane-bound proteins, including some within the 20-24 kDa range. Guanosine triphosphate-stimulated PIP_2 hydrolysis was inhibited by PKA-induced phosphorylation in these cells.

Finally, the B_2-receptor itself might be a target of PKA. Inspection of the amino acid sequence of this receptor subtype reveals a PKA phosphorylation consensus segment. However, whether or not phosphorylation by PKA of this receptor would cause altered ability to either bind its ligand or associate with G-proteins remains unknown. Each of these possibilities requires further investigation and could be tested with purified forms of these

proteins in the presence of catalytic fragments of PKA. One could assay for altered activity and phosphorylation of each of the phospholipases mentioned. Additionally, reconstituted membranes containing various combinations of purified B_2-receptors, phospholipases, and G-proteins could be incubated with PKA catalytic subunits and assayed for the increases in phosphorylation, altered activity, ligand binding, and GTP content.

7.5.6 *Other cross-talk mechanisms in MDCK cells*

A previous investigation established that PKC activators and BK, acting via PKC stimulation, have an inhibitory effect on AVP-induced cAMP formation in MDCK cells (Friedlander and Amiel, 1987). This means that a possibility for bidirectional cross-talk between two major signal transduction pathways, i.e., PKC-coupled and adenylyl cyclase-coupled, exists in these kidney-derived cells. The physiological significance of the observations described herein requires further examination; however, it is not unlikely that cross-talk between signalling pathways which are mutually inhibitory is very important for mediating the marked opposing effects of hormones such as AVP and BK on renal functions such as those involved in water flow and Na+ transport (Schuster et al., 1984; Tomita et al., 1985).

7.6 Summary

The main contributions of this thesis are summarized in **Figure 7.1** which is a schematic illustration of the various factors influencing BK-induced AA release from MDCK-

D1 cells. Bradykinin, through the interaction with its putative B_2-receptor activates a G-protein(s) which subsequently initiates a series of signalling cascades. A receptor-operated-G-protein -linked Ca^{2+} channel allows for an influx of extracellular Ca^{2+} which causes the translocation of $cPLA_2$ from the cytosol to membranes. Both PC-PLC and PLD are also activated at the onset by BK, possibly by a G-protein-linked mechansim, and this increases DAG and PA in the membrane. DAG and PA exert a physical effect on bilayer structure and increase substrate accessibility to $cPLA_2$. Additionally, a PKC-independent activation of MAPK would serve to enhance $cPLA_2$ activity through a phosphorylation event. Finally, agents which cause an elevation of intracellular cAMP levels, leading to increased PKA activation, can inhibit BK-stimulated phospholipase activation, including AA release, and DAG and PA production, as well as several other activities, including that of RAF-1.

Figure 7.1 **Signalling pathways involved in the bradykinin stimulation of arachidonic acid release in MDCK-D1 cells**

BK
B2R
G
?
ras
RAF1
MAPKK
MAPK
AC
cAMP
PKA
PC-PLC
PLD
PA
DAG
Ca2+
Ca2+
cPLA2
cPLA2
AA
+
+
-
-
-

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9

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PUBLICATIONS in REFEREED JOURNALS:

1. **Kennedy, C.R.J.**, Slack R., Xin Ding L., Aubry H. and Proulx P.R. Transfer of arachidonyl groups within the lipids of two human neuroblastoma cell lines. *Biochim. Biophys. Acta.* **1211**: 326-334, 1994

2. **Kennedy, C.R.J.**, Proulx, P.R. and R.L. Hébert. Regulation of bradykinin-stimulated phospholipase C activity by protein kinase A in MDCK-D1 cells. *Biochim. Biophys. Acta.* **1258**: 206-214, 1995.

3. **Kennedy, C.R.J.**, Hébert, R.L. and P.R. Proulx. The role of phospholipases A_2,
C and D in the bradykinin-induced release of arachidonic acid in MDCK cells. *Am. J. Physiol.***271** (*Cell physiol. 40*): C1064-C1072, 1996.

PAPERS SUBMITTED or in PREPARATION

1. **Kennedy, C.R.J.,** Proulx, P.R., and R.L. Hébert. Bradykinin-induced translocation of $cPLA_2$ in MDCK cells. Accepted, August 1996 to the *Canadian Journal of Physiology and Pharmacology.*

2. **Kennedy, C.R.J.**, Do, M.T., Hébert, R.L. and P.R. Proulx. Bradykinin-stimulated arachidonic acid release in MDCK-D1 cells does not require acute PKC activation. In preparation.

ABSTRACTS and PRESENTATIONS at SCIENTIFIC MEETINGS:

1. **Kennedy, C.R.J.**, Slack, S., Aubry, H., Xin Ding, L., and P. Proulx. Redistribution of arachidonyl groups within the lipids of human neuroblastoma cells. Poster presented at the *36th Annual Meeting of the Canadian Federation of Biological Societies*, Windsor, Ontario, June 1993 (p50, Program Proceedings).

2. **Kennedy, C.R.J.**, Naismith, C., Proulx, P., and R.L. Hébert. Cross-talk between protein kinase A and phospholipase C signalling pathways in MDCK-D1 cells. *J. Am. Soc. Nephrol.* **4**: (3), p489, 1993.

3. Hébert, R.L., **Kennedy, C.R.J.**, and P. Proulx. Régulation de la bradykinine par l'activation simultané des phospholipases A_2 et C chez les cellules MDCK-D1. *Médecine/Sciences* **10**: suppl. 1, p41, 1994.

4. **Kennedy, C.R.J.**, Proulx, P., and R.L. Hébert. Regulation of bradykinin-stimulated phospholipases A_2 and C activities by protein kinase A in MDCK-D1 cells. Poster presented at the *Annual Meeting of the Canadian Society for Clinical Investigation*, Toronto, Ontario, September, 1994. *Clin. and Invest. Med.* **17**: (4), B99, 1994.

5. **Kennedy, C.R.J.**, Proulx, P., and R.L. Hébert. Regulation of bradykinin-stimulated phospholipases C and A_2 by protein kinase A in MDCK-D1. Poster presented at the *27th Annual Meeting of the American Society of Nephrology*, Orlando, Florida, October 1994. *J. Am. Soc. Nephrol.* **5**: (3), p661, 1994.

6. **Kennedy, C.R.J.**, Proulx, P., and R.L. Hébert. The regulation of arachidonic acid release in MDCK cells by protein kinase A. Poster presented at the *Annual Meeting of the Canadian Society for Clinical Investigation*, Montreal, Québec, September, 1995. *Clin. and Invest. Med.* **18**: (4), B102, 1995.

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