Vasoconstrictor-stimulated Phosphoinositide Signalling in Caveolae/Lipid Rafts of Intact Small Arteries.

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medicine and Human Sciences.

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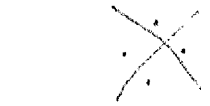

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Abbreviations

AA	-	Arachidonic Acid
AngII	-	Angiotensin II
ANOVA	-	Analysis of variance
CaM	-	Calmodulin
CCE	-	Capacitative calcium entry
CD	-	Cyclodextrin
CDP	-	Cytidine Diphosphate
CDS	-	CDP-diacylglycerol synthase
CSD	-	Caveolin scaffolding domain
DAG	-	Diacyglycerol
DGK	-	Diacylglycerol kinase
ECE	-	Endothelin-converting enzyme
ECL	-	Enhanced chemiluminescence
EDRF	-	Endothelium-derived relaxing factor
eNOS	-	Endothelial nitric oxide synthase
ER	-	Endoplasmic reticulum
ET-1	-	Endothelin-1
ET-2	-	Endothelin-2
ET-3	-	Endothelin-3
FRET	-	Fluorescence resonance energy transfer
GPI	-	Glycosylphosphatidylinositol
HRP	-	Horseradish peroxidase
IP_3	-	Inositol-1,4,5-trisphosphate

L_o	-	Liquid-ordered
LPP	-	Lipid phosphate phosphatase
LysoPA	-	Lysophosphatidic acid
MBS	-	MES-buffered saline
mTOR	-	Mammalian target of rapamycin
MW	-	Molecular weight
N_2	-	Nitrogen
Na_2CO_3	-	Sodium carbonate
NA	-	Noradrenaline
NO	-	Nitric oxide
PA	-	Phosphatidic acid
PAP	-	Phosphatidic acid phosphatase
PC	-	Phosphatidylcholine
PE	-	Phenylephrine
PEth	-	Phosphatidylethanolamine
PH	-	Pleckstrin homology
PhE	-	Phorbol esters
PI	-	Phosphatidylinositol
PIP	-	Phosphatidylinositol 4-monophosphate
PIP_2	-	Phosphatidylinositol 4,5,-bisphosphate
PI3K	-	Phosphatidylinositol 3-kinase
PKB/Akt	-	Protein kinase B
PKC	-	Protein kinase C
PL	-	Phospholipids
PLA	-	Phospholipase A

PLC	-	Phospholipase C
PLD	-	Phospholipase D
PO_4	-	Phosphate
PP1c	-	Protein phosphatase 1 catalytic subunit
PS	-	Phosphatidylserine
PSS	-	Physiological salt solution
RMSA	-	Rat mesenteric small arteries
SDS	-	Sodium dodecyl sulphate
SEM	-	Standard error of the mean
SL	-	Sphingolipids
SM	-	Sphingomyelin
SR	-	Sarcoplasmic reticulum
TCA	-	Trichloroacetic acid
TBS	-	Tris-buffered saline
TLC	-	Thin-layer chromatography
TTBS	-	Tween TBS
VSM	-	Vascular smooth muscle

Abstract

Caveolae/rafts, specialised plasma membrane microdomains, were recently implicated in vascular smooth muscle (VSM) contraction but all work to date has focused on protein signalling pathways. Vasoconstrictors such as noradrenaline (NA) and endothelin-1 (ET-1) signal through phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) producing the second messengers inositol-1,4,5-trisphosphate and diacylglycerol (DAG). DAG is phosphorylated by DAG kinases (DGK) to produce phosphatidic acid (PA) – also a second messenger with distinct cellular targets.

Both NA and ET-1-stimulated PIP_2 hydrolysis was localised to caveolae/rafts. This involved the PLC-δ_1 isoform in an extracellular calcium-dependent manner for NA but not for ET-1 suggesting agonist-specific activation of PLC isoforms. Both NA and ET-1-stimulated DGK activity and DGK-derived PA production was localised to caveolae/rafts although this was not exclusive for ET-1. Whilst DGK activity in response to either agonist was unaffected by extracellular calcium removal, PA levels were reduced suggesting calcium-dependent regulation of PA metabolism.

NA-stimulated DGK activity and PA production was partially dependent on phosphoinositide 3-kinase (PI3K) with the PI3K-dependent DGK activity localising to caveolae/rafts. However, ET-1-stimulated DGK activity and PA production were independent of PI3K suggesting agonist-specific activation of DGK isoforms. The PI3K-dependent DGKθ isoform and the downstream PI3K target protein kinase B (PKB/Akt) were both present in caveolae/rafts. Furthermore, PKB/Akt levels in caveolae/rafts were increased by NA but not ET-1. This suggests differential activation of DGKθ and this could be a result of different effects of NA and ET-1 on PKB/Akt levels in caveolae/rafts.

Cholesterol depletion is reported to disrupt caveolae/rafts and enhanced the contractile response to NA in intact RMSA. Cholesterol depletion enhanced NA but not ET-1-stimulated PIP_2 hydrolysis and had no effect on DGK activity with either agonist. However, PA levels for both NA and ET-1 were increased. Whilst this suggests that caveolae/raft disruption affects vasoconstrictor-stimulated PI signalling in VSM, it was unclear if caveolae/rafts were disrupted in RMSA as evidenced by different effects of cholesterol depletion on caveolae/raft markers. Additionally, cholesterol depletion is now known to have effects other than disruption of caveolae/rafts, which could also account for the differences observed.

In conclusion, the data presented in this thesis supports a role for caveolae/rafts in regulation of vascular contractility as sites where agonist-stimulated PI turnover couples to intracellular signalling pathways through localised production of second messengers. The agonist-specific activation of enzyme isoforms and spatial differences in production of lipid second messengers are likely to be important for agonists that utilise a common signalling system.

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Dedication

I would like to dedicate this thesis to my parents. I want to thank them for believing in me, for picking me up when I needed it and for their ongoing support. I could not have made it this far without them.

Acknowledgements

Firstly, I would like to thank Dr. Jacqueline Ohanian for her excellent supervision and support over the course of my PhD and for her seemingly limitless patience in dealing with my numerous questions and demands on her time. I would also like to thank Dr. Vasken Ohanian for helpful discussion and for constructive criticism throughout the past three years. Special thanks to Dr. Alan Curry for the electron microscopy of caveolae/raft preparations and also to Fiona for being such a good friend and sounding board.

CHAPTER 1

Background Introduction

1.0 Lipid signalling

The vasculature is a complex network of arteries, capillaries and veins whose main function other than the movement of blood is the maintenance of cellular homeostasis (Pugsley & Tabrizchi, 2000) Small arteries (arterioles) are the main resistance vessels of the vascular network, important in the maintenance of blood pressure and the distribution of blood flow. This is regulated by the vascular tone of arterial vascular smooth muscle (VSM), dependent on a number of stimuli including circulating hormones, neurotransmitters and endothelium-derived factors (Levick, 2000). Research spanning the past 15 years has implicated small arteries in the pathogenesis of diseases such as hypertension wherein they undergo structural remodelling partly as a result of chronic vasoconstriction (Intengan & Schiffrin, 2001). Therefore, the mechanisms regulating vasoconstriction are of interest.

Signal transduction is the process by which an external stimulus is converted into a cellular response. Binding of a ligand to its receptor, or physical stimuli such as stress or ultraviolet light activates a complex network of signalling cascades that carry the signal to the appropriate subcellular compartments and facilitate the cellular response. Although intracellular signalling pathways are ubiquitous, they can produce a diverse array of responses and are an area of active research. An understanding of the normal cellular response to external stimuli would be useful for determining any signalling abnormalities in pathological states.

The process of signal transduction involves a variety of intracellular effectors and second messengers to transmit and amplify the signal. Second messengers are essential intermediates that link extracellular stimuli to intracellular responses and are defined by specific criteria: 1) Changes in the messenger level in response to physiological stimuli must be observed; 2) a direct *in vitro* target must be identified; 3) the ability of the messenger to activate this target in cells must be confirmed; 4) a role of this pathway in mediating a cellular response must be established (Khan *et al.*, 1995). One such group of molecules are lipid-derived second messengers, generated on agonist stimulation of phospholipases that selectively hydrolyse complex lipids such as phosphatidylinositol (PI), phosphatidylcholine (PC) and sphingomyelin (SM) to produce biologically active molecules.

1.1 Signalling by phospholipid metabolism

The plasma membrane is a major source of phospholipids. One of the most well-characterised responses to a variety of stimuli is the phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) producing the second messengers inositol-1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) (**Fig. 1.1**).

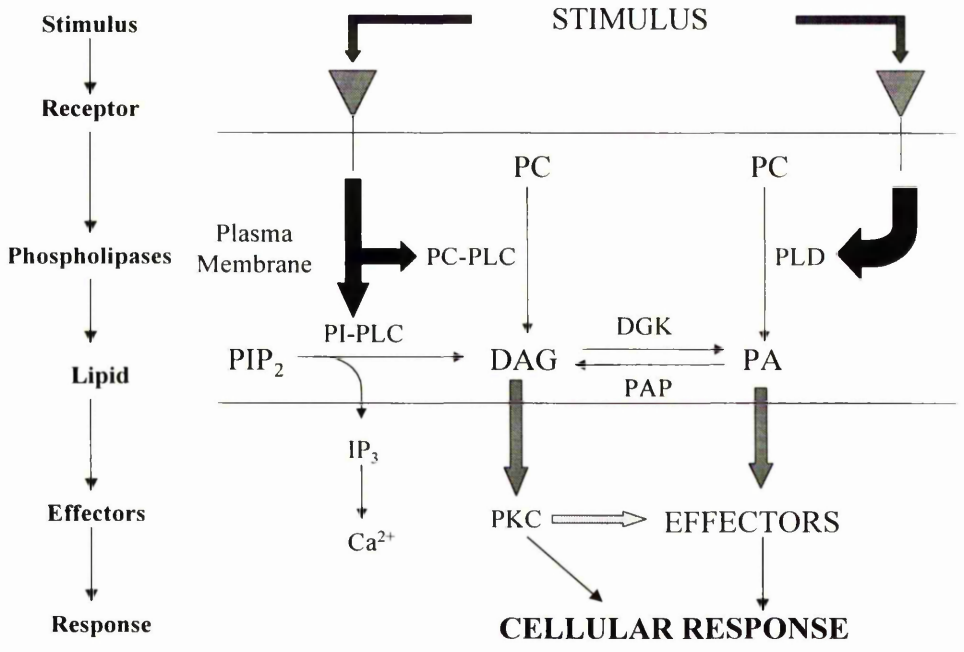


Figure 1.1: Signalling by Phospholipid Metabolism. Receptor activation by hormones/growth factor/physical stimuli activates phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), producing diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). IP_3 mobilises calcium from intracellular stores and DAG activates protein kinase C (PKC). DAG Kinase (DGK)-mediated phosphorylation of DAG to phosphatidic acid (PA) attenuates PKC-mediated responses but may elicit PA-mediated responses. DAG and PA can also be produced from phosphatidylcholine (PC) by PC-PLC and phospholipase D (PLD) respectively. PA can also be metabolised to DAG by PA Phosphatase (PAP; adapted from Ohanian & Ohanian, 2001)

1.1.1 The phosphoinositide effect

It is over 50 years since the discovery of the phosphoinositide effect by Hokin and Hokin who reported the incorporation of [^{32}P] into PI on the cholinergic stimulation of pigeon pancreas slices (Hokin & Hokin, 1953). It was some time later before this was proposed to reflect receptor-mediated signal transduction (Durell *et al.*, 1969) and a common mechanism of PI turnover was suggested (Michell, 1975). Evidence accumulated indicating that PIP_2 was the immediate substrate of PI

hydrolysis (Abdel-Latif *et al.*, 1977;Schact & Agranoff, 1972) and it was subsequently proposed that PI turnover occurred indirectly to replenish levels of PIP_2. This process was speculatively linked to calcium mobilisation (Michell *et al.*, 1981).

The hydrolysis of PIP_2 results in a bifurcating signal: IP_3 is released into the cytosol, stimulating calcium mobilisation from intracellular stores (Somylo *et al.*, 1985;Streb *et al.*, 1983) by binding to its receptor on the sarcoplasmic reticulum (SR) (Mignery *et al.*, 1990) and promoting calcium influx from the extracellular space by modulating membrane potential to allow the opening of voltage-gated calcium channels (McCarron *et al.*, 2002). The second messenger role of DAG was established when it was found to be the physiological activator of protein kinase C (PKC) (Kishimoto *et al.*, 1980), a family of ubiquitous serine/threonine kinases initially isolated from rat brain and bovine cerebellum (Takai *et al.*, 1977;Inoue *et al.*, 1977), providing an important link between PI turnover and cellular signalling cascades. IP_3 and DAG were proposed to have unique temporal roles – the former in the early cellular response and the latter in the sustained cellular response (Rasmussen & Barrett, 1984). Subsequent studies on DAG reported biphasic production in hepatocytes (Bocckino *et al.*, 1985) and VSM cells (Griendling *et al.*, 1986) significantly showing that DAG production is not necessarily coupled to IP_3 generation. Further evidence indicated that PIP_2 hydrolysis produced quantitatively minor amounts of DAG correlating with the first peak of production (Pessin & Raben , 1989;Sunako *et al.*, 1989;Kennerly, 1987) and it was suggested that PC was a likely source of sustained DAG production.

1.1.2 Phosphatidylcholine hydrolysis

In a number of mammalian cells, PC is preferentially localised to the external leaflet of the plasma membrane (Zachowski, 1993) and is the most abundant of the cellular phospholipids comprising up to 50% compared to the phosphoinositides comprising <0.1% of total phospholipid (Billah & Anthes, 1990). Hydrolysis of PC stimulated by tumour-promoting phorbol esters (PhE) was first reported in 1981 (Mufson *et al.*, 1981) and later found to occur in response to hormones (Besterman *et al.*, 1986;Bocckino *et al.*, 1985). PC metabolism was subsequently reported by both phospholipase D (PLD) (Cabot *et al.*, 1988) and PC-specific PLC (Besterman *et al.*, 1986) and has been observed in numerous tissues in response to many different agonists suggesting that it is of physiological importance in production of lipid second messengers (Exton, 1994;Exton, 1990).

1.2 Second messengers

1.2.1 IP_3 in vascular smooth muscle contraction

Vasoconstrictors such as noradrenaline (NA) and endothelin-1 (ET-1) stimulate smooth muscle contraction by the activation of PLC-mediated PI turnover and subsequent production of IP_3 and DAG (Heagerty & Ollerenshaw, 1987;Takuwa & Rasmussen, 1987). In smooth muscle, IP_3 production is rapid – it has been observed within 1 second (Duncan *et al.*, 1987), peaking within 1-2 minutes (Griendling *et al.*, 1986). An increase in cytoplasmic calcium concentration is the primary modulator of smooth muscle contraction. Free calcium in the cytosol associates with the protein calmodulin (CaM) resulting in activation of myosin-light chain kinase and subsequent phosphorylation of the regulatory light chains of myosin II, which facilitates the development of force (reviewed in Ganitkevich *et al.*,

2002;Sanders, 2001). This initial component of contraction is attributed to IP_3-mediated calcium release from the SR (Somylo *et al.*, 1985) and calcium influx through voltage-gated channels opened by membrane depolarisation (McCarron *et al.*, 2002). However, IP_3 and calcium levels increase transiently whereas many agonists can stimulate a sustained phase of contraction (Takuwa & Rasmussen, 1987) and this was proposed to involve DAG activation of PKC (Griendling *et al.*, 1986).

1.2.2 Diacylglycerol

DAG is a membrane-associated hydrophobic molecule that, in addition to its role as PKC activator, occupies a central position in glycerolipid synthesis (Cases *et al.*, 2001;McMaster & Bell, 1997a;McMaster & Bell, 1997b). The PKC family consists of twelve isoforms subdivided into three groups – the classical (α, βI, βII, γ), novel (δ, ε, η, θ and μ) and atypical (ξ, ι, λ) (reviewed in Webb *et al.*, 2000) of which the classical and novel families are activated by DAG. Through this pathway, DAG has been implicated in a number of signalling pathways that control cell proliferation, differentiation, survival, transformation and apoptosis (Dempsey *et al.*, 2000). In VSM, PKC has been suggested to enhance contraction by a process termed calcium sensitisation, the regulation of myosin phosphorylation independently of a change in intracellular calcium concentration (reviewed in Ganitkevich *et al.*, 2002) and has also been implicated in regulating the interaction between actin and myosin filaments through phosphorylation of the actin-binding proteins caldesmon and calponin, a process known as thin-filament regulation (Gorenne *et al.*, 2004;Je *et al.*, 2001;Gerthoffer *et al.*, 1997).

Cellular effects of DAG have usually been attributed solely to PKC but recently this assumption has been challenged with the discovery of alternative DAG "receptors". Currently, five groups of alternative DAG effectors are known that all bind DAG with PKC-like affinity resulting in their translocation to the plasma membrane or intracellular compartments: the chimaerins, protein kinase D, Ras guanyl nucleotide-releasing protein, munc13s and the DAG kinases (DGK) β and γ (Yang & Kazanietz, 2003;Brose & Rosenmund, 2002). Thus, the signal from DAG can be carried independently of PKC adding a level of complexity to DAG signalling.

1.2.3 Phosphatidic acid

A second product of PC and PI turnover is phosphatidic acid (PA), the phosphorylated form of DAG and a key intermediate in lipid synthesis (reviewed in (Athenstadt & Daum, 1999). Although representing only a minor portion of membrane phospholipids at rest (Fukami & Takenawa, 1992), PA was found to accumulate on stimulation with certain agonists leading to the theory that it functions as a second messenger in its own right. It has subsequently been implicated in a number of cellular processes in addition to being a source of other lipid messengers such as arachidonic acid (AA) (Kitatani *et al.*, 2000), DAG (Sciorra & Morris, 2002;Sciorra & Morris, 1999) and lysophosphatidic acid (lysoPA) (Hiramatsu *et al.*, 2003).

Early studies implicated endogenous PA as a mediator of calcium influx (Salmon & Honeyman, 1980) and linked exogenous PA to calcium mobilisation although subsequent work showed that this was due to contamination with oxidised fatty acids (Holmes & Yoss, 1983) and lysoPA respectively (Jalink *et al.*, 1990). However, later

work with non-contaminated systems indicated that exogenous PA does stimulate calcium mobilisation in cardiomyocytes and VSM cells with results suggesting this occurs through PLC-mediated IP_3 production (Bhugra *et al.*, 2003;Liu *et al.*, 1999b;Chuang *et al.*, 1993). The exogenous roles of PA are important physiologically as activated macrophages and cells of the immune system can release PA (Lim *et al.*, 2003), platelets are reported to release PA during clotting (English *et al.*, 2001) and disrupted cells release PA during inflammation (Lim *et al.*, 2003). In addition to being a potent mitogen (Schatter *et al.*, 2003;Reeves *et al.*, 2000;Fukami & Takenawa, 1992), exogenous PA is also reported to induce hormone release (Siddhanta *et al.*, 2000;Zheng *et al.*, 1997), platelet aggregation (Xiao *et al.*, 2001), actin polymerisation (Porcelli *et al.*, 2002;Siddiqui & English, 1997), and superoxide release in neutrophils (Erickson *et al.*, 1999). PA may also be produced on the outer leaflet of the plasma membrane as extracellular release of choline suggests (Jones *et al.*, 1993;Gu *et al.*, 1992;Huang & Cabot, 1990) with PA generated in this manner in neutrophils found to increase permeability of the endothelial monolayer (English *et al.*, 1999). However, the direct constrictor action of exogenous PA on vascular smooth muscle have yet to be reported.

Substantial research has identified a number of possible cellular targets through which endogenous PA may act (**table 1.1**). Further analysis of PA binding has led to the identification of short amino acid regions accounting for the interaction – an 11 residue TAPAS-1 (Tryptophan Anchoring Phosphatidic Acid-Selective) domain in the phosphodiesterase PDE4A1 (Baillie *et al.*, 2002) and primarily through three conserved cationic residues in Raf-1 (Rizzo *et al.*, 2000). However, to date there has been no discovery of a PA-binding domain common to many different proteins

analogous to other lipid binding domains e.g. PH domains, suggesting that structural requirements for such a domain may be simple and heterogenous.

Targets	Potential Functions	References
Raf-1 Ras	Cell Growth DNA Synthesis	(Rizzo *et al.*, 2000;Tsai *et al.*, 1991)
PLC-β, -γ	Positive Feedback, Amplification of Responses.	(Litosch, 2000;Jones & Carpenter, 1993)
Sphingosine kinase	Sphingolipid signalling	(Delon *et al.*, 2004;Olivera *et al.*, 1996)
Rho GDI-proteins PI-4-P 5-Kinase	Cytoskeletal Regulation	(Jones *et al.*, 2000;Chuang *et al.*, 1993)
Dynamin	Endocytosis	(Burger *et al.*, 2000)
PKC Myosin phosphatase	Calcium sensitisation	(Lopez-Andreo *et al.*, 2003;Limatola *et al.*, 1994)
PP1c PA-dependent kinase	Protein phosphorylation	(Jones & Hannun, 2002;McPhail *et al.*, 1999)
Opi1p	Gene expression in yeast	(Loewen *et al.*, 2004))
mTOR	Cell growth	(Avila-Flores *et al.*, 2005)

Table 1.1: Proposed PA targets and potential functional consequences.

Abbreviations – PP1c – protein phosphatase 1 catalytic subunit, PLC – phospholipase C, PKC – protein kinase C, PI-4-P 5-Kinase –phosphatidylinositol-4-monophosphate 5-kinase, mTOR – mammalian target of rapamycin.

Despite such a broad spectrum of PA targets, the majority of these observations are *in vitro* and there has been no confirmation of their *in vivo* roles, hampered by an inability to control cellular PA levels. Thus, the second messenger functions of endogenous PA accumulation remain unresolved.

1.2.4 DAG and PA in vascular smooth muscle

Despite considerable research, mostly focused on PKC, the evidence concerning an essential role for DAG in VSM contraction is conflicting. Whilst early studies reported biphasic production of DAG in response to vasoconstrictors, these were primarily carried out in cultured or dispersed cells (Griendling *et al.*, 1989;Slivka *et al.*, 1988;Griendling *et al.*, 1986;Bocckino *et al.*, 1985) with subsequent studies in intact tissue reporting that DAG accumulation was not required in response to agonists that produce sustained contraction (Liu *et al.*, 1999a;Ward *et al.*, 1995;Ohanian *et al.*, 1990;Rembold & Weaver, 1990). Furthermore, although angiotensin II (AngII) stimulated DAG accumulation, the resultant contraction was transient (Ohanian *et al.*, 1993;Lasseque *et al.*, 1993). A later study in the same tissue system reported that maximal agonist-induced contraction was unaffected by downregulation of DAG-activated PKC isoforms (Ohanian *et al.*, 1996) whereas PKC inhibitors reduced ET-1-stimulated contraction of aortic rings (Danthuluri & Brock, 1990). Whilst this suggests agonist-specific involvement of PKC, more recently, AA-mediated activation of atypical PKCs has been suggested to be more important with DAG-activated PKCs playing a minor role (Guo *et al.*, 2003;Gailly *et al.*, 1997). Thus, DAG may not have an essential role in regulation of sustained VSM contraction and it has been proposed that regulation of DAG metabolism is more significant (Ohanian & Heagerty, 1994;Ohanian *et al.*, 1993).

Many studies have reported accumulation of PA in response to a number of vasoconstrictors in both cultured smooth muscle cells (Pyne & Pyne, 1995;Ye *et al.*, 1994;Lasseque *et al.*, 1993) and intact vascular tissue (Liu *et al.*, 1999a;Ward *et al.*, 1995;Ohanian *et al.*, 1990) leading to the theory that it plays a role in regulation of vascular tone. This is supported by the observation that PA-induced calcium mobilisation via production of IP_3 has been reported in VSM cells (Bhugra *et al.*, 2003) and the time course for PA changes was closely related to calcium-dependent tonic contraction of the rat aorta (Jones *et al.*, 1993). However, although this suggests a role, the direct constrictor actions of exogenous PA have yet to be reported and the manner in which endogenous PA may regulates vascular tone is unclear. This could solely involve calcium mobilisation or could be more complex involving regulation of other signalling cascades. With such a wide array of potential endogenous targets (**section 1.2.3**), considerable research is required to determine their *in vivo* significance before more definite conclusions can be made.

In summary, although DAG has been implicated in a number of cellular processes, its involvement in regulation of vascular tone is still a matter of debate and whilst evidence suggests a role for PA in regulation of vascular tone, the signalling pathways and downstream effectors involved are still to be elucidated.

1.3 Phosphatidic acid production

The second messenger functions of PA would require a precise regulation of cellular levels to ensure the correct responses to appropriate stimuli and differences in production and metabolism of PA are likely to be important in signal specificity for the number of stimuli that signal via phospholipid turnover. PA can be produced

through three major routes (**Fig. 1.2**): 1) PLD-mediated PC hydrolysis, 2) DGK-mediated DAG phosphorylation, and 3) *De novo* synthesis

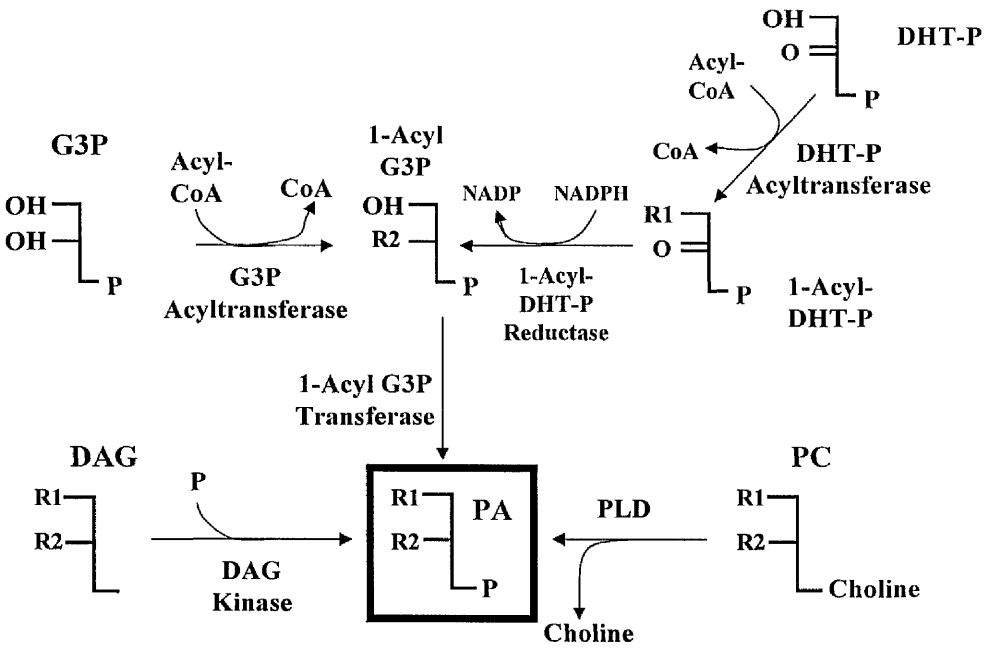


Fig. 1.2: PA is produced by three major pathways.

Abbreviations: 1-Acyl G3P – 1-acyl glycerol 3-phosphate; P – phosphate; DHT-P – dihydroxyacetone phosphate; 1-Acyl-DHT-P – 1-acyl-dihydroxyacetone phosphate; G3P – glycerol 3-phosphate.

1.3.1 Phospholipase D

PLD catalyses the breakdown of PC to produce PA and choline and such activity was first observed in mammalian cells over 25 years ago (Saito & Kanfer, 1975). The subsequent discovery that PLD catalyses a unique transphosphatidylation reaction in the presence of a primary alcohol produced an experimental tool used to determine PLD involvement in cellular processes (Kanfer, 1980). Currently, two mammalian PLD isoforms have been cloned – PLD1 and 2 – that differ in regulatory properties (reviewed in Exton, 2002) and activation of PLD has been reported in numerous cell types in response to a variety of stimuli including hormones, growth

factors, neurotransmitters and physical stimuli (Liscovitch *et al.*, 2000). In smooth muscle, PLD has been reported to be activated by NA (Ward *et al.*, 1995), ET-1 (Liu *et al.*, 1999a), AngII (Lasseque *et al.*, 1993) and bradykinin (Pyne & Pyne, 1995).

1.3.2 Diacylglycerol Kinase

DGK is a key enzyme, occupying a major step in the PI turnover cycle and is considered to be a major pathway of attenuating PKC activation. First identified in brain microsomes, DGK utilises ATP to phosphorylate DAG (Hokin & Hokin, 1959). A number of DGK activities were subsequently characterised (see Kanoh *et al.*, 1990) although it was some time before the first DGK was cloned from white blood cells (Schaap *et al.*, 1990). Currently, nine mammalian DGK isoforms have been cloned, all with conserved catalytic domains and two cysteine rich domains. Isoforms are divided into five groups according to functional domains and substrate specificity (**Table 1.2**) (reviewed in Van Blitterswijk & Houssa, 2002).

1.3.2.1 Regulation of DGK

Regulation of DGK enzymes is complex, appears unique for subtype or isoform and is not clearly understood. Translocation has been suggested as a necessary first step for activation and has been shown for a number of DGK activities (Van Blitterswijk & Houssa, 1999). However, this has only been demonstrated for three cloned isoforms involving movement to the plasma membrane (α, θ and ζ) (Santos *et al.*, 2002;Walker *et al.*, 2001;Schaap *et al.*, 1993) or nucleus (ζ) (Topham *et al.*, 1998) and the trigger for translocation is not clear. Some studies have discounted substrate availability as a factor, reporting that exogenously increasing DAG levels at the membrane did not affect DGK activity

(Monaco *et al.*, 2002;Van der Bend *et al.*, 1994). However, the C1 (cysteine-rich) domains of β and γ isoforms bound PhE with high affinity causing their translocation to the plasma membrane (Shindo *et al.*, 2003) and it was suggested that DAG binding to the same domains could translocate the enzymes *in vivo* although this has not been effectively demonstrated. Calcium has also been implicated in regulation of DGKα translocation and activity by binding to the EF-hands (Abe *et al.*, 2003;Jiang *et al.*, 2000a) but this has not been demonstrated *in vivo* and is still a matter of debate (Van Blitterswijk & Houssa, 2002).

Type	Isoforms	Characteristics
I	α, β, γ	EF-Hand motifs, recoverin homology domain
II	δ,η	N-terminal PH domain
III	ε	Arachidonate-specific, no specialised domains
IV	ζ,ι	MARCKS homology domain, ankyrin repeats, nuclear localisation signal
V	θ	3rd cysteine-rich domain, PH domain overlaps with Ras-associating domain, proline-rich region

Table 1.2: Classification of DGK enzymes.

Following translocation, a second activation step is proposed to occur such as phosphorylation (Van Blitterswijk & Houssa, 2002). A number of studies have reported phosphorylation of DGK enzymes. DGKα is phosphorylated by protein kinase A (Soling *et al.*, 1989), PKC (Schaap *et al.*, 1993), the epidermal growth factor receptor and Src (Cutrupi *et al.*, 2000) with the latter increasing DGK activity

in vitro. DGK-ζ is also phosphorylated by PKC causing its movement out of the nucleus (Topham *et al.*, 1998). In T-cells, this is required for translocation to the plasma membrane and inhibits enzyme activity (Luo *et al.*, 2003b). PKC-mediated phosphorylation has also been implicated in regulating DGK activity in fibroblasts (Van Blitterswijk *et al.*, 1991) and regulating translocation in guinea pig taenia coli (Nobe *et al.*, 1997). Other regulators of DGK have been reported. DGKθ is negatively regulated by RhoA and, in small arteries, is activated in a PI 3-kinase (PI3K)-dependent manner (Walker *et al.*, 2001;Houssa *et al.*, 1999). DGKα can be activated by PI3K products in the absence of intracellular calcium (Cipres *et al.*, 2003) and can be inhibited by free fatty acids in VSM (Du *et al.*, 2001). Finally, arachidonyl-specific DGK-ε can be inhibited by PIP_2 and, to a lesser extent, PA suggesting a way in which PI turnover could be feedback regulated (Walsh *et al.*, 1995).

1.3.2.2 Contribution of PLD and DGK to PA production

The relative contribution of these two pathways to PA levels has been a matter of debate and is likely agonist and tissue-dependent. PLD was reported as the major contributor in ET-1-stimulated cardiomyocytes (Ye *et al.*, 1994), AngII-stimulated VSM cells (Lasseque *et al.*, 1993), NA-stimulated rat aorta (Jones *et al.*, 1993) and the ischaemic rat heart (Kurz *et al.*, 1999) whereas DGK pathways predominate in muscarinic-stimulated neuroblastoma cells (Pacini *et al.*, 1993), thrombin-stimulated platelets (Huang *et al.*, 1991) and vasopressin-stimulated hepatocytes (Allan & Exton, 1993). This is likely to be important for signalling specificity owing to the proposed differential effects of PLD and DGK-derived PA species (**section 1.5**).

1.3.3 *De Novo* synthesis

PA is synthesised *de novo* by two different acylation pathways with distinct precursors (glycerol-3-phosphate and dihydroxyacetone) that converge on production of lysoPA (1-Acyl-3-glycerophosphate). This is subsequently acylated by lysoPA transferase to produce PA. In both cases, the first step is rate-limiting and intermediates usually do not accumulate at rest (reviewed in Athenstadt & Daum, 1999). Whilst PA synthesis has been examined in some studies and is reported in response to insulin and platelet-activating factor (Kester, 1993;Farese *et al.*, 1987) but not AngII or phenylephrine (PE) (Vila *et al.*, 1990), the predominant localisation of these pathways to the endoplasmic reticulum (ER) and other intracellular organelles suggests that they are not relevant in the context of agonist-induced signal transduction at the plasma membrane.

1.3.4 Origin of DAG for the DGK pathway

Whilst PA produced from PLD is derived from PC, the origin of PA from the DGK pathway could come from PC- or PI-derived DAG with two major pathways contributing to this (**Fig. 1.3**): 1) PI-PLC-mediated PIP_2 hydrolysis and 2) PC-PLC/SM Synthase-mediated PC hydrolysis. Although production of DAG from PA phosphatase (PAP) has been observed (Sciorra & Morris, 1999;Exton, 1994), this would create a futile cycle with active DGK and the two pathways are likely to be spatially segregated.

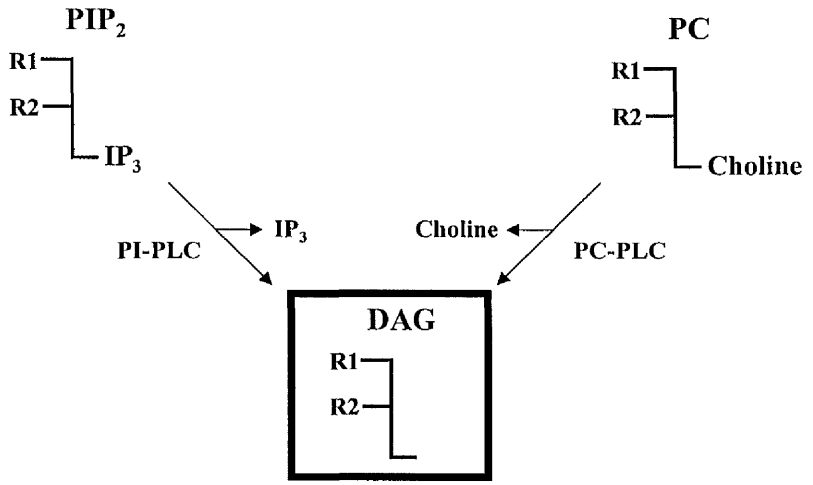


Fig. 1.3: DAG can originate from two major pathways.

Abbreviations: PIP_2 – phosphatidylinositol-4,5-bisphosphate, IP_3 – inositol-1,4,5-trisphosphate, DAG – diacylglycerol, PC- phosphatidylcholine, PC-PLC; PC-specific phospholipase C, PI-PLC – phosphoinositide-specific phospholipase C.

1.3.4.1 Phosphoinositide-specific phospholipase C

Identified as the key enzyme in agonist-stimulated PIP_2 hydrolysis, PI-PLC was first purified in 1981 (Takenawa & Nagai, 1981) with subsequent research revealing the existence of multiple isoforms (Rhee *et al.*, 1989). Although eleven isoforms have been identified, separated into four classes - β (1-4), γ (1-2), δ (1-4) and ε (**Fig. 1.4**), a recent study reported that bovine PLC-δ_2 is a homologue of human PLC-δ_4 suggesting there are only three PLC-δ isoforms (Irino *et al.*, 2004). The PLC isoforms are relatively non-conserved with the exception of the X and Y domains comprising the catalytic core.

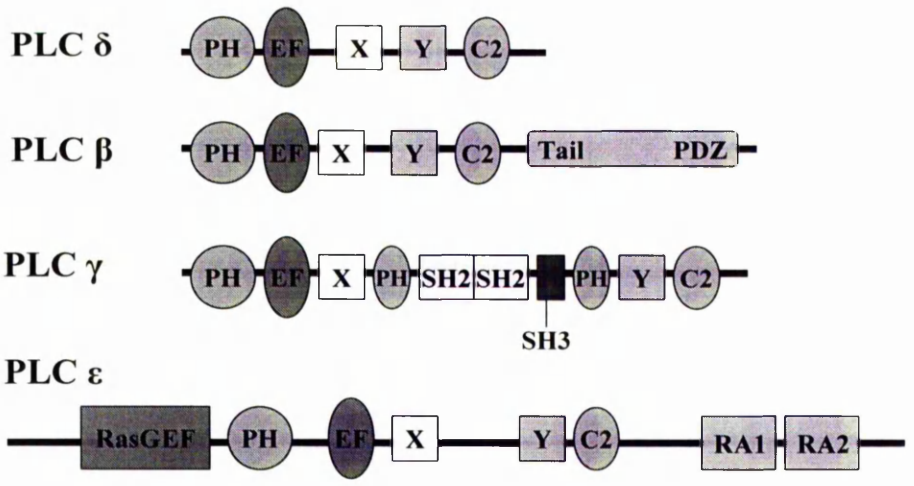


Fig. 1.4: Domain organisation of the four PLC subtypes.

Catalytic (X and Y) and regulatory domains are shown. PH – pleckstrin homology domain, EF – EF-hand motif, PDZ – PDZ-binding motif, SH – src-homology domain, RasGEF – Ras guanine nucleotide exchange factor-like domain, RA – Ras associating domain (adapted from Fukami, 2002).

1.3.4.2 Regulation of PLC isoforms

The PLC isoforms are differentially regulated according to their subtype. (**Table 1.3**) Despite this, the agonist-induced anchoring of PLC to the membrane is considered an important first step of activation (Fukami, 2002). The predominant regulation of the β isoforms by heterotrimeric G-proteins suggests they are likely candidates to be involved in PIP_2 hydrolysis stimulated by G-protein-coupled agonists. However, as can be seen from the table, all four subtypes of PLC can be regulated by G-proteins suggesting that other isoforms may also be involved.

Subtype	Regulation	References
β (1-4)	Gq subtype of heterotrimeric G-proteins, βγ dimers. Also activated by Rac, PA and CaM Inhibited by PKC	(Snyder *et al.*, 2003;Litosch, 2003;McCullar *et al.*, 2003;Litosch, 2000;Runnels & Scarlata, 1999;Smreka & Sternweis, 1993;Taylor *et al.*, 1991)
γ (1,2)	Receptor tyrosine kinases, non-receptor tyrosine kinases Src and Syk. Activity enhanced by PA and PLD2. Inhibited by glycosphingolipids	(Jang *et al.*, 2003;Wetzker & Bohmer, 2003;Shu *et al.*, 2002;Rhee, 2001;Jones & Carpenter, 1993;Rhee, 1991;Kim *et al.*, 1990)
ε	Stimulated by Ras, $G_{12/13}$ subtype of heterotrimeric G-proteins, βγ dimers. Inhibited by G_i-coupled receptors.	(von Dorp *et al.*, 2004;Song *et al.*, 2001;Lopez *et al.*, 2001;Wing *et al.*, 2001)
δ (1-4)	Activity enhanced by PIP_2 and calcium. Inhibited by IP_3. Activated by Gi-coupled receptors and a high molecular weight G-protein Gh. Activated by p122Rho GTPase-activating protein. Inhibited by RhoA.	(Murthy *et al.*, 2004;Hodson *et al.*, 1998;Allen *et al.*, 1997;Lomasney *et al.*, 1996;Feng *et al.*, 1996;Homma & Emori, 1995)

Table 1.3: Regulation of PLC isoforms.

Abbreviations – AA – arachidonic acid, PA – phosphatidic acid, PKC – protein kinase C, PIP_2 – phosphatidylinositol-4,5-bisphosphate, IP_3 – inositol-1,4,5-bisphosphate, CaM – calmodulin.

1.3.4.3 PI-PLC in vascular smooth muscle

Despite current knowledge of PLC regulation, it is unclear which isoforms are involved in vasoconstrictor-stimulated PIP_2 hydrolysis as VSM contains at least four isoforms - β_1, β_2, γ_1 and δ_1 (LaBelle *et al.*, 2002). As mentioned above, the β isoforms are likely contenders owing to their regulation by heterotrimeric G-proteins and supporting this, activation of PLC-β_2 and β_1 has been reported in tracheal smooth muscle (Coburn *et al.*, 1997) and cultured aorta cells (Ushio-Fakai *et al.*, 1998). Furthermore. a recent study in NA-stimulated rat-tail artery reported that PLC-β_2 was the major participant (LaBelle *et al.*, 2002). However, as G-protein

coupled receptor agonists can also activate PLC-γ_1 (Marrero *et al.*, 1994) and PLC-δ_1 (Kim *et al.*, 1999) this suggests that they could also be involved in vasoconstrictor-stimulated PIP_2 hydrolysis.

1.3.4.4 PC-specific phospholipase C

Considerably less is known about PC-PLC than of its PI-specific counterpart Whilst a number of bacterial PC-PLCs have been cloned that require calcium and zinc ions for activity (Preuss *et al.*, 2001), to date no mammalian PC-PLC have been cloned. However, there is direct evidence that such a PC-PLC is expressed in mammalian cells. A PC-PLC has been partially purified from canine myocardium and from bull seminal plasma (Sheikhnejad & Srivastava, 1986;Wolf & Gross, 1985). Also, a recent study in fibroblasts reported detection of PC-PLC and translocation to the plasma membrane on agonist stimulation (Ramoni *et al.*, 2004). A recent study demonstrating that the PC-PLC inhibitor D609 also inhibited sphingomyelin synthase, an enzyme that carries out the same reaction as PC-PLC has led to the suggestion that the two enzymes are the same (Luberto & Hannun, 1998) and this was found to be the case with a bacterial PC-PLC (Luberto *et al.*, 2003). However, whilst two mammalian sphingomyelin synthase isoforms were recently cloned (Huitema *et al.*, 2004), whether these account for the observed PC-PLC activities in mammalian tissue has yet to be resolved.

Previous research in ET-1-stimulated RMSA reported PC-PLC-mediated DAG production but this was not subsequently metabolised to PA (Liu *et al.*, 1999a) yet in bradykinin-stimulated airway smooth muscle, PC-PLC-derived DAG was converted

to PA (Pyne & Pyne, 1995). Thus, the role of PC-PLC in PA production is likely to be agonist-specific.

1.4 Phosphatidic acid metabolism

Breakdown of PA will terminate its signalling activities but can also produce other bioactive lipids. There are three major pathways by which this occurs (**Fig. 1.5**): 1) Phospholipase A (PLA)-mediated hydrolysis, 2) PAP-mediated dephosphorylation, and 3) Conversion to cytidine-diphosphate (CDP)-DAG by CDP-DAG Synthase (CDS).

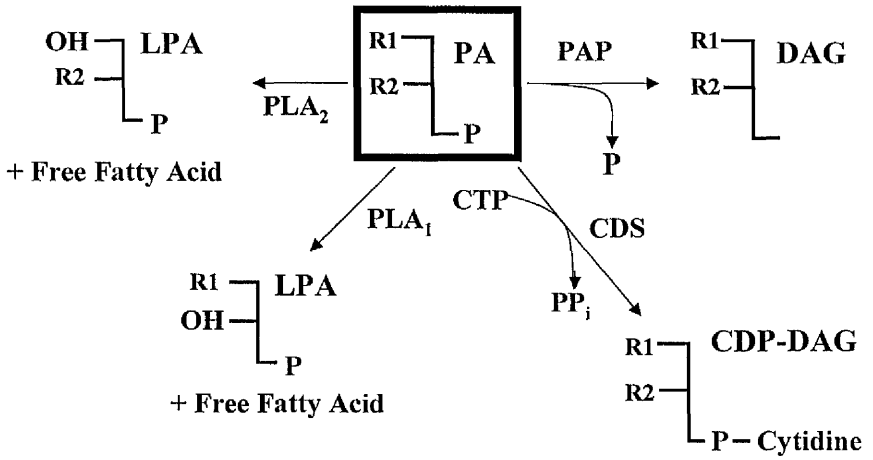


Fig. 1.5: Routes of PA Metabolism.

Abbreviations: CTP – cytidine triphosphate, PP_i – pyrophosphate, P – phosphate, LPA – lysophosphatidic acid, PAP – phosphatidate phosphatase, CDP-DAG – cytidine diphosphate diacylglycerol, CDS – CDP-DAG synthase,

1.4.1 Phospholipase A – PLA_1 and PLA_2

The PLA enzymes catalyse phospholipid hydrolysis to produce lysophospholipids and a free fatty acid. PLA_2 and PLA_1 act at the sn-2 and sn-1 positions respectively to generate different lysophospholipid species. The PLA_2 family consists of three subtypes – secretory, classical and calcium-independent, that

usually remove arachidonate from PA. Cytosolic PLA_2 is thought to be primarily responsible for AA release and in VSM, has been observed in response to α_1-adrenergic agonists (Guo *et al.*, 2003;LaBelle & Polyak, 1998), endothelins (Trevisi *et al.*, 2002), vasopressin (Lehman *et al.*, 1993) and AngII (Ford & Gross, 1989) suggesting that it is a major pathway for PA metabolism. AA and its downstream metabolites have been implicated in processes such as inflammation and apoptosis (Balsinde *et al.*, 2002).

PLA_1 enzymes are less well characterised but are divided into two groups according to their structure and cellular localisation - intracellular or extracellular. Whilst studies have indicated that lysoPA generated from these enzymes is more biologically active (Sonoda *et al.*, 2002), there is insufficient research to determine a role for these enzymes in breakdown of endogenous agonist-stimulated PA and they may act preferentially on serum PA (Miyazawa *et al.*, 2003;Hiramatsu *et al.*, 2003).

1.4.2 Phosphatidate phosphatase

PAP is part of a family of lipid phosphate phosphatases (LPP) that catalyse the dephosphorylation of a number of bioactive lipids such as lysoPA and sphingosine-1-phosphate. PAP – also known as type 1 phosphatases – are mainly found in the cytosol and are thought to function primarily at the ER on activation although a broader specificity LPP that dephosphorylates other lipids in addition to PA was localised to the plasma membrane where it is proposed to regulate signal transduction (reviewed in Sciorra & Morris, 2002;Brindley *et al.*, 2002). PA breakdown from this pathway has been reported in fibroblasts and embryonic kidney cells (Sciorra & Morris, 1999), AngII- and NA-stimulated VSM cells (Parmentier *et*

al., 2001;Freeman, 2000) and PhE-stimulated WISH cells (Balboa *et al.*, 1998) but does not always occur such as in ET-1-stimulated RMSA (Liu *et al.*, 1999a) and carbachol-stimulated neuroblastoma cells (Pacini *et al.*, 1993).

1.4.3 CDP-DAG synthase

A key enzyme in PI turnover, CDS utilises PA and cytidine triphosphate to produce CDP-DAG, the liponucleotide precusor of PI. Ubiquitously distributed among mammalian tissues, CDS is predominantly localised to the ER with the nature of the PI cycle implying that PI-derived PA is the predominant substrate. However, little is known about the regulation of CDS (reviewed in Heacock & Agranoff, 1997).

1.5 Lipid second messengers: are different species important?

The existence of different production pathways utilising distinct phospholipid sources results in DAG and PA containing different fatty acid species at the sn-1 and sn-2 position; those derived from PI are primarily polyunsaturated, usually arachidonate enriched whereas those of PC origin are predominantly monounsaturated or saturated fatty acids (Pettitt *et al.*, 1997). Recently, it was proposed that the distinct species have different functional consequences – with PI-DAG and PC-PA being the important signalling species and PC-DAG and PI-PA being inactive metabolites (Hodgkin *et al.*, 1998). However, both *in vitro* and *in vivo* evidence suggests that different DAG species activate different PKC isoforms (Madani *et al.*, 2001;Ha & Exton, 1993;Leach *et al.*, 1991). The situation with PA is unclear, as very few studies have examined the potencies of different PA species in activating potential targets. However, early studies suggest a similar picture as PLD-

derived PA has been implicated in actin stress-fibre formation and MAP kinase activation whereas a PI-derived PA is reported to activate a cyclic-AMP phosphodiesterase (Hodgkin *et al.*, 1998;El-Bawab *et al.*, 1997;Cross *et al.*, 1996). However, whilst activation of distinct cellular targets by different lipid species is plausible, the compartmentalisation of different lipid species with different cellular targets would also ensure activation of specific targets.

2.0 Contractile agonists and lipid mediators

In VSM, vasoconstrictors signal through the PI signalling pathway. Noradrenaline (NA) and endothelin-1 (ET-1) are potent vasoconstrictors and are physiological regulators of small arteries (Liu *et al.*, 1999a;Ohanian *et al.*, 1993). Experiments in this thesis utilised NA and ET-1 to further investigate PI signalling in small arteries. These agonists will be described in more detail in the following sections.

2.1 Noradrenaline

NA is the major neurotransmitter of the sympathetic nervous system and was first identified by von Euler in 1946. Belonging to the catecholamines, NA is also secreted by cells in the adrenal medulla and can function as a circulating hormone (Rang *et al.*, 1999;Nicholls *et al.*, 1992). NA-stimulated VSM contraction occurs with two distinct phases – a rapid, transient contraction followed by a longer sustained phase (Ohanian *et al.*, 1993;Takuwa & Rasmussen, 1987).

2.1.1 Synthesis

Derived from L-tyrosine, NA (**Fig. 1.6**) is synthesized by the action of three enzymes producing the catecholamine intermediates DOPA and dopamine. In this pathway, the first enzyme tyrosine hydroxylase is the major control point and is inhibited by NA in a negative feedback loop. Conversion of tyrosine to DOPA, and subsequently to dopamine occurs in the cytoplasm, with dopamine transported to synaptic vesicles where the conversion to NA occurs. The majority of NA is stored in these vesicles with any escaping NA subject to degradation in the cytoplasm by the enzyme monoamine oxidase (see Rang *et al.*, 1999;Nicholls *et al.*, 1992).

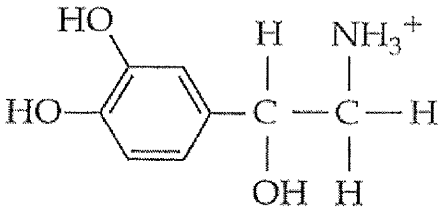


Fig. 1.6: Structure of Noradrenaline (Nicholls *et al.*, 1992)

2.1.2 Adrenoreceptors

2.1.2.1 Subtypes

Adrenoreceptors are the cell membrane sites through which NA and adrenaline, the main endogenous catecholamines, exert their effects. At present, nine different subtypes have been cloned and pharmacologically classified (Guimaraes & Moura, 2002): $3\alpha_1$ (A, B, D), $3\alpha_2$ (A, B, C) and 3β (1, 2, 3). These receptors belong to the family of G-protein coupled receptors characterised by seven transmembrane domains (Pierce *et al.*, 2002). The α_1-adrenoreceptors are mainly coupled to PLC by $G_{q/11}$ proteins (Theroux *et al.*, 1996;Wu *et al.*, 1992), the α_2-adrenoreceptors

predominantly couple to G_i proteins, inhibiting adenylyl cyclase activity (Cotecchia *et al.*, 1990) whilst the β-receptors couple to G_s proteins to stimulate adenylyl cyclase although some have been reported to couple to G_i as well (Chaudhry *et al.*, 1994;Asano *et al.*, 1984).

2.1.2.2 Function in vascular smooth muscle

In most mammalian species, the α_1-adrenoreceptors mediate VSM contraction (Guimaraes & Moura, 2002) but the question as to which α_1 receptor subtype is involved is difficult to answer as VSM tissues express multiple subtypes and contributions are likely to be tissue-specific (Rudner *et al.*, 1999;Piascik *et al.*, 1997). For example, in RMSA, only α_{1B} receptors played a functional role in the response to PE whereas in the aorta, femoral, iliac and superior mesenteric arteries, α_{1D} is implicated (Piascik *et al.*, 1997) with α_{1B} receptors playing a minor role in the mesenteric (Hussain & Marshall, 2000). However, a study with NA in the isolated superior mesentery of the rat concluded that α_{1A} was the major isoform involved (Williams & Clarke, 1995). Additionally, differential activation of subtypes in responses to exogenous and neurally released NA has also been reported (Zacharia *et al.*, 2004).

Although functional studies indicate the presence of α_2-adrenoreceptors on VSM, their contribution to contraction seems to be restricted to small arteries/arterioles and veins (Leech & Faber, 1996). However, the α_2-adrenoreceptors are found at the prejunctional level in every vascular tissue studied where they mediate a negative modulation of NA release (Guimaraes & Moura, 2002). This is thought to be their primary physiological function as supported by work in receptor-null animals (Kable

et al., 2000) although they are also found on the endothelium where they can mediate release of nitric oxide (NO) (Richard *et al.*, 1990).

β-adrenoreceptor-mediated vasodilation is thought to play an important role in regulation of vascular tone. Stimulation of β-receptors relaxes VSM, thus controlling peripheral tone and distribution of blood around the body (Brawley *et al.*, 2000b). The β_2 receptors are the predominant subtype in most VSM although β_1- and β_3-subtypes can also contribute to vasodilation (Vatner *et al.*, 1985). Data from receptor null animals is consistent with β_1-receptors regulating heart rate and contractility, β_2 mediating vasodilation and β_3-receptors controlling lipolysis in adipose tissue although this is an oversimplified picture (Guimaraes & Moura, 2002). β-adrenoreceptors have also been found prejunctionally (the β_2 subtype) where they were observed to have a facilitatory role on NA release (Coppes *et al.*, 1995) and on the endothelium, where they mediated relaxing responses involving NO (Brawley *et al.*, 2000a).

2.1.3 Evidence for NA-stimulated phospholipid hydrolysis

Like other calcium-mobilising hormones, NA was found to stimulate turnover of phosphoinositides (Berridge, 1984) resulting in rapid production of IP_3 and calcium release from intracellular stores (Ollerenshaw *et al.*, 1988). This was a consequence of PI-PLC activation in a G-protein dependent manner (LaBelle & Murray, 1990), later confirmed to be mediated by the G_q subtype (Wu *et al.*, 1992). NA also stimulates hydrolysis of PC and this has been shown in rabbit saphenous artery (Nally *et al.*, 1992), rat aorta (Jones *et al.*, 1993), rat tail artery (Gu *et al.*, 1992) and RMSA (Ward *et al.*, 1995). This was due to PLD activation in rat aorta

and RMSA, whereas in rat-tail artery and rabbit saphenous artery, activation of both PLD and PC-PLC was reported.

2.1.4 NA-stimulated lipid second messenger production

NA stimulation of both PIP_2 and PC turnover suggests the production of the second messengers DAG and PA. Although accumulation of DAG in response to α_1-adrenoreceptor activation was reported in MDCK cells (Slivka *et al.*, 1988), in RMSA total DAG did not increase on NA stimulation (Ohanian *et al.*, 1990) as a result of NA-stimulated DGK activation (Ohanian & Heagerty, 1994;Ohanian *et al.*, 1993). NA-stimulated PA production has been reported in a number of tissues including rabbit ventricular myocytes (Ye *et al.*, 1994), RMSA (Ward *et al.*, 1995;Ohanian *et al.*, 1990) and rat aorta (Jones *et al.*, 1993) occurring by both PLD-mediated hydrolysis of PC and DGK-mediated phosphorylation of DAG.

2.2 The endothelins

The concept of endothelium regulation of vascular function first emerged on the discovery of endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980), later characterised as NO (Palmer *et al.*, 1987). This was followed by report of an endothelium-derived constricting factor (Hickey *et al.*, 1985), subsequently isolated as a peptide and named endothelin (Yanagisawa *et al.*, 1988). There are now three known members of the endothelin family (reviewed in (Masaki, 1998;Mateo & De Artinato, 1997) with the original peptide renamed endothelin-1 (ET-1) on the discovery of related genes encoding endothelin-2 (ET-2) and –3 (ET-3) (Inoue *et al.*, 1989).

2.2.1 Structure, expression and synthesis

The three ET family members are structurally very similar. They are all 21 amino acid peptides, 2.49kD in size with two disulphide bridges. ET-2 and ET-3 differ from ET-1 by two and six amino acids respectively (Inoue *et al.*, 1989). ET-1 (**Fig. 1.7**) is the only form detected in vascular endothelium although it is also in non-vascular tissue such as brain, kidney and lung (Sakurai *et al.*, 1991). Both ET-2 and ET-3 are detected in brain, adrenal gland and intestine whilst ET-3 is abundant in neuronal tissues (Shiba *et al.*, 1992).

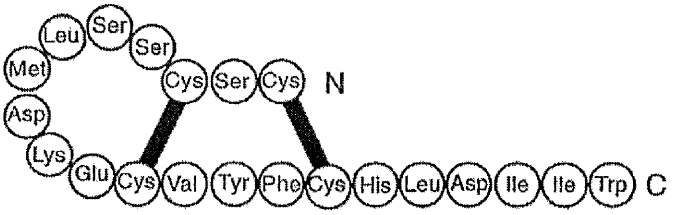


Fig. 1.7: Schematic of Endothelin-1 (Masaki, 1998)

ETs are synthesised from 'Preproendothelin' precursors that undergo cleavage by endopeptidases to produce a 37-41 amino acid precursor named Big ET (or proET). Further breakdown by specific endothelin-converting enzyme (ECE) results in production of mature endothelins. The majority of this occurs in the vascular endothelium where ECE activity is localised to plasma membrane and intracellular compartments. Regulation of ET-1 occurs at the level of biosynthesis. Expression of the gene is induced by a number of factors such as adrenaline, thrombin, vasopression, AngII, cytokines and physical stimuli and decreased by NO, prostacyclin and heparin (see Masaki, 1998;Mateo & De Artinato, 1997).

2.2.2 Endothelin receptors

Currently, two mammalian subtypes of endothelin receptor have been cloned that belong to the G-protein coupled receptor family. Cloned in 1990, the receptors have distinct affinity profiles for the three peptides: ET_A has high affinity for ET-1 (Arai *et al.*, 1990) whereas ET_B has roughly equal affinities for all three ET peptides (Sakurai *et al.*, 1990). The ET_B receptor has been subdivided into B1 and B2 types according to functional distinction and affinity for the compound IRL1038 (Karaki *et al.*, 1994) but this does not apply to the human vasculature (Maguire & Davenport, 1995). Additional receptor subtypes have been described in some tissues: an ET-3 specific ET_C receptor (Karne *et al.*, 1993) and an ET_{AX} receptor with ET_A characteristics but insensitive to the antagonist BQ-123 have been isolated from *Xenopus laevis* (Kumar *et al.*, 1994). However, it was recently concluded that, in the absence of molecular and ligand-binding evidence, there is no justification to classify mammalian receptors beyond ET_A and ET_B (Davenport, 2002).

ET_A and ET_B can couple to G_s, G_i, and G_q proteins depending on the cell type. In CHO cells, ET_A couples to G_s and G_q with ET_B to Gi and Gq (Eguchi *et al.*, 1993) yet in atrial myocytes ET_A couples to G_i and inhibits cAMP production (Ono *et al.*, 1994) and in rat liver ET_B couples to G_s and G_q (Jouneaux *et al.*, 1994). Recently, ET_A and ET_B were reported to have similar affinity for G_q but differences in extent of activation whereas ET_B had greater affinity for G_i but it was activated to a similar degree by both receptors (Doi *et al.*, 1999). This differential coupling was suggested to be due to receptor differences in various physiological conditions.

2.2.3 Effects in vascular smooth muscle

Endothelin receptors are widely distributed; the ET_A receptor is localised to smooth muscle and mediates vasoconstriction (Maguire & Davenport, 1995) whereas the ET_B receptors are on the endothelium and mediate release of EDRFs (Takayunagi *et al.*, 1991). The B2 receptors are distinct in being found on VSM, initially the rabbit jugular and saphena, and also mediate vasoconstriction but this is proposed to occur mainly in the veins and microcirculation (Warner *et al.*, 1993). In humans, ET_A receptors predominate on the VSM with the low density of ET_B receptors contributing little to vasoconstriction in normal or disease states (Maguire & Davenport, 1995).

ET-1 is the most potent vasconstrictor known and produces slow, exceptionally long-lasting vasoconstriction (Yanagisawa *et al.*, 1988). Although it is currently accepted that ET-1 is a vasodilator at physiological concentrations acting through the ET_B receptors as demonstrated in the isolated rat mesentery (Warner *et al.*, 1989), in pathological states such as hypertension, ET-1 levels are proposed to increase such that the vasoconstrictor effect is manifested (Inagami *et al.*, 1995). In addition to constrictor and dilator responses, ET-1 also promotes proliferation of endothelial cells, VSM and fibroblasts that can result in structural changes to the vascular wall (Bobik *et al.*, 1990).

2.2.4 Evidence of ET-1-stimulated phospholipid hydrolysis

Following isolation of the ET peptide, a number of early studies reported that ET-1 stimulates phospholipid turnover. IP_3 production was rapid (<30s), coinciding with PIP_2 breakdown suggesting activation of PI-PLC (Van Renterghem *et al.*,

1988;Resnik *et al.*, 1988) and resulted in calcium release from intracellular stores (Marsden *et al.*, 1989). ET-1 also stimulates PC turnover by both PLC and PLD pathways with activation of PLD by ET-1 reported in VSM cells (Wilkes *et al.*, 1993), rat aorta (Liu *et al.*, 1992) and ventricular myocytes (Fahimi-Vahid *et al.*, 2002) whilst other studies reported ET-1-stimulated activation of both PLD and PC-PLC in mesangial cells (Baldi *et al.*, 1994) and RMSA (Liu *et al.*, 1999a). Thus, ET-1 can stimulate both PC and PI hydrolysis at the plasma membrane.

2.2.5 ET-1-stimulated lipid second messenger production

ET-1 stimulation of phospholipid turnover suggested production of the messengers DAG and PA. As with other stimuli (**section 1.1.1**), ET-1 was reported to stimulate biphasic production of DAG – with an early PI-derived peak (30s) and a later PC-derived peak (5 min) (Sunako *et al.*, 1990;Griendling *et al.*, 1989). Consistent with this, ET-1-stimulated PKC activity was reported and PKC inhibitors prevented tonic responses of rat aortic rings to ET-1 (Danthuluri & Brock, 1990;Griendling *et al.*, 1989). ET-1-stimulated production of PC-derived DAG was also reported to occur via PC-PLC but was suggested to be unnecessary for ET-1-mediated contraction of intact RMSA (Liu *et al.*, 1999a). ET-1-stimulated PA production has been reported in a wide number of tissues including mesangial cells (Baldi *et al.*, 1994), rat aorta (Liu *et al.*, 1992) cardiac myocytes (Ye *et al.*, 1994) and RMSA (Liu *et al.*, 1999a) with results in all these studies suggesting that PLD-mediated PC hydrolysis makes a significant contribution to PA accumulation. However, ET-1 stimulation of DGK-mediated PA production has not been assessed directly in the literature.

2.3 Summary

The activation of phospholipid signalling pathways can lead to the generation of lipid second messengers. The existence of different pathways that produce and metabolise these second messengers results in the generation of distinct lipid species dependent on the phospholipid source and temporal differences in lipid second messenger production. In VSM, vasoconstrictors such as NA and ET-1 can activate these pathways regulating the production of DAG and PA. Therefore, specificity of response to NA and ET-1 could come from the generation of distinct lipid species and pattern of production of these second messengers. However, an alternative mechanism for specificity could be the compartmentalisation of lipid second messenger production within specific membrane domains.

3.0 Membrane microdomains

The plasma membrane bilayer contains a variety of phospholipids, glycerolipids and sphingolipids with proteins that traverse the membrane or interact with either leaflet. The landmark "fluid mosaic" model proposed a mosaic of alternating globular transmembrane proteins and lipid bilayer (Singer & Nicolson, 1972) and was the accepted view for some time. However, more recently, research has caused a shift to the idea that membrane lipids are not randomly distributed but show local heterogeneity with different lipid types associating in distinct domains. Two such domains have attracted considerable interest over the past two decades – lipid rafts and caveolae.

3.1 Lipid rafts

3.1.1 The raft hypothesis

The existence of membrane microdomains was suggested as far back as 1988 to explain the selective delivery of sphingolipids (SL) to the apical domain of polarised cells (Simons & Van Meer, 1988) with subsequent work reporting these domains were insoluble in cold-detergent (Hooper & Turner, 1988) and exploiting this to determine the canonical 'raft mixture' for model membrane studies of 34:36:32 phospholipids: SL: cholesterol (Brown & Rose, 1992). However, it wasn't until 1997 that Simons and Ikonen, in their seminal paper, first put forward the concept that SL-cholesterol rafts are generally used as platforms for membrane-linked functions including membrane traffic, cell morphogenesis and cell signalling through the recruitment of a specific set of membrane proteins and the exclusion of others (Simons & Ikonen, 1997).

3.1.2 Raft formation

The principle underlying raft formation lies in the difference in properties between SL and phospholipids (PL). Both SL and glycosphingolipids have longer, more saturated acyl chains (**Fig. 1.8A**) and a higher melting temperature than PL and so can pack more tightly together (Ramstedt & Slotte, 2002;Brown, 1998). Thus, SL were proposed to exist in a different phase to PL in the membrane and this was well characterised in model PL/SL bilayers (Silvius, 1992). However, the plasma membrane also contains a high level of cholesterol which has important effects (Ohno-Rekila *et al.*, 2002). The addition of cholesterol to pure PL bilayers abolishes the sharp transition between gel and liquid-disordered phases producing a liquid-ordered (L_o) phase with intermediate properties (**Fig. 1.8B**) – tight packing of acyl

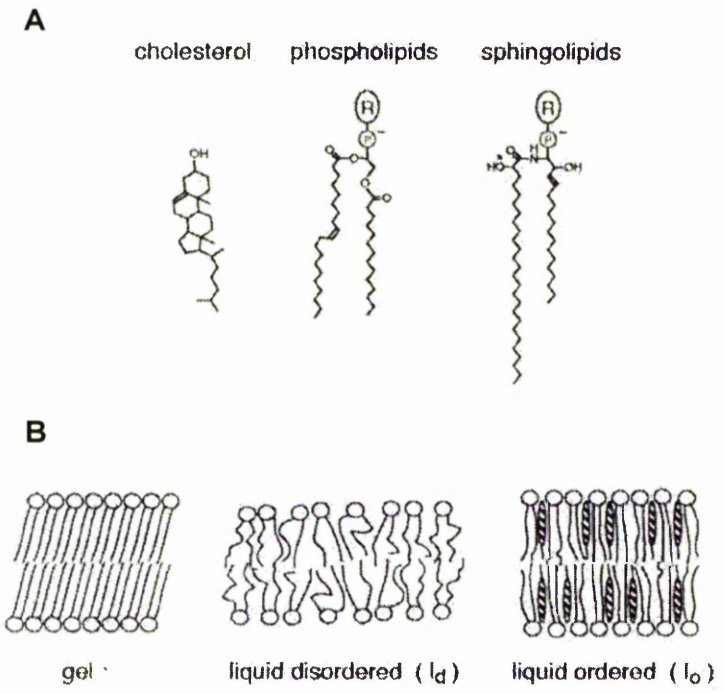


Fig. 1.8: Lipid structure and membrane phases. A) The structure of cholesterol (left) shown with a typical phospholipid (middle) and a typical sphingolipid (right). **B)** A representation of membrane phases: the gel phase (left), the liquid disordered phase (middle) and the intermediate liquid-ordered phase (right). (Munro, 2003)

chains but high lateral mobility (McMullen *et al.*, 1993). Additionally, other studies demonstrated that cholesterol interacts more favourably with SL over membrane PL (Sankaram & Thompson, 1990). In the raft hypothesis, it was proposed that SL associate laterally with each other with cholesterol functioning as a spacer to fill the voids between adjacent molecules - resulting in a L_o-raft floating in a liquid-disordered PL bilayer (Simons & Ikonen, 1997). Supporting this, subsequent *in vitro* studies in model membranes utilising the 'raft mixture' (34:36:32 PL: SL: cholesterol) (Brown & Rose, 1992) reported formation of L_o microdomains and, furthermore, this was replicated in model membranes prepared from lipids of renal brush border membranes (Dietrich *et al.*, 2001a;Dietrich *et al.*, 2001b;Ahmed *et al.*, 1997). However, despite evidence in model membranes, a major reason that the raft

hypothesis has remained controversial is the difficulty in proving definitively that rafts exist in living cells.

3.1.3 Visualisation of rafts in living cells

Considerable research has been directed towards the *in vivo* visualisation of rafts with the most direct way being to analyse distribution of putative raft components, such as glycosylphosphatidylinositol (GPI)-anchored proteins, in the cell. Whilst a number of early studies reported a uniform distribution of GPI-anchored proteins in resting cells (Harder *et al.*, 1998;Mayor *et al.*, 1994), this was subsequently attributed to insufficient resolution and sensitivity of the techniques used (Simons & Toomre, 2000). However, even with more sophisticated techniques such as fluorescence-energy resonance transfer (FRET) and single particle/fluorophore-tracking, there were mixed results. Some studies reported no clustering of lipid or protein reporters at steady state (Kenworthy *et al.*, 2000;Kenworthy & Edidin, 1998) whereas others reported clustering but raft diameters calculated varied widely from 26 ± 13 nm (Subczynski & Kusumi, 2003), 44 ± 8nm (Prior *et al.*, 2003) and <70nm (Varma & Mayor, 1998) at the low end to 200 nm (Dietrich *et al.*, 2001c) and 700 nm (Schutz *et al.*, 2000) at the higher end of the scale. Whilst this may reflect the different techniques used, more recent studies have suggested that rafts are smaller still. Recent analysis of earlier FRET data reported that GPI-anchored proteins were predominantly monomers with a fraction (20-40%) existing in cholesterol-dependent clusters (<5 nm) of no more than four proteins (Sharma *et al.*, 2004). This is consistent with observations of 'condensed complexes' of 15-30 molecules of sphingolipid and cholesterol (Radhakrishnan *et al.*, 2000) and the recently proposed 'lipid shell' hypothesis wherein raft proteins are

encased in a shell of a few cholesterol-sphingolipid molecules conferring their affinity for liquid-ordered phases (Anderson & Jacobson, 2002). Thus, the current view of rafts is as small, dynamic and unstable at rest but where subtle changes can translate rapidly into bigger localised effects.

3.1.4 Summary

The raft hypothesis has received considerable attention since its proposal and rafts have been implicated in a large number of processes yet unequivocal evidence for their existence remains elusive. However, with renewed interest in lipid-lipid and lipid-protein interactions and the ongoing development of more sophisticated techniques, it is hoped that this issue will be resolved in the near future.

3.2 Caveolae

First discovered in the 1950s by electron microscopy, caveolae ("little caves") were defined as "a small pocket, vesicle, cave or recess communicating with the outside of a cell and extending inward" (Yamada, 1955;Palade, 1953) and have become synonymous with flask-shaped membranes of 50-80nm diameter (**Fig. 1.9**). However it is now known that caveolae can exist as flat, vesicular or tubular and be open or closed at the cell surface (Anderson, 1998). The lack of molecular markers for caveolae restricted research within the domain of electron microscopy but this was resolved in the early 1990s by two independent groups.

3.2.1 The caveolins

In 1992, Kurzchalla *et al.* identified a 21-kD membrane protein as a component of Golgi-derived detergent-insoluble complexes. The protein, VIP21, was

localised to Golgi and uncoated pits in tissue and cells (Kurzchalla *et al.*, 1992). Simultaneously, Rothberg *et al.* analysed the caveolar coat using an antibody directed against a 22-kd protein isolated as a substrate of v-Src in transformed cells (Rothberg *et al.*, 1992). The protein was named caveolin and subsequent cDNA sequencing confirmed that VIP21 and caveolin were identical (Glenney Jr, 1992).

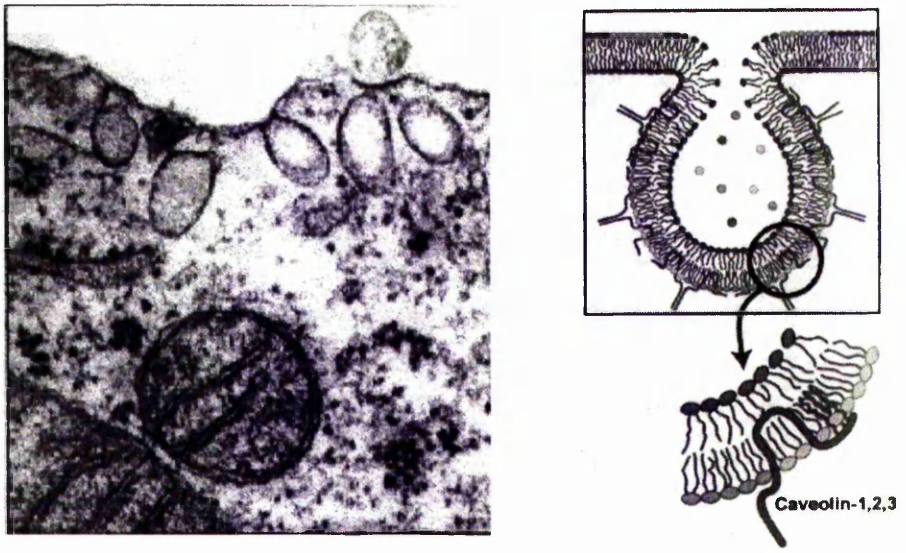


Fig. 1.9: The Caveolae Membrane System. The classical flask shaped caveolae observed at the membrane of a fibroblast (left) and a schematic of caveolae showing the unique topology of the caveolin proteins (right). (From Parton, 2001)

Caveolin was found to encode two forms - α and β (Scherer *et al.*, 1995) and was renamed caveolin-1 on the discovery of two other caveolin genes and proteins. Caveolin-2 has similar distribution to caveolin-1 being most abundantly expressed in adipocytes, endothelial cells and fibroblasts (Scherer *et al.*, 1996) whilst caveolin-3 is muscle-specific and most closely related to caveolin-1 being 65% identical and 85% similar (Tang *et al.*, 1996b;Way & Parton, 1995). The caveolins assume an unusual 'hairpin' membrane topology with both the C- and N-termini facing the cytoplasm (**Fig. 1.9**) anchored to the membrane by a 33 amino acid sequence of hydrophobic residues (Glenney Jr, 1992).

3.2.2 Caveolae formation

The discovery of the caveolins as major structural components of the caveolar coat suggests a key role in caveolar biogenesis. Evidence supporting this theory comes from studies where caveolin-1 expression is sufficient to drive formation of caveolae-like vesicles in lymphocytes and other cells normally devoid of caveolae (Engelman *et al.*, 1997;Li *et al.*, 1996b) and, furthermore, null mutant mice lacking caveolin-1 and caveolin-3 lack morphologically identifiable caveolae. The process of caveolae biogenesis was proposed to involve the self-assembly properties of caveolin as both caveolin-1 and -3 form homo-oligomers and caveolin-1 can form hetero-oligomers with caveolin-2 (Sargiacomo *et al.*, 1995). This occurs in two steps: the first step occurs in the ER, requires the caveolin oligomerisation domain (61-101) and results in formation of discrete oligomers. In step two, occurring in the Golgi, adjacent homo-oligomers interact with each other through contacts between C-terminal domains (Das *et al.*, 1999) resulting in an interlocking network of molecules that gives rise to the striated coat of caveolae (reviewed in (Couet *et al.*, 2001). However, whilst a recent study concluded that a heptameric oligomer of caveolin is the basic building block of the caveolar coat (Fernandez *et al.*, 2002), it was also concluded that nothing in the structure of caveolin suggests it functions directly in shaping the caveolae membranes. Furthermore, evidence against caveolin involvement in caveolae formation comes from studies showing that morphologically similar structures can exist in cells lacking caveolin expression with proteins such as flotillin-1 and dynamin able to induce formation of smooth vesicles (Le *et al.*, 2002;Volonte *et al.*, 1999). In studies expressing caveolin-1 in cells lacking caveolae, the caveolae-like vesicles formed accumulated in the cytoplasm (Li *et al.*, 1996b) and the existence of caveolin-1 associated lipid rafts (Scheiffele *et al.*, 1998) suggest

that caveolin-1 alone may not be sufficient for producing the classical caveolae flask-shape. Finally, more recent studies have concluded that caveolin-1 (and caveolin-3 in muscle tissue) stabilise the caveolae at the membrane (Le *et al.*, 2002) and function to organise caveolae lipids (Fernandez *et al.*, 2002) and the ganglioside GM_1 was recently implicated in membrane curvature and was suggested to help shape caveolae by localising to the neck regions (Pei & Chen, 2003)

Like lipid rafts, caveolae are also enriched in cholesterol and sphingolipids and the evidence supporting a role for cholesterol in caveolae formation is more favourable. Caveolae were found to be sensitive to cholesterol depletion and oxidation, with caveolin-1 retreating to internal membrane compartments until cholesterol is restored (Conrad *et al.*, 1995;Smart *et al.*, 1994). Caveolin-1 and caveolin-3 both bind cholesterol in order for proper transportation to the membrane, a process requiring palmitoylation in the C-terminal, and caveolin-1 incorporates into reconstituted lipid vesicles only when they contain high cholesterol concentrations (Li *et al.*, 1996c;Murata *et al.*, 1995). These studies all suggest a major role for cholesterol in transporting caveolins to the membrane and caveolar biogenesis. Given the sensitivity of caveolar morphology to cholesterol, it is perhaps unsurprising that they have been implicated in cholesterol homeostasis and trafficking (Fielding & Fielding, 2001)

3.2.3 Caveolin knockout animals

Caveolae research recently received a major boost by the development of the first caveolin (-/-) knockout animals. Surprisingly, caveolin-1 (-/-) knockouts were viable despite lacking caveolae in numerous tissues. Further analysis found a loss of

caveolin-2 expression, defects in endocytosis and a hyperproliferative phenotype, particularly in the alveoli of the lung (Razani *et al.*, 2001). Also, aortas of caveolin-1 (-/-) knockout mice exhibited enhanced acetylcholine-induced vasorelaxation and blunted vasoconstriction to PE (Drab *et al.*, 2001;Razani *et al.*, 2001). Subsequent studies showed dramatic increases in microvascular permeability (Schubert *et al.*, 2002) and, more recently, caveolin-1 (-/-) knockout mice were reported to have a dramatic reduction in lifespan secondary to pulmonary fibrosis, hypertension and cardiac hypertrophy (Park *et al.*, 2003). Similarly, caveolin-3 and caveolin-2 (-/-) knockout mice were viable but the former developed mild myopathic changes in skeletal muscle similar to patients with a form of limb-girdle muscular dystrophy (Galbiati *et al.*, 2001) whereas in caveolin-2 (-/-) knockouts, the lungs displayed a similar phenotype to caveolin-1 (-/-) knockouts (Razani *et al.*, 2002). As caveolin-2 expression was reduced in caveolin-1 (-/-) knockouts, this suggests that caveolin-2 is functionally more important in this instance. Finally, caveolin-1/caveolin-3 (-/-) double knockout mice were also viable and fertile but completely lacked caveolae in all tissues examined. Lack of caveolin expression resulted in the development of a severe cardiomyopathic phenotype within 2 months (Park *et al.*, 2002).

The development of caveolin (-/-) knockout mice has provided a major boost in caveolae research. As the loss of caveolin expression causes a concomitant loss in morphologically identifiable caveolae, such animals allow the exploration of the physiological roles of both caveolae and the caveolins *in vivo*.

3.3 Rafts and caveolae: are they related?

The similarity in lipid composition and operational definitions of caveolae and lipid rafts led to the theory that the two are related yet matters were confused by uncertainty in the literature where the distinction between lipid rafts, flattened caveolae or caveolae proper was unclear (Simons & Toomre, 2000). Whilst an early review proposed rafts were 'pre-caveolae' (Okamoto *et al.*, 1998) supported by the ability of the caveolins to drive caveolae formation from raft components (Engelman *et al.*, 1997), the reported existence of lipid rafts containing caveolin-1 suggested otherwise (Scheiffele *et al.*, 1998). A recent review aimed to clear this up and it is currently accepted that caveolae are a highly specialised subset of rafts requiring the caveolar morphology for a distinct set of cellular functions (Simons & Ehehalt, 2002).

3.4 Rafts, caveolae and signal transduction

Research over the past decade has yielded a number of proposed caveolae/raft-associated proteins. This list includes many signalling proteins such as H-Ras, Src tyrosine kinases, Rho, PKC, endothelial nitric oxide synthase (eNOS), G-protein coupled receptors, growth factor receptors and G-protein α subunits amongst others (see Frank *et al.*, 2003). A recent proteomic analysis reported a large proportion of signalling molecules were enriched in caveolae/rafts (Foster *et al.*, 2003) although few proteins are localised exclusively, the exception being several putative calcium regulatory proteins (Fujimoto *et al.*, 1992). The presence of signalling proteins in caveolae/rafts led to their proposed involvement in signal transduction.

3.4.1 Rafts and signal transduction

The original raft hypothesis proposed that lipid rafts could be functional units involved in signal transduction based largely on enrichment of signalling molecules within them (Simons & Ikonen, 1997). In principle, rafts could modulate signalling in a number of ways: 1) The localisation of specific signalling components to rafts would enable efficient signalling, 2) the translocation of molecules in and out of rafts could control signalling specificity and control the cell's ability to react to various stimuli, 3) the concentration of proteins within rafts could facilitate cross talk between pathways and 4) conversely, could also prevent unnecessary interaction of other pathways (Simons & Toomre, 2000). The role of rafts in signal transduction has primarily been investigated in cultured cells and studies have implicated these domains in immunoglobulin E signalling in mast cells and basophils (Simons & Toomre, 2000), Ras signalling (Parton & Hancock, 2004) and glial-cell-derived neurotrophic factor signalling (Simons & Toomre, 2000). However, whilst considerable work has also suggested a role for rafts in T-cell receptor signalling (Janes *et al.*, 2000;Kabouridis *et al.*, 2000), some more recent studies have challenged this model and the role of rafts in this process remains a matter of debate (Glebov & Nichols, 2004;Pizzo *et al.*, 2002).

3.4.2 Caveolae and signal transduction

In 1994, the caveolae signalling hypothesis was proposed stating 'compartmentalization of certain cytoplasmic signalling molecules within caveolae could allow efficient and rapid coupling of activated receptors to more than one effector system', suggested by the isolation of signal transduction molecules and GPI-anchored proteins in a caveolin-rich complex (Lisanti *et al.*, 1994a). It was

subsequently found that a region of caveolin-1 termed the caveolin scaffolding domain (CSD) (residues 82-101) interacts with Ras and G_α subunits (Song *et al.*, 1996;Li *et al.*, 1995). The CSD was used to screen phage display libraries and resulted in the deduction of two binding motifs – XAXAAAAX and XAAAAXAAX (X=aromatic residue, A = any residue) that are present in Gα subunits and highly specific for interaction with caveolin-1 (Couet *et al.*, 1997). This took on greater significance when it was reported that binding of the CSD resulted in suppression of protein activation for eNOS (Garcia-Cardena *et al.*, 1997), Gα subunits (Li *et al.*, 1995), Ras and Src tyrosine kinases (Li *et al.*, 1996a) suggesting that caveolin-1 (and caveolin-3 in muscle) act as scaffolding proteins with caveolae functioning as 'a platform on which different signalling components can congregate' (Parton, 2001). However, some studies that failed to find enrichment of signalling proteins in caveolae raised doubt as to their involvement; subsequently, functions were assigned to the biochemically similar lipid rafts (Simons & Toomre, 2000). Whilst difficulties in purifying caveolae away from rafts have hampered analysis of specific functions associated with the caveolar morphology, more recent studies have attempted this with some success. Caveolae and the caveolins have been implicated in NO generation by eNOS with the process found to require both caveolins and the caveolar morphology (Sowa *et al.*, 2001) and this suggests that specific caveolar functions do exist.

3.4.3 Rafts, caveolae and lipid signalling

Although considerable work has suggested caveolae/rafts are sites of protein signalling, more recently caveolae/rafts are emerging as sites of lipid signalling. Caveolae/rafts were found enriched in PIP_2 (Pike & Casey, 1996) and are major sites

of PI turnover in bradykinin-stimulated A431 cells (Pike & Miller, 1998) and activated T-cells (Parmryd *et al.*, 2003) although subsequent studies concluded the majority of PIP_2 was in non-caveolar rafts and the PIP_2 producing PI-4-phosphate kinase was localised there also (Watt *et al.*, 2002;Waugh *et al.*, 1998). Additionally, other studies have reported the production of the lipid second messengers PA and DAG in rafts of thrombin-stimulated platelets (Bodin *et al.*, 2001) and stimulated Jurkat T-cells respectively (Rouquette-Jazdanian *et al.*, 2002). Finally, the enzyme phospoinositide 3-kinase (PI3K) and its products such as phosphatidylinositol 3,4,5-trisphosphate (PIP_3) have been localised to caveolae/rafts of thrombin-stimulated platelets and Jurkat T-cells (Parmryd *et al.*, 2003;Bodin *et al.*, 2001). As PIP_3 is also thought to be a lipid second messenger with distinct cellular targets (reviewed in Cantrell, 2003), this further suggests that caveolae/rafts are also important for lipid signalling pathways.

3.5 Rafts, caveolae and vascular contractility

Caveolae are abundant in both endothelial cells and smooth muscle (Parton, 2003) and in vascular tissue have been implicated in the regulation of vascular contractility. There is strong evidence of a role for caveolae in vasodilation through caveolin-1-mediated regulation of eNOS and NO production (Garcia-Cardena *et al.*, 1997). Furthermore, a CSD peptide inhibited the acetylcholine-induced relaxation of mice aortic rings (Bucci *et al.*, 2000) and caveolin-1 (-/-) knockout mice have increased basal NO levels and exhibit enhanced NO-dependent vasodilation (Razani *et al.*, 2001). Additionally, the exposure of vascular endothelial cells to shear stress or increased flow activates localised eNOS signalling and NO production in caveolae (Rizzo *et al.*, 2003). Finally, caveolae are reported to be necessary for the generation

of calcium sparks – localised calcium release events caused by ryanodine receptors in the SR (Lohn *et al.*, 2000). In arterial VSM, calcium sparks indirectly lead to vasodilation through the activation of plasma membrane potassium channels and regulation of membrane potential (Bergdahl & Sward, 2004;Lohn *et al.*, 2000). In addition to a role in vasodilation, other studies have suggested a role for caveolae in agonist-induced vasoconstriction.

An increase in cytoplasmic calcium concentration is the primary modulator of smooth muscle contraction with research implicating caveolae as important sites of calcium homeostasis and signalling. Early studies have reported caveolae as sites of calcium entry in smooth muscle (for review see Isshiki & Anderson, 2003) and calcium regulatory proteins have been localised to caveolae (Fujimoto *et al.*, 1992). Furthermore, caveolin-1 interacts and colocalises with TRPC1, TRPC3 and TRCP4, members of the transient receptor potential family of channels reported to be involved in capacitative calcium entry (CCE), a process of extracellular calcium influx in response to depleted intracellular calcium stores (Bergdahl & Sward, 2004;Bergdahl *et al.*, 2003). Consistent with this, disruption of caveolae/rafts in rat caudal artery reduced ET-1-stimulated contraction through delocalisation of TRPC1 (Bergdahl *et al.*, 2003). As extracellular calcium influx is reported to be the major source of increased cytoplasmic calcium in agonist-stimulated smooth muscle (Nilsson *et al.*, 1994), this further supports a role for caveolae in vasoconstriction.

In addition to a role in calcium signalling, research also suggests caveolae may play a role in calcium sensitisation – the process by which agonists increase contractile force independently of a change in intracellular calcium concentration. This is

reported to involve RhoA, its downstream effector Rho kinase and also PKC (Somylo & Somylo, 2003;Ganitkevich *et al.*, 2002). RhoA was localised to caveolae of endothelial cells (Gingras *et al.*, 1998), PKC-α, -ε and -ξ isoforms are reported to interact with caveolae in COS cells (Oka *et al.*, 1997) and a translocation of Rho kinase to caveolae has been reported in rabbit arteries (Urban *et al.*, 2003). Furthermore, carbachol-stimulated redistribution of PKC-α and RhoA was prevented by a CSD peptide (Taggart *et al.*, 2000). Additionally, carbachol-induced contraction and PKC-dependent increases in contractility in response to α_1-adrenergic agonists were inhibited by a similar CSD peptide (Je *et al.*, 2004; Lee *et al.*, 2001).

Finally, disruption of caveolae by cholesterol depletion was found to differentially impair agonist-induced contraction – affecting responses to ET-1 and vasopressin but not α_1-adrenergic agonists (Dreja *et al.*, 2002) whilst caveolin-1 (-/-) knockout mice show evidence of reduced VSM contractile responses to AngII, ET-1 and PE (Razani & Lisanti, 2001). These studies all suggest a role for caveolae/rafts in regulation of agonist-induced contraction.

4.0 Purpose of study

The spatial regulation of signalling pathways is now understood to be a major component of signal transduction. Caveolae/rafts, specialised membrane microdomains, are thought to function as 'platforms on which different signalling components can congregate' and, more recently, have been implicated in regulation of vascular tone but previous work to date has focused on protein-signalling pathways. However, a major cellular response to vasoconstrictors is the turnover of membrane phospholipids and production of lipid second messengers but the

localisation of this is unknown. Presented in this thesis is an investigation of the role of caveolae/rafts in phosphoinositide signalling during agonist-induced small artery contraction. The use of intact arteries where VSM cells are in contact with non-proliferative endothelial cells provides a more physiologically accurate system as opposed to dispersed, proliferative VSM cells. Furthermore, the effects of both NA and ET-1 were investigated to study any spatial differences to agonists acting through the same transmembrane signalling system.

CHAPTER 2

Materials and Methods

1.0 Introduction

The methods described in this chapter were employed in the investigation of NA and ET-1-stimulated phosphoinositide signalling in the caveolae/lipid rafts of rat mesenteric small arteries (RMSA).

2.0 Materials and methods

2.1 Animals and incubation conditions

Adult female Sprague Dawley rats (approx. 200-300g weight, age range 6-14 weeks) were killed by stunning and cervical dislocation. All procedures were carried out in accordance with institutional guidelines and the U.K. Animals (Scientific Procedures) Act 1986. The mesentery was excised and placed in ice-cold physiological salt solution (PSS, **Table 2.1**). Mesenteric small arteries (internal diameter <300μm) (**Fig. 2.1**) were cleaned of adherent fat and connective tissue, and dissected from the mesenteric bed. Unless stated otherwise, dissected arteries were equilibrated in 1ml of culture media (GIBCO Medium 199 – 25mM HEPES with Hank's salts, L-Glutamine, L-amino acids) at 37°C for one hour prior to stimulation with NA (15μM final concentration), ET-1 (100nM final concentration) or dH_2O as vehicle.

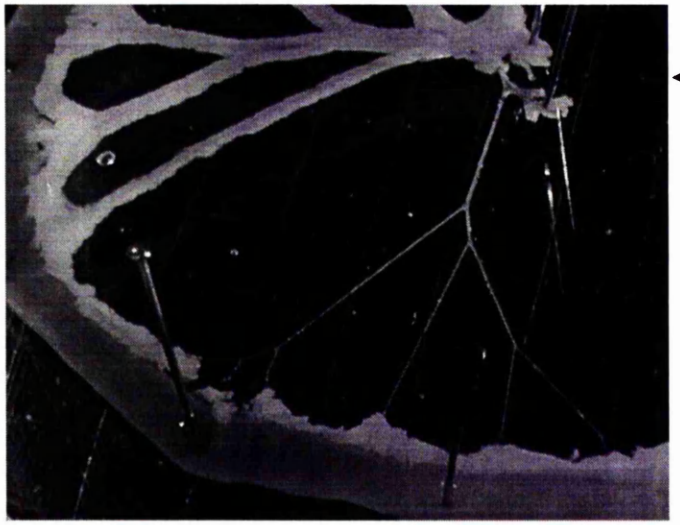


Fig. 2.1:Rat mesenteric small arteries. Small arteries shown are cleaned of adjoining fat and connective tissue but remain connected to the mesentery. Arteries were dissected below the superior mesenteric artery for use.

2.2 Caveolae/lipid raft preparation

2.2.1 Detergent-free extraction

Caveolae/raft-enriched microdomains were purified from RMSA according to the detergent-free method of Song *et al.* with the following modifications (Song *et al.*, 1996). Arteries were homogenised on ice in 400µl of 0.5M sodium carbonate (Na_2CO_3) pH 11. Homogenate was transferred to a 1.5ml microfuge tube, the homogeniser rinsed with 100µl of Na_2CO_3 (0.5M, pH 11) and the rinse pooled with the homogenate. This was further processed on ice with an Omni homogeniser (3 x 10s 20,000rpm bursts at 10 s intervals) and sonication (3 x 20s bursts at 20s intervals) with 3µl aliquots subsequently removed for protein determination by Bradford Assay (**section 2.10.1**).

2.2.2 Detergent extraction

For some experiments, caveolae/raft-enriched microdomains were isolated in cold detergent according to the method of Sargiacomo *et al.* (Sargiacomo *et al.*, 1993). The procedure followed was the same as that for detergent-free isolation with 0.5M Na_2CO_3 pH 11 replaced with 1% Triton X-100 in MES-buffered saline (MBS; 25mM MES pH 6.5, 0.15M NaCl) pH 6.5 containing protease inhibitors.

2.2.3 Isolation of caveolae/raft microdomains

Microdomains were isolated using a discontinuous sucrose gradient. Homogenate volume was adjusted to 450µl with appropriate homogenisation buffer, mixed with 450µl 80% sucrose in MBS and placed at the bottom of a polyallomer centrifuge tube (11mm x 34mm). 700µl of 35% sucrose in MBS+ 0.25M Na_2CO_3 (MBS + 1% Triton X-100 and protease inhibitors for detergent extraction) was layered on top followed by 625µl of 5% sucrose in MBS+ 0.25M Na_2CO_3 (MBS + 1% Triton X-100 and protease inhibitors for detergent extraction). The gradient was centrifuged at 55,000 rpm (160,000 g) for sixteen hours at 4°C in a TLS-55 rotor using a Beckman TL100 centrifuge. Following centrifugation, 12 fractions of 175µl were removed from the top of the gradient with the non-caveloar/raft pellet resuspended in the remaining gradient as fraction 13.

2.2.4 Electron microscopy of purified caveolae/rafts

Membrane fractions were prepared as described (**section 2.2**) and pooled as fractions 2-5 (caveolae/raft), fractions 6–9 (non-caveolae/raft 1) and fractions 10-13 (non-caveolae/raft 2). Membranes were concentrated by diluting 3-fold in MBS followed by centrifugation at 100,000 xg, 4°C for 2 hours. Supernatant was discarded

and membranes were fixed in gluteraldehyde. Following a wash with sodium cacodylate, samples were immersed in osmium tetroxide and dehydrated in ascending grades of alcohol. After two washes with propylene oxide, samples were embedded in araldite CY212 resin and this was left to polymerise overnight. Samples were cut on an ultramicrotome, stained with uranyl acetate and lead citrate and pictures obtained with a Phillips CM 10 electron microscope.

2.3 Protein analysis

2.3.1 Analysis of total protein

Equilibrated arteries were homogenised by ground glass on ice in 80µl 0.5M Na_2CO_3 pH 11. Homogenisers were rinsed with 50µl 0.5M Na_2CO_3 pH 11 and pooled with the homogenate. 3µl aliquots were removed for protein estimation by the Bradford Assay (**section 2.10.1**) and appropriate amounts of 5-fold concentrated Laemmlli sample buffer were added (**Table 2.1**) (Laemmli, 1970). Samples were then heated at 90°C for 5 min and stored at –20°C.

2.3.2 Analysis of caveolae/raft proteins

Proteins from membrane fractions were precipitated on ice for 30 minutes in 5% trichloroacetic acid (TCA - 175µl of 10%TCA added to each fraction), pelleted by centrifugation at 12000g for 5 minutes in a MicroCentaur centrifuge and resuspended in 2% sodium-dodecyl-sulphate (SDS, 12µl), 1M Tris pH 8.8, (4µl) and 5-fold concentrated Laemelli sample buffer (4µl). Samples were vortex mixed for 10 min, heated to 90°C for 5 min and stored at –20°C.

2.3.3 SDS-PAGE

Protein samples were analysed by discontinuous SDS-PAGE. Stacking gel was layered onto separating gel (**Table 2.1**). Gels were run at approximately 100-110V, 25-40A in running buffer (25mM Tris, 200nM glycine, 3.5mM SDS). Molecular weight markers (Sigma SDS-6H 30-200kDa) were included on all gels.

Solution	Composition
Physiological Salt Solution (PSS)	119mM NaCl, 4.7mM KCl, 1.17mM $MgSO_4.7H_2O$, 1.18mM KH_2PO_4, 0.026mM K_2EDTA, 5.5mM glucose, 2.5mM $CaCl_2.2H_2O$, pH 7.2.
x5 Laemelli Sample Buffer	320mM Tris pH 6.8, 25% glycerol, 5% β-mercaptoethanol and a trace of bromophenol blue.
Separating Gel (9%)	3.75ml 1M Tris pH 8.8, 3ml 30% acrylamide, 50µl 20% SDS, 12.5µl TEMED, 300µl 10% APS, 2.89ml dH_2O.
Stacking Gel	625µl 1M Tris pH 6.8, 700µl 30% acrylamide, 25µl 20% SDS, 12.5µl TEMED, 3.49ml dH_2O.
$[PO_4]_o$ Buffer	129mM NaCl, 5.8mM KCl, 0.8mM $MgSO_4.7H_2O$, 4.2 mM $NaHCO_3$, 5.6 mM Glucose, 1.26mM $CaCl_2.2H_2O$, 0.002mM $Fe(NO_3)_3.9H_2O$, 25mM HEPES (For experiments with D609, HEPES was substituted with 20mM MOPS).
Calcium-Free HEPES Buffer	129mM NaCl, 5.8mM KCl, 0.8mM $MgSO_4.7H_2O$, 4.2 mM $NaHCO_3$, 5.6 mM Glucose, 0.44mM KH_2PO_4, 0.34mM $Na_2HPO_4.2H_2O$, 0.002mM $Fe(NO_3)_3.9H_2O$, 25mM HEPES, 1mM EGTA.

Table 2.1: Composition of solutions used.

2.3.4 Western blotting

Following SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose membrane according to the method of Towbin *et al.* (Towbin *et al.*, 1979). Transfer was carried out at 40V in blot buffer (25mM Tris, 200mM glycine,

10% methanol). Membranes were stained in 5% Ponceau S (w/v) in 1% acetic acid (v/v), molecular weight markers marked with ink and membranes destained in 0.1% tween 20 in tris buffered saline (15mM Tris pH 8, 150mM NaCl) (TTBS).

2.3.5 Probing of western blots

Membranes were incubated for at least 30 min in 5% teleostean gelatin in TTBS to block non-specific binding sites. After blocking, membranes were incubated in 0.1% gelatin in 0.1% TTBS supplemented with primary antibody (**Table 2.2**). Following primary antibody, membranes were washed (3 x 15 minutes, TTBS) and incubated in 0.1% gelatin in TTBS supplemented with the appropriate secondary antibody (**Table 2.2**) conjugated to horseradish-peroxidase (HRP). Membranes were washed (3 x 15 minutes, TTBS) and incubated in enhanced chemiluminescence (ECL) (Pierce SuperSignal West Pico) for 5-6 minutes. Proteins were visualised by exposure to X-ray film (Kodak X-Omat AR) for times varying between 5 seconds to 30min. Blots were quantified using a Model GS-690 Laser densitometer and band intensities analysed using the densitometer software.

2.3.6 Stripping of membranes

When stripping of membranes was required, proteins were crosslinked to the membrane by incubation in 0.1% glutaraldehyde for 10 min and washed in dH_2O for 5 minutes prior to staining of membranes with Ponceau S. Membranes were stripped by incubation in 0.7% β-mercaptoethanol in strip buffer (62.5mM Tris-HCl pH 6.8, 2% SDS) at 80°C for 45 minutes and washed in large volumes of dH_2O followed by TTBS for 30 min. Membranes were then re-probed with primary antibody and the normal procedure followed as detailed above.

Antibody	Type	1^{o} Conc.	1^{o} Time	2^{o}	2^{o} Time	2^{o} Conc.
Caveolin-1	Mono	1:10000	O/N 4°C	Mouse	30 min	1:10000
c-Src	Poly	1:200	O/N 4°C	Rabbit	30 min	1:15000
Caveolin-3	Mono	1:5000	O/N 4°C	Mouse	60 min	1:5000
PLC-δ_1	Mono	1:200	O/N 4°C	Mouse	30 min	1:5000
PLC-γ_1	Poly	1:1000	O/N 4°C	Rabbit	30 mins	1:15000
Paxillin	Mono	1:10000	O/N 4°C	Mouse	60 min	1:5000
Hic-5	Mono	1:3000	O/N 4°C	Mouse	30 min	1:5000
Spectrin	Poly	1:2500	O/N 4°C	Rabbit	60 min	1:15000
α-Actinin	Poly	1:1000	O/N 4°C	Rabbit	30 min	1:10000
DGKθ	Poly	1:1000	O/N 4°C	Rabbit	30 min	1:15000
PKB/Akt	Poly	1:5000	1hr. 37°C	Sheep	30 min	1:5000
Raf-1	Poly	1:1000	O/N 4°C	Rabbit	30 min	1:10000
MYPT1	Poly	1:500	O/N 4°C	Goat	30 min	1:10000
PP1c	Poly	1:1000	O/N 4°C	Rabbit	30 min	1:10000

Table 2.2: Primary antibodies and corresponding secondary antibodies.
Abbreviations: Mono = monoclonal, Poly = polyclonal, 1^{o} = primary, 2^{o} = secondary, O/N = overnight.

2.3.7 Calculation of band molecular weights

To calculate the molecular weight (MW) of bands on western blots, a standard curve of log MW versus distance migrated was constructed in Prism data software with measurements taken from the standard molecular weight markers run on each gel.

2.3.8 Ganglioside detection

The ganglioside GM_1 was detected by the dot-blot method of Tkachenko and Simons using HRP-conjugated β-cholera toxin (Tkachenko & Simons, 2002). 4μl of each gradient fraction was spotted on nitrocellulose membrane, air-dried and blocked for 90 min in 2% non-fat milk in TTBS. The membrane was incubated for two hours in HRP-conjugated β-cholera toxin (1:2000) and washed in TTBS (4 x 10 min) before incubation in ECL for 5-6 minutes. GM_1 was visualised by exposure of X-Ray film (Kodak X-OMAT AR) over a range of 2-60 seconds. Films were quantified using a Model GS-690 Laser densitometer and band intensities analysed using the densitometer software.

2.4 Lipid Analysis

2.4.1 Cholesterol analysis

2.4.1.1 Extraction of total cholesterol

Lipids were extracted according to the method of Bligh and Dyer (Bligh & Dyer, 1959). Following equilibration, arteries were removed into 0.5ml ice-cold chloroform:methanol (2:1, v/v) and homogenised. Homogenate was left on ice for ten minutes and transferred to a 1.5ml microfuge tube before the addition of 0.5ml chloroform and 0.5ml distilled water. The mixture was agitated and centrifuged for 5 minutes at 12000xg. The upper aqueous phase was removed and discarded, the lower organic phase transferred to a glass vial and dried under a stream of oxygen-free nitrogen (N_2) gas. Dried samples were reconstituted in 15μl chloroform and the residual protein pellet was air dried prior to determination of protein content by Lowry assay (**section 2.10.2**).

2.4.1.2 Extraction of fraction cholesterol

Fraction lipids were extracted according to the method of Bligh and Dyer (Bligh & Dyer, 1959). To each fraction removed from the sucrose density gradient, 1ml chloroform-methanol (1:1 v/v) was added and fractions left on ice for 10 min. 200µl dH_2O was added, samples were agitated and spun at 12000xg for 5 min. Aqueous layers were discarded, organic layers transferred to glass vials and dried under a stream of oxygen-free N_2. Dried samples were reconstituted in 15µl chloroform-methanol (1:1 v/v).

2.4.1.3 Isolation of Cholesterol by thin-layer chromatography

Reconstituted samples were spotted on a heat-activated Silica Gel 60 thin-layer chromatography (TLC) plate (Merck 5721). To separate out cholesterol from other lipids, the plate was developed as described by Macala *et al.* in a solvent mix of chloroform: methanol: acetic acid: formic acid: dH_2O (70:30:12:4:2 v/v) to 7 cm from baseline followed by hexane: di-isopropylether: acetic acid (130:70:4 v/v) (Macala *et al.*, 1983).

2.4.1.4 TLC Staining and cholesterol determination

To visualise cholesterol, plates were immersed in TLC stain (8% cupric acetate in 3% phosphoric acid w/v), air-dried for 5-10 minutes and charred at 120°C for 30-40 minutes. Lipids were identified using co-chromatographed lipid standards and intensity of spots analysed by densitometry. To determine absolute cholesterol levels, varying concentrations of cholesterol standard were co-chromatographed. Following denstiometric analysis, a cholesterol standard curve was constructed using Prism computer software and cholesterol content of samples determined.

2.4.2 Phosphatidic acid and phosphoinositide analysis

To measure PA and PIs in intact small arteries, the incorporation of [^{33}P]-phosphate (PO_4) into phospholipids was determined according to the method of Mallows and Bolton (1987) modified by Ohanian *et al.* (Ohanian *et al.*, 1990;Mallows & Bolton, 1987).

2.4.2.1 Radiolabelling of arteries

Tissues were depleted of PO_4 by incubating in PO_4-free buffer ($[PO_4]_0$) (**Table 2.1**) for 1 hour at 37°C. Tissue was then transferred to 100µl ^{33}P-$[PO_4]_0$ buffer (6.8µl $^{33}P_i$ in 1ml $[PO_4]_0$ buffer, 2.5 MBq, pre-warmed at 37°C) for 1 hour at 37°C before the addition of 768µl of $[PO_4]_0$ buffer to reduce PO_4 concentration to 0.78mM – that of the normal culture medium (M199) used - and samples were left to equilibrate for a further 10 min prior to stimulation with NA (15µM), ET-1 (100nM) or dH_2O as required.

2.4.2.2 Extraction of total phospholipid

Lipids were extracted according to the method of Bligh and Dyer (Bligh & Dyer, 1959). Arteries were removed into 0.5ml ice-cold chloroform/methanol/HCl (20:40:1, v/v) and homogenised. Homogenate was left on ice for ten minutes and transferred to 1.5ml microfuge tubes before the addition of 0.5ml chloroform and 0.5ml distilled water. The mixture was agitated and spun for 5 minutes at 12000xg. The upper aqueous phase was removed and discarded, the lower organic phase transferred to a glass vial and dried under a stream of oxygen-free N_2. Dried lipid samples were reconstituted in 15µl chloroform. The residual protein pellet was air dried prior to determination of protein content by Lowry assay (**section 2.10.2**).

2.4.2.3 Extraction of phospholipids from membrane fractions.

Fraction lipids were extracted according to the method of Bligh and Dyer (Bligh & Dyer, 1959). To each fraction removed from the sucrose density gradient, 1ml chloroform-methanol (1:1 v/v) was added and fractions left on ice for 10 minutes. 200µl dH_2O was added, samples were agitated and spun at 12000xg for 5 minutes. Aqueous layers were discarded, the organic layers transferred to glass vials and dried under a stream of oxygen-free N_2. Dried samples were reconstituted in 15µl chloroform-methanol (1:1 v/v). To ensure clear results for analysis of PIP and PIP_2, organic layers were pooled into fractions 2-5 (caveolae/raft), fractions 6-9 (non-caveolae/raft 1) and fractions 10-13 (non-caveolae/raft 2).

2.4.2.4 Separation of [^{33}P]-PA

Reconstituted samples were spotted onto a heat-activated oxalate-coated (**section 2.4.2.6**) Silica Gel 60 TLC plate. The plate was developed in a solvent mix of ethyl acetate: acetic acid: 2,2,4 trimethyl pentane (9: 2: 5 v/v) separating the ^{33}P-PA from other radiolabelled phospholipids as previously described in RMSA (Ohanian *et al.*, 1990). Following air-drying, radioactivity incorporated into PA was counted directly by autoradiography (Packard Electronic Autoradiographer) and PA was identified by use of a co-chromatographed standard following visualisation with I_2 vapour.

2.4.2.5 Separation of [^{33}P]-PIP and [^{33}P]-PIP_2

Reconstituted samples were spotted on a heat-activated oxalate-coated (**section 2.4.2.6**) Silica Gel 60 TLC plate. The plate was developed according to the method of Gonzalez-Sastre and Folch-Pi (1968) in a solvent mix of chlorofom:

methanol: dH_2O: ammonium hydroxide (17:13.2:2.8:1 v/v) (Gonzalez-Sastre & Folch-Pi, 1968). Following air-drying, radioactivity incorporated into the phospholipids was counted directly by autoradiography (Packard Electronic Autoradiographer).

2.4.2.6 Oxalate-coating of TLC plates

2% potassium oxalate (w/v) in dH_2O was mixed with an equal volume of methanol (HiperSolv grade). Silica gel 60 TLC plates (Merck 5721) were immersed in the solution for a few seconds and air-dried for at least one hour before use.

2.5 Conditions for use of inhibitors

2.5.1 Inhibition of PI3K

To inhibit PI3K activity, the inhibitor LY294002 was used at 10 μM, a concentration sufficient to block NA-stimulated phosphorylation of protein kinase B (PKB/Akt) in RMSA (Walker *et al.*, 2001). Stock solution was 100mM in DMSO stored at –20°C. To minimise final DMSO concentration, 5μl of 100mM stock was added to 495μl $[PO_4]_0$ buffer or M199. For a 10μM final concentration, this stock was further diluted by 1 in 100 (0.01% DMSO final concentration). Arteries were incubated for 60 min in 10μM LY294002 prior to addition of vasoconstrictor or vehicle.

2.5.2 Butan-1-ol

To determine involvement of PLD, radiolabelled arteries were incubated in 2% pre-warmed butan-1-ol for 10 min prior to addition of vasoconstrictor or vehicle as previously described in RMSA (Liu *et al.*, 1999a;Ward *et al.*, 1995)

2.5.3 Inhibition of PC-PLC

D609 has been used for many years as a specific inhibitor of PC-specific phospholipase C (Muller-Decker, 1989) and at a concentration of 2.5μg/ml was sufficient to inhibit ET-1 stimulated PC-derived DAG production in RMSA (Liu *et al.*, 1999a). Stock solution was 2.5mg/ml in dH_2O and diluted 1 in 1000 prior to use. When used in HEPES buffers, D609 is toxic (Calbiochem data sheet). Accordingly, MOPS was substituted for HEPES in all buffers for these experiments. Prior to addition of vasoconstrictor or vehicle, arteries were incubated for at least 60 min in 2.5μg/ml D609.

2.6 Conditions for use of receptor antagonists and agonists

2.6.1 Prazosin

Prazosin is a selective antagonist for the α_1-adrenoreceptors. In the isolated perfused mesentery of the rat, 30nM prazosin was sufficient to inhibit 1μM NA-stimulated vasoconstriction (Williams & Clarke, 1995). To ensure sufficient antagonist was present, prazosin was used at a final concentration of 10μM. Stock solution was 10mM in DMSO and diluted 1 in 1000 for use. Arteries were incubated for 1 hour in 10μM prazosin prior to stimulation with NA (15μM).

2.6.2 Phenylephrine

Phenylephrine (PE) is a pharmacological agonist specific for α_1-adrenoreceptors. In cultured rat ventricular myocytes, 50μM PE stimulates MAPK activity to 90% of the levels stimulated by 50μM NA and is antagonised by 5μM prazosin (Bogoyevitch *et al.*, 1996). PE stock was 1.5mM in dH_2O and diluted 1 in 100 for a final concentration of 15 μM.

2.6.3 BQ-123

BQ-123 (cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu)) is a selective antagonist for the ET_A class of ET receptors. 10μM BQ-123 inhibited ET-1-stimulated IP_3 formation in cultured rat VSM cells (Eguchi *et al.*, 1992) and cardiomyocytes (Clerk *et al.*, 1994). Stock solution was 1mM in dH_2O and was diluted 1 in 100 for use. Arteries were incubated for 60 min in 10μM BQ-123 prior to stimulation with ET-1 (100nM).

2.7 Conditions for removal of extracellular calcium

To determine the effect of calcium removal on signal transduction pathways, RMSA were incubated in a calcium-free HEPES buffer containing 1mM EGTA (**Table 2.1**) for 10 min prior to stimulation as described previously in RMSA (Ohanian *et al.*, 1997)

2.8 Conditions for cholesterol depletion

To determine the effect of cholesterol depletion on signal transduction pathways, RMSA were incubated in 2% 2-hydroxylpropy-β-cyclodextrin (CD) (w/v) in M199 or $[PO_4]_0$ buffer for 1 hour. To replenish cholesterol of depleted samples, tissue was incubated in pre-complexed CD (0.4%) and cholesterol (16μg/ml) for 1 hour as described previously in A431 cells (Pike & Miller, 1998).

2.9 Diacylglycerol kinase assay

2.9.1 Membrane-associated DGK activity

The method used is that described by Ohanian and Heagerty for determination of membrane-associated DGK activity in rat subcutaneous small

arteries (Ohanian & Heagerty, 1994). Arteries were homogenised in 100µl ice-cold homogenisation buffer (20mM MOPS pH 7.2, 0.25M sucrose, 1mM dithiothreitol, 1mM EGTA, 1mg/ml leupeptin) and centrifuged at 300,000xg for 10 min (Beckman TL100 centrifuge). Supernatant was removed as the soluble fraction; the pellet washed with 200µl homogenisation buffer and resuspended in 40µl homogenisation buffer as the membrane fraction.

2.9.2 Caveolae/raft DGK activity

Membrane fractions were prepared as described (**section 2.2**) and pooled as fractions 2-5 (caveolae/raft), fractions 6–9 (non-caveolae/raft 1) and fractions 10-13 (non-caveolae/raft 2). Membranes were concentrated by diluting 3-fold in MBS followed by centrifugation at 100,000 xg, 4°C for 2 hours. Supernatant was discarded, membrane pellets washed once in 0.2M MES pH 6.0 and resuspended in 20µl homogenisation buffer for caveolae/raft fractions and 40µl for non-caveolae/raft fractions.

2.9.3 Extraction of DGK activity

DGK activity was extracted by the method of Lemaitre *et al.* (Lemaitre *et al.*, 1990). Equal volumes of extraction buffer (20mM MOPS pH7.2, 4% octyl β-glucopyranoside, 0.6M KCl, 20% glycerol, 1mM dithiothreitol) were added to resuspended fractions and the mixture left on ice for 30 min with occasional tapping. Samples were centrifuged at 300,000g for 10 min and supernatant transferred to a 1.5ml screw cap microfuge tube. Previous use of this method in subcutaneous small arteries resulted in 90-100% of DGK activity being recovered in the supernatant (Ohanian & Heagerty, 1994).

2.9.4 Assay of DGK activity

DGK activity was assayed using the OBG mixed-micelle assay of Lemaitre and Glomsett (Lemaitre & Glomsett, 1992). A mixed-micelle solution containing 10.15mM DAG, 11.55mM PS and 222mM OBG was prepared. Calculated amounts of 18:1/18:1 DAG and PS were dried in a glass vial under O_2-free N_2 gas and resuspended in freshly prepared 222mM OBG. The solution was sonicated for 30s and vortex-mixed at frequent intervals until a clear solution was formed. The reaction mixture contained 10µl of mixed micelle solution, 5µl of assay buffer (0.375mM MOPS pH 7.2, 0.125 mM $MgCl_2$, 6mM dithiothreitol) and 10µl of DGK extract. The reaction was started by the addition of 10µl of ^{32}P-ATP (1.82mM, 100-200 dpm/pmol) and left for 15 min at room temperature.

2.9.5 Extraction and separation of [^{32}P]-PA

Reactions were terminated by the addition of 300µl of chloroform: methanol: 10M HCl (66:33:1 v/v), samples agitated and centrifuged at 12000g with the resultant organic phase washed twice with 250µl of pure upper phase (chloroform: methanol: dH_2O, 3:48:47 v/v). The resultant organic phase was transferred to glass vials, dried under oxygen-free N_2 and reconstituted in 12µl chloroform. Samples were spotted on to a heat-activated silica gel 60 TLC plate, developed in chloroform: acetone: methanol: acetic acid: dH_2O (10:4:3:2:1 v/v) and [^{32}P]-PA quantified by autoradiography. Spots were visualised by iodine staining and PA identified by comparison with co-chromatographed PA standard.

2.10 Determination of protein concentration

2.10.1 Bradford assay

The method used was that of Bradford (1976). 200µl of Biorad reagent was added to 800µl aliquot of diluted sample (in dH_2O), mixed and the absorbance read at 595 nm. Protein concentration was determined by comparison to a five-point standard curve (0-20µg bovine serum albumin) (Bradford, 1976).

2.10.2 Lowry assay

The method used was that of Lowry *et al.* (1951) modified by Shakir *et al.* (1994). Dried protein samples were dissolved in 2M NaOH. Solution C was prepared by mixing 1ml solution A (189mM Na_2CO_3, 100mM NaOH) with 49mls Solution B (2% Sodium potassium tartrate, 1% copper sulphate). 1ml of Solution C was added to 300µl of diluted sample (in dH_2O), mixed and incubated at 37°C for 3 minutes. 100µl of Folin & Ciocalteau's reagent (10:9 reagent: dH_2O v/v) was added and incubated at 37°C for 3 minutes. Samples were mixed and absorbance read at 750 nm. Protein concentration was determined by comparison to a five point standard curve (0-40µg bovine serum albumin) (Shakir *et al.*, 1994;Lowry *et al.*, 1951).

2.11 Materials

2.11.1 Reagents

^{33}P-phosphate (specific activity 370 MBq/ml) was purchased from Amersham (Buckinghamshire, U.K) and ^{32}P-γ-ATP (specific activity 370 MBq/ml) from ICN (UK). Tissue-culture media M199 was from Gibco (Paisley, Scotland). Protogel (30% w/v acrylamide) was from National Diagnostics (Hull, U.K.) and Hybond-C extra nitrocellulose paper from Amersham (U.K). West Pico enhanced

chemiluminescence was from Pierce (Rockford, IL, USA). Silica gel 60 TLC plates (Merck 5721) and all 'HiPerSolv' and 'AnalaR' solvents were from VWR International (U.K.). Inhibitors LY294002, D609, MG-132, the antagonists' prazosin and BQ-123 and endothelin-1 were from Calbiochem (UK). Phenylephrine, trichloroacetic acid, noradrenaline, β-cholera toxin and all other reagents used were from Sigma (Poole, Dorset, U.K).

2.11.2 Antibodies

Monoclonal anti-caveolin-1 (clone 2297), monoclonal anti-caveolin-3, monoclonal anti-paxillin and monoclonal anti-hic-5 were all purchased from Transduction Laboratories (U.S.A.). Monoclonal anti-PLC-δ_1 (clone S-11-2) and polyclonal anti-PKB/Akt were purchased from Upstate (U.S.A.). Polyclonal anti-c-Src, polyclonal anti-Raf-1 (C-12), polyclonal anti-MYPT1 (E-19) and polyclonal anti-PP1c (FL-18) were all from Santa Cruz Biotechnology (U.S.A.). Polyclonal anti-DGKθ was a gift from W. van Blitterswijk (Amsterdam, Netherlands). Polyclonal anti-PLC-γ_1 was a gift from M. Katan (London, U.K.). Polyclonal anti-α-actinin was a gift from D. Critchley (Leicester, U.K.). Polyclonal anti-spectrin was a gift from J. Pinder (London, U.K.).

2.12 Data presentation and statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined using students t-test or analysis of variance (ANOVA) where indicated with $P < 0.05$ considered statistically significant and 'n' referring to the number of animals used for each experimental procedure.

CHAPTER 3

Validation of Caveolae/Lipid Raft Isolation Protocols

1.0 Introduction

Lipid rafts are operationally defined as detergent-insoluble microdomains that are cholesterol-dependent (Simons and Ikonen, 1997). The detergent-insolubility of caveolae/lipid rafts was exploited to produce a protocol utilising the non-ionic detergent Triton X-100 that enabled the rapid isolation of these domains (Brown and Rose, 1992). However, later studies called into question the use of this technique suggesting that the detergent had effects on the membrane composition (Heerklotz, 2002) and this led to the development of detergent-free caveolae/raft isolation techniques such as that produced by Song et al. (1996) utilising high pH carbonate extraction (Song *et al.*, 1996). A detergent-free protocol was used to isolate caveolae/rafts from RMSA and this was validated by examining distribution of three caveolae/raft markers – caveolin-1, caveolin-3 and the ganglioside GM_1 by western blotting and dot blots. Marker distribution was also compared to that obtained using detergent isolation of caveolae/rafts to confirm the efficiency of the protocol.

The depletion of cellular cholesterol with agents such as cyclodextrin (CD) is reported to disrupt rafts, 'flatten' the caveolar invagination and cause redistribution of caveolae/raft associated proteins (Conrad *et al.*, 1995; Smart *et al.*, 1994). To further validate the isolation of caveolae/rafts from RMSA, CD treatment was used to deplete cholesterol and the effect of cholesterol depletion on distribution of the caveolae/raft proteins caveolin-1, caveolin-3 and GM_1 was investigated using western blotting.

2.0 Methods

The protocols for the experiments detailed in this chapter are described in sections 2.1-2.4, 2.8 and 2.10 of chapter 2.

3.0 RESULTS

3.1 Validation of detergent-free isolation protocol

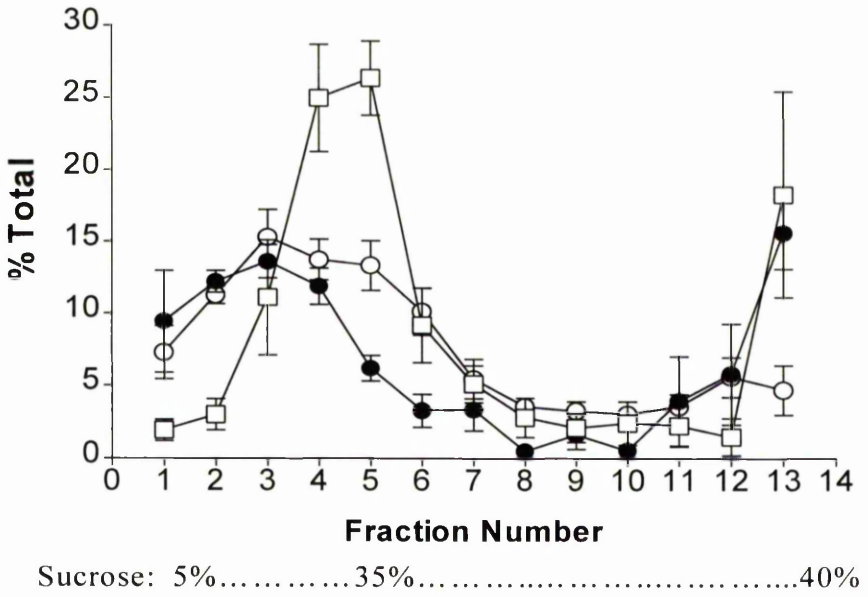
3.1.1 Distribution of caveolae/raft markers

To confirm reproducible isolation of caveolae/rafts, membrane fractions were isolated from unstimulated RMSA by detergent-free extraction and analysed for the content of three caveolae/raft markers – caveolin-1 (caveolae), the ganglioside GM_1 (rafts) and cholesterol (caveolae and rafts) as described above. To obtain a clearer picture for cholesterol, fractions were pooled as pairs. Results with detergent-free extraction show that 66 ± 5% of caveolin-1 (n=5), 44 ± 2% of cholesterol (n=4) and 54 ± 4% of GM_1 (n=4) are localised to fractions 2-5 as can be seen in the representative pictures for each marker (**Fig. 3.1**).

3.1.2 Electron microscopy of purified caveolae/rafts

To further confirm isolation of caveolae/rafts, membrane fractions were isolated from unstimulated RMSA, pooled as caveolae/raft (fractions 2-5) and concentrated by centrifugation (100,000g for 2 hours). Concentrated caveolae/raft membranes were then processed for electron microscopy as described above (**Fig. 3.2**).

A



B

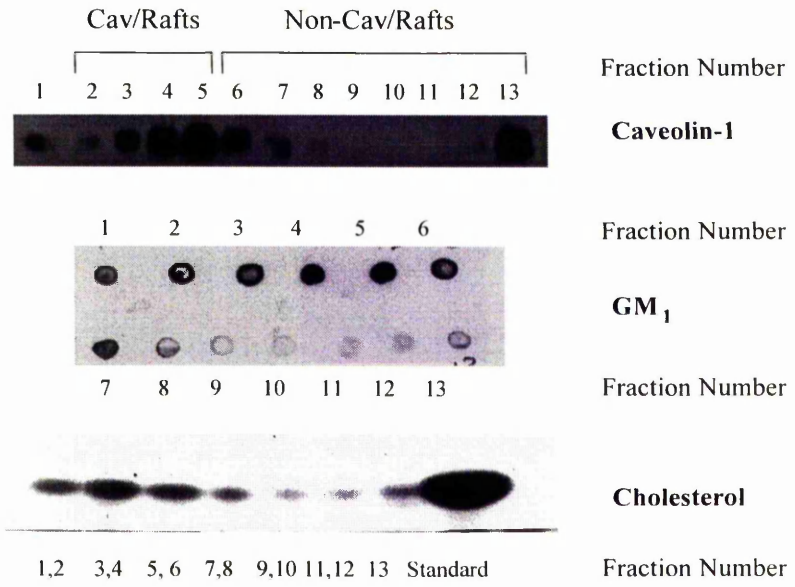


Fig. 3.1: Isolation of caveolae/rafts from rat mesenteric small arteries. RMSA were processed for detergent-free isolation of caveolae/rafts and fraction content analysed for caveolin-1 (open square), GM_1 (open circle) and cholesterol (closed circle) as described in sections 2.3 and 2.4 1. **A)** Data are expressed as mean ± SEM % total marker (n=4) **B)** Representative caveolin-1 immunoblot, GM_1 dot-blot and cholesterol TLC plate.

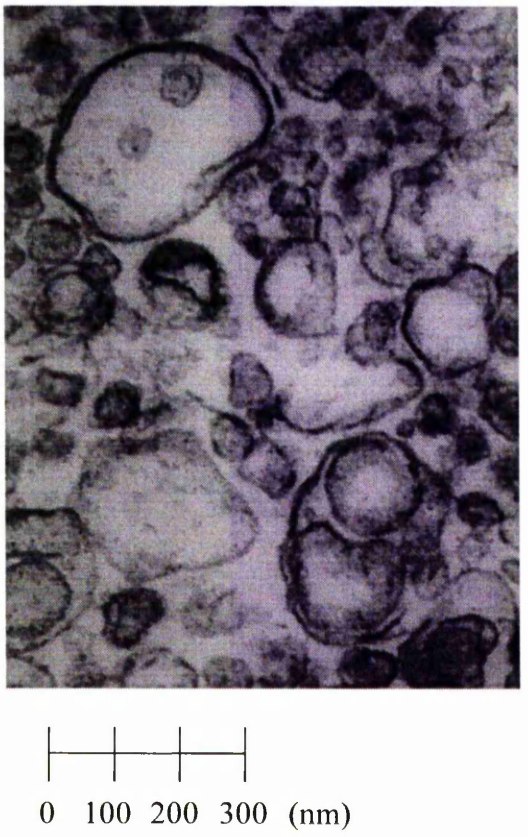


Fig. 3.2: Electron microscopy of purified caveolae/rafts from intact RMSA. Membrane fractions were isolated from unstimulated RMSA, caveolae/raft fractions (fractions 2-5) were pooled and processed for electron microscopy as described in section 2.2.4. Picture shown is magnified approximately x100,000.

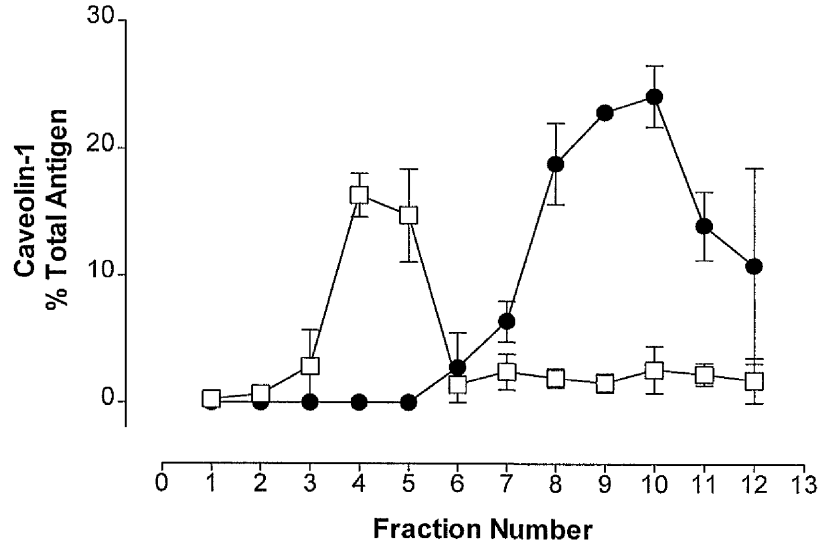
3.1.3 Comparison of detergent and detergent-free isolation

To compare caveolae/raft isolation methods, membrane fractions were prepared from RMSA by detergent- and detergent-free extraction, with cholesterol and caveolin-1 content analysed as described above. Data are expressed as % total marker. Triton extraction resulted in 36.9% cholesterol in fractions 2-5 (52.6% for Na_2CO_3) with the majority of cholesterol found in fractions 4-8 (73.6%, n=1, **Fig. 3.3A**). Furthermore, the presence of Triton X-100 affected running of the TLC plate. Similarly, detergent isolation resulted in 97.2% of total caveolin-1 remaining in fractions 7-12 of the gradient (n=2, **Fig. 3.3B**). Additionally, the presence of 1% Triton X-100 in the homogenisation buffer resulted in smearing of blots (**Fig. 3.4**).

3.2 Validation of Cholesterol separation

To confirm identification of cholesterol on TLC separation, membrane fractions were isolated from RMSA, lipids extracted and cholesterol content analysed as described above. Known lipid standards for PC, SM, triglycerides and cholesterol were co-chromatographed, spots were visualised by staining and lipids identified. The use of co-chromatographed lipid standards identifies cholesterol as the seventh band from the baseline (**Fig. 3.5**). Results also indicate the corresponding bands for PC (band 3), SM (band 2) and triglycerides (band 9).

A



B

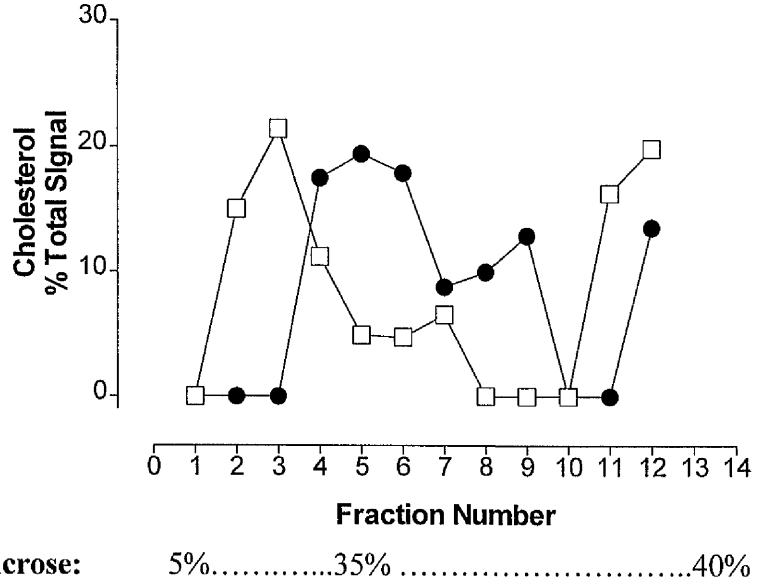


Fig. 3.3: Sodium carbonate is more effective at isolating caveolae. RMSA were homogenised in either 0.5M Na_2CO_3 (open squares) or 1% Triton X-100 (closed circles), caveolae/rafts isolated and analysed for **A)** caveolin-1 or **B)** cholesterol content as described in sections 2.2 and 2.3. Data are expressed as mean ± SEM % total cholesterol or caveolin-1 and are from at least one paired experiment.

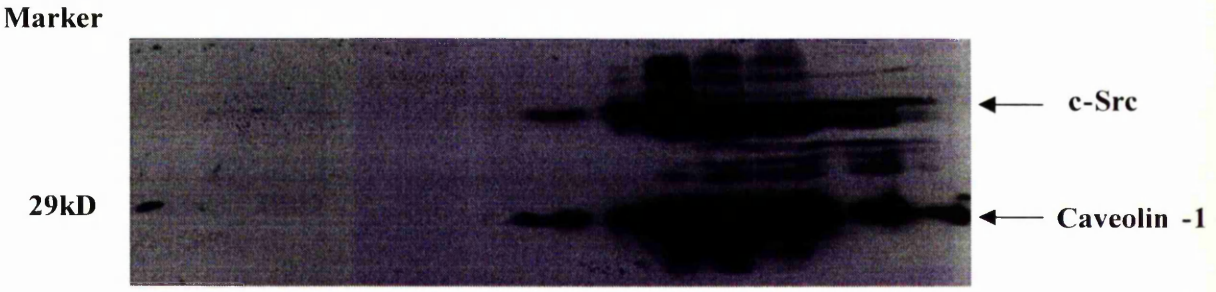


Fig. 3.4: Triton extraction resulted in smearing of blots. RMSA were homogenised in 1% triton X-100, caveolae/rafts isolated and caveolin-1 and c-src detected by immunoblot as described in section 2.2. Shown is a representative immunoblot of two experiments.

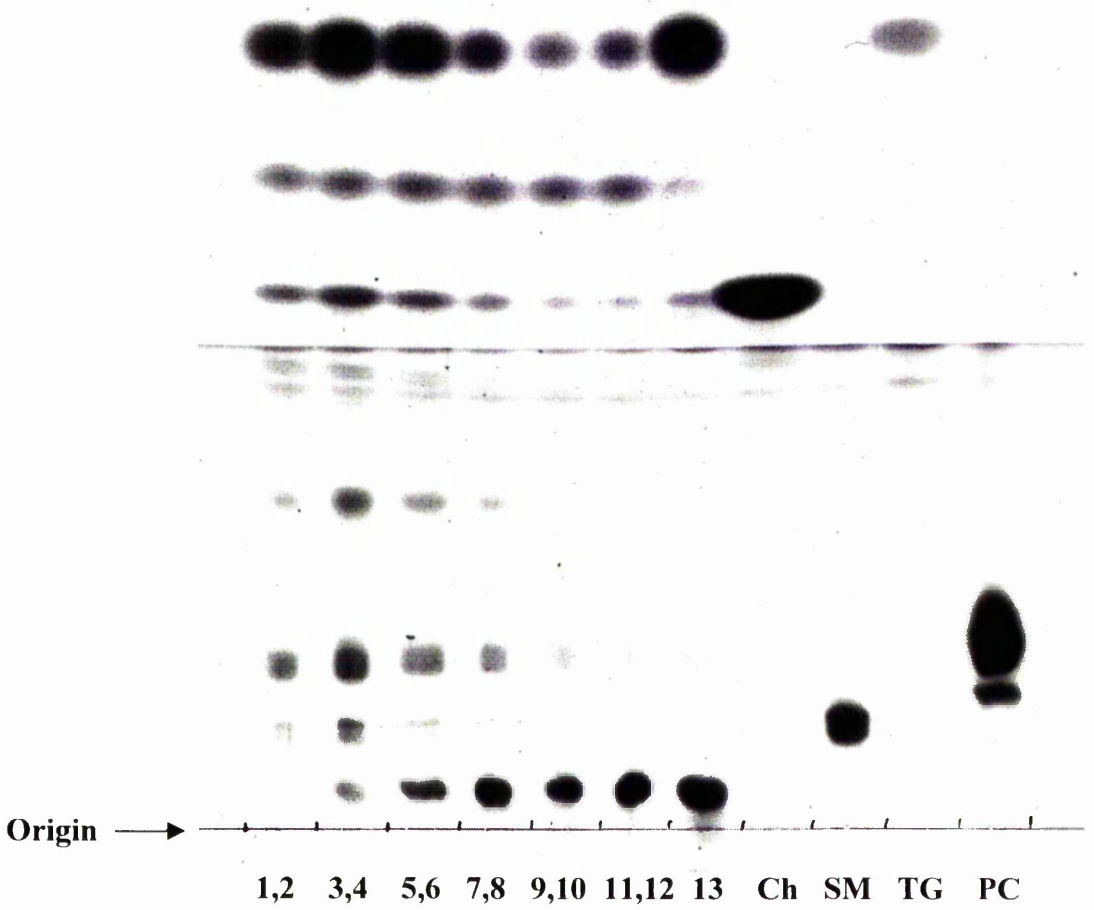


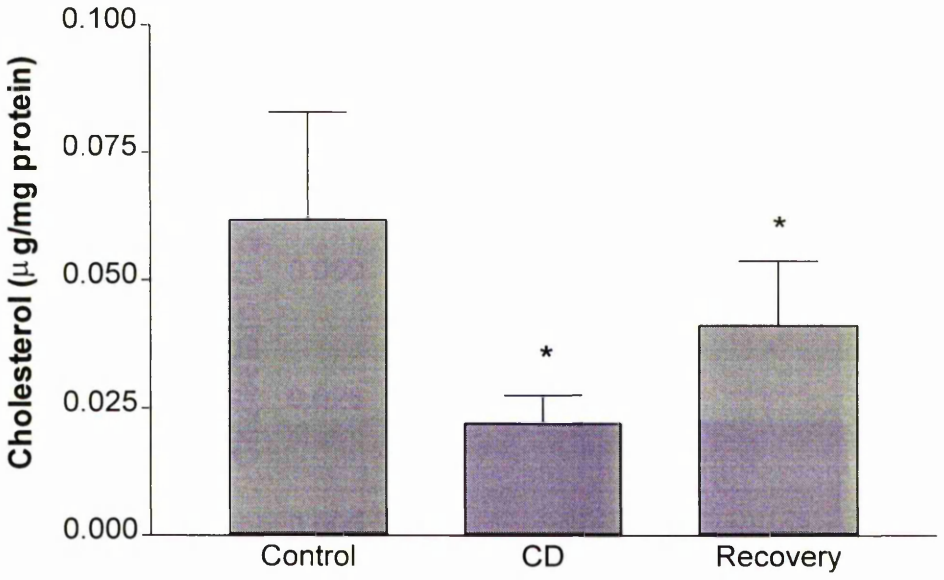
Fig. 3.5: Identification of cholesterol using co-chromatographed standards. Membrane fractions were prepared from RMSA, lipids extracted and cholesterol content analysed as described in section 2.4.1. Co-chromatographed standards for cholesterol (Ch), sphingomyelin (SM), phosphatidylcholine (PC) and triglycerides (TG) were used to identify lipids following staining. TLC plate shown is representative of four experiments.

3.3 Validation of cyclodextrin effect on cholesterol levels

To confirm that CD treatment depleted cholesterol, RMSA were incubated in the presence or absence of 2% CD in culture medium (M199) for 1 hour followed by culture medium (M199) or CD: cholesterol (0.4%: 16μg/ml) for 1 hour. Total lipids were extracted and cholesterol analysed as described above. Cholesterol was quantified using a standard curve of cholesterol standard concentrations run in parallel and results normalised for protein content of samples. Data are expressed as mean ± SEM μg cholesterol/mg protein.

CD treatment significantly reduced cholesterol levels to 35.6 ± 8.4 % of control levels (P <0.04, n=6). Treatment of depleted samples with pre-complexed cholesterol/CD (recovery) significantly increased cholesterol to 66.6 ± 11.5% of controls (P <0.02, n=5, **Fig. 3.6**).

A



B

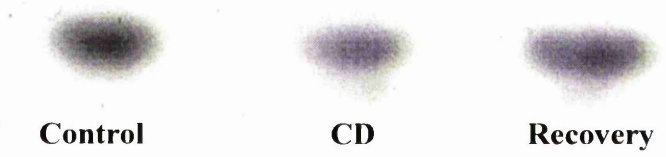


Fig. 3.6: Effect of CD on total cholesterol levels. RMSA were incubated in M199 or 2% CD in M199 for 1 hour at 37°C followed by M199 (control and CD) or 0.4%CD/16µg/ml cholesterol (recovery) for 1 hour at 37°C. Total lipids were extracted and cholesterol analysed by TLC as described in section 2.4.1. **A)** Data is expressed as mean ± SEM µg cholesterol/mg protein (* P <0.05, t-test, CD compared to basal, recovery compared to CD, n=5) **B)** Representative cholesterol TLC plate of five experiments

3.4 Effect of cholesterol depletion on distribution of caveolae/raft markers

3.4.1 Caveolin-1

RMSA were incubated in 2% CD for 1 hour, membrane fractions prepared and caveolin-1 content analysed by immunoblot as described in chapter 2. Analysis by ANOVA indicated that cholesterol depletion had no significant effect on caveolin-1 distribution in membrane fractions (P >0.70, n=5, **Fig. 3.7**). In unstimulated RMSA, 44.3 ± 4.0% of caveolin-1 was localised to fractions 2-5 compared with 42.3 ± 7.8% in cholesterol-depleted arteries (P >0.35, n=5).

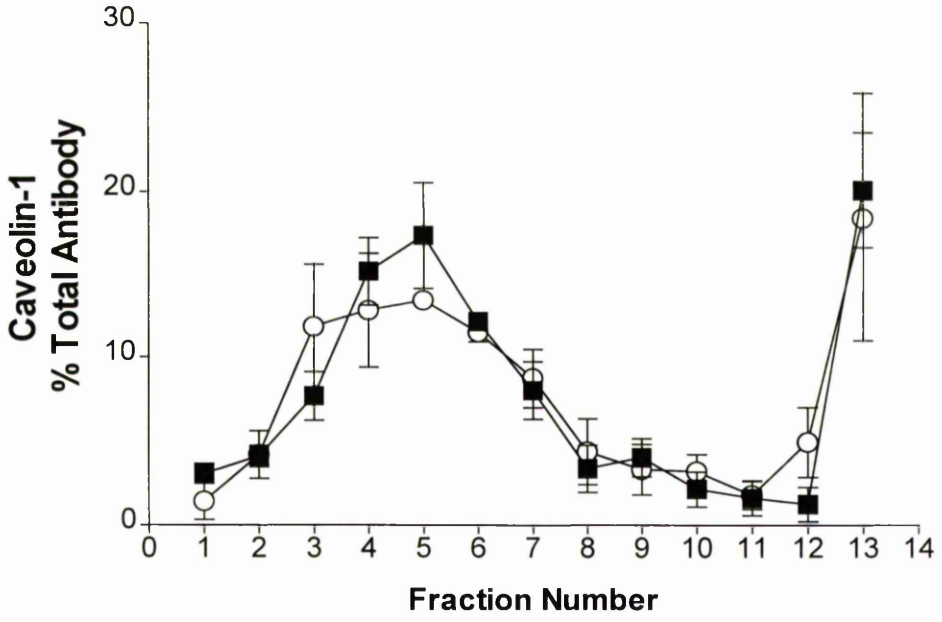
3.4.2 Caveolin-3

RMSA were incubated in 2% CD for 1 hour, membrane fractions prepared and caveolin-3 content analysed by immunoblot as described in chapter 2. Analysis by ANOVA suggested that cholesterol depletion had no effect on caveolin-3 distribution (P >0.20, n=2, **Fig. 3.8**). However, the amount of caveolin-3 in caveolae/raft fractions significantly decreased from 24.4 ± 1.1% to 10.3 ± 0.03 % following cholesterol depletion (P <0.03, n=2).

3.4.3 Ganglioside GM_1

RMSA were incubated in 2% CD for 1 hour, membrane fractions prepared and GM_1 content analysed by dot-blot as described in chapter 2. Analysis by two-way ANOVA indicated that cholesterol depletion had no significant effect on the distribution of GM_1 (P >0.9, n=4, **Fig. 3.9**). In unstimulated RMSA, 53.6 ± 4.3% of GM_1 localised to caveolae/rafts compared with 48.8 ± 5.7% following cholesterol depletion (P >0.25, n=4).

A



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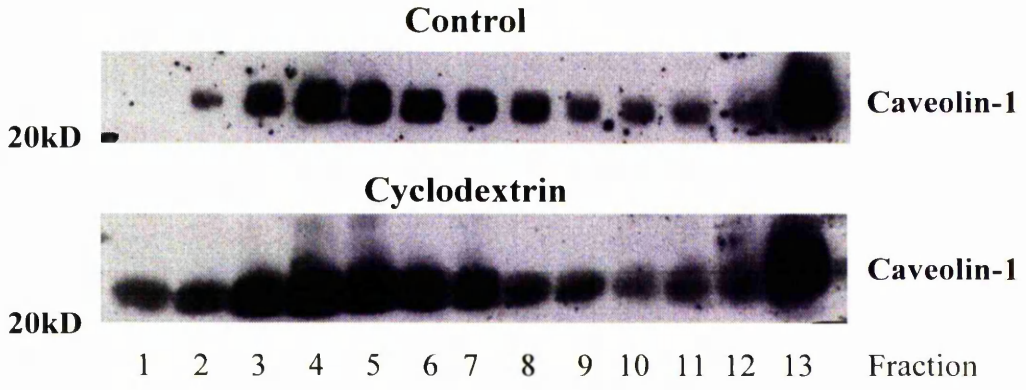
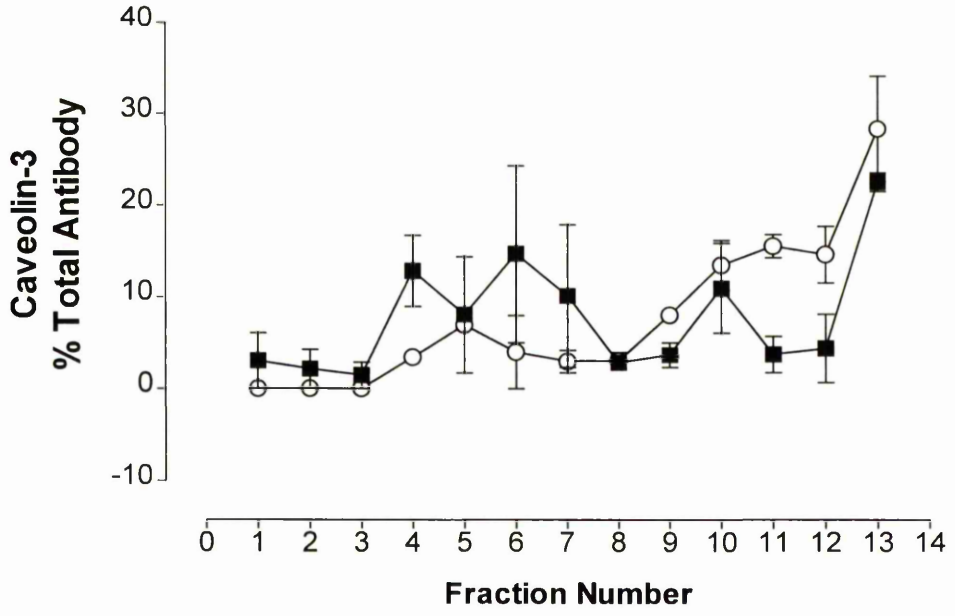


Fig. 3.7: Effect of cholesterol depletion on caveolin-1 distribution. RMSA were incubated in the presence (open circles) or absence of 2% CD (closed squares) for 1 hour, membrane fractions prepared and caveolin-1 content determined by immunoblot as described in chapter 2. **A)** Results are expressed as mean ± SEM % total caveolin-1 (n=5) **B)** Representative caveolin-1 immunoblots for control (top) and cholesterol-depleted samples (bottom).

A



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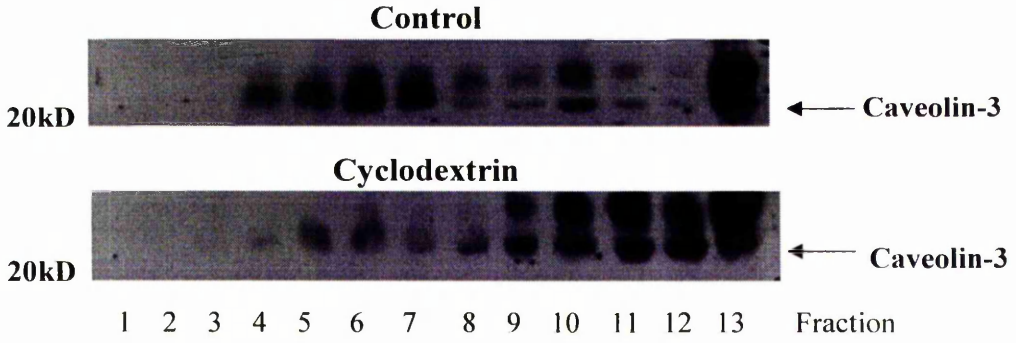
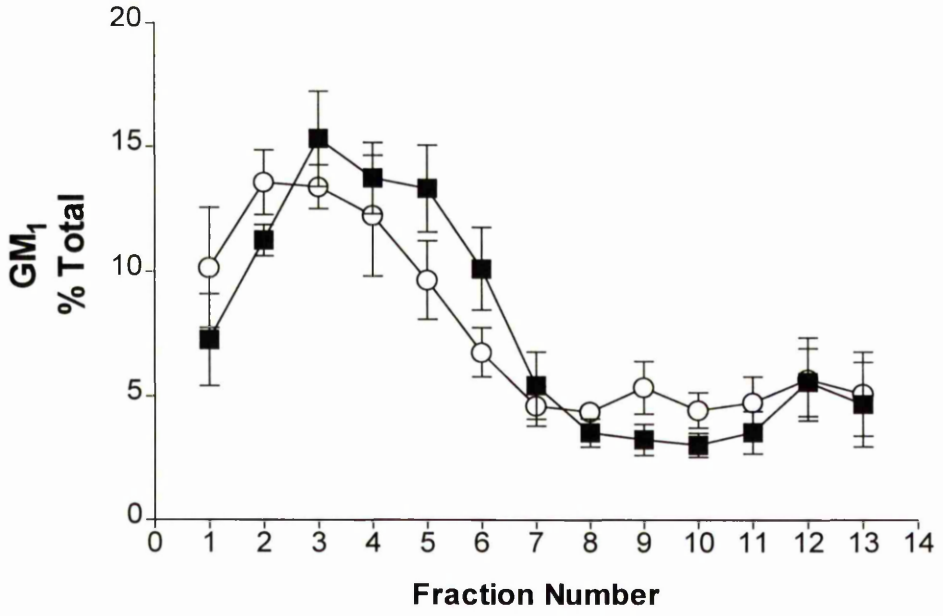


Fig. 3.8: Effect of cholesterol depletion on caveolin-3 distribution. RMSA were incubated in the presence (open circles) or absence of 2% CD (closed squares) for 1 hour, membrane fractions prepared and caveolin-3 content determined by immunoblot as described in chapter 2. **A)** Results are expressed as mean ± SEM % total caveolin-3 (n=2) **B)** Representative caveolin-3 immunoblots for control (top) and cholesterol-depleted samples (bottom).

A



B

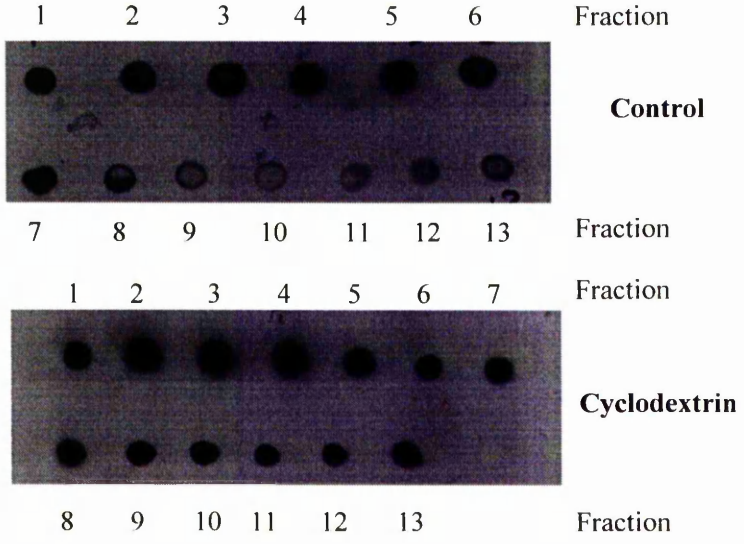


Fig. 3.9: Effect of cholesterol depletion on GM_1 distribution. RMSA were incubated in the presence (open circles) or absence of 2% CD (closed squares) for 1 hour, membrane fractions prepared and GM_1 content determined as described in chapter 2. A) Results are expressed as mean ± SEM % total GM_1 (n=4) B) Representative GM_1 immunoblots for control (top) and CD-treated samples (bottom).

4.0 Discussion

4.1 Isolation of caveolae/raft domains

As a consequence of their light buoyant density, caveolae/raft domains float to the 5-35% sucrose interface of a discontinuous sucrose gradient, approximately fraction 4. Results show marked enrichment of the caveolae/raft markers cholesterol, caveolin-1 and GM_1 within fractions 2-5, in and around the 5-35% sucrose interface, confirming the isolation of caveolae/raft domains from RMSA in agreement with previous studies utilising this protocol (Song *et al.*, 1996). For subsequent experiments, fractions 2-5 were designated the caveolae/raft fractions, fractions 6-9 termed non-caveolae/raft 1 and fractions 10-13 termed non-caveolae/raft 2.

Electron microscopy was used to further confirm the isolation of caveolae/rafts from RMSA by detergent-free extraction. Pooled caveolae/rafts (membrane fractions 2-5) were concentrated by centrifugation and processed for electron microscopy. The pictures obtained (**Fig. 3.2**) show caveolae/rafts as vesicular structures closely resembling plasma membrane caveolae seen in pictures of whole cells (**Chapter 1, Fig. 1.8**) or as curved membrane sheets. Importantly, there are no visible contaminants of other cellular organelles or membranes. These pictures obtained are similar to the morphology observed for caveolae/rafts purified from other cell types in previous studies (Lisanti *et al.*, 1994; Rybin *et al.*, 1999).

4.2 Comparison of detergent and detergent-free extraction

Results indicated that detergent-free extraction was a more efficient method of isolating caveolae/rafts from RMSA in comparison to extraction with the non-ionic detergent Triton X-100. Furthermore, the presence of Triton interfered with

analysis by both chromatography and immunoblotting resulting in obscuring of results. The detergent-insolubility of caveolae/raft domains provided the basis for a rapid and useful technique for purifying these domains from the membrane (Simons & Ikonen, 1997;Sargiacomo *et al.*, 1993). However, more recently, this has been called into question with detergent extraction reported to affect protein association with caveolae/rafts and the native state of cell membranes (Foster *et al.*, 2003;Heerklotz, 2002). Therefore, detergent-free extraction was used to isolate caveolae/rafts in all subsequent experiments.

4.3 Identification of cholesterol

The use of lipid standards confirms the isolation of cholesterol from other lipids in intact RMSA. These results correspond to previous results utilising this method with lipids extracted from brain tissue (Macala *et al.*, 1983). Furthermore, results also indicate that caveolae/raft fractions contained SM and PC (**Fig. 3.5**) in agreement with previous studies in cultured platelets and T-cells (Rouquette-Jazdanian *et al.*, 2002;Bodin *et al.*, 2001).

4.4 Depletion of cholesterol with cyclodextrin

Treatment of RMSA with the cholesterol-binding sugar cyclodextrin significantly reduced cholesterol levels whilst subsequent treatment of depleted samples with precomplexed cyclodextrin/cholesterol significantly restored cholesterol levels consistent with previous studies utilising this technique (Hailstones *et al.*, 2004;Ushio-Fakai *et al.*, 2001;Furuchi & Anderson, 1998).

4.5 Effects of cholesterol depletion on localisation of caveolae/raft markers

Whilst the treatment of RMSA with 2% CD significantly depleted cellular cholesterol levels, little effect was observed on caveolin-1 distribution in membrane fractions. This is contrary to a number of early studies reporting redistribution of caveolin-1 to higher density fractions in cholesterol-depleted A431 cells (Pike & Miller, 1998) and fibroblasts (Furuchi & Anderson, 1998) and translocation of caveolin-1 to intracellular organelles on cholesterol oxidation (Conrad *et al.*, 1995;Smart *et al.*, 1994). The reason for this discrepancy is not clear as caveolin-1 is reported to require cholesterol binding for full transport to the plasma membrane (Murata *et al.*, 1995), particularly as cholesterol depletion did cause some redistribution of the muscle-specific caveolin-3 away from caveolae/rafts in RMSA. This could reflect different effects of CD treatment on cultured cells and intact tissue and consistent with this, a study in intact rat tail artery indicated no apparent effect of cholesterol depletion on caveolin-1 distribution (Dreja *et al.*, 2002). As intact tissue contains both VSM and endothelial cells, this differential effect observed could be due to CD extracting cholesterol preferentially from muscle tissue over endothelial cells. Alternatively, caveolin-1 and caveolin-3 may have different requirements for cholesterol in order to oligomerise and reach the plasma membrane. Previously, it was reported that approximately 40 mol% of cholesterol was required to form the caveolar invagination (Hailstones *et al.*, 2004). It is possible that caveolin-3 may require a higher level of cholesterol to reach the membrane. Alternatively, there may exist distinct populations of caveolae containing caveolin-3 and caveolin-1 with different cholesterol levels required for their formation and so less cholesterol needs to be removed to cause caveolin-3 redistribution. As with caveolin-1, cholesterol depletion had no significant effect on GM_1 distribution disagreeing with model

membrane studies reporting the cholesterol-dependent association of GM_1 with cholesterol- and sphingolipid-enriched domains (Pei & Chen, 2003;Dietrich *et al.*, 2001a;Dietrich *et al.*, 2001b) although this could reflect differences between model membranes and intact tissue. The lack of effect of CD on GM_1 agrees with a previous study reporting that CD extracts cholesterol preferentially from outside of sphingolipid microdomains and has no effect on the buoyant density property of GM_1, leaving sphingolipid rafts intact (Ilangumaran & Hoessli, 1998).

Although redistribution of caveolin-3 was observed, the lack of effect of CD treatment on caveolin-1 and GM_1 suggests that caveolae/rafts may remain intact in cholesterol-depleted RMSA. This is supported by previous work reporting that caveolae of VSM were disrupted in caveolin-1 (-/-) knockout mice, suggesting that caveolin-1 may be more important for structural integrity of caveolae in VSM (Drab *et al.*, 2001). However, a study utilising electron microscopy in rat tail artery showed that CD treatment had no effect on caveolin-1 distribution but did result in less numerous caveolae (Dreja *et al.*, 2002). Thus, the structural integrity of caveolae/rafts in cholesterol depleted RMSA remains uncertain.

4.6 Use of Female Rats

As female rats were used for all experiments, the possibility that the oestrous cycle may have influenced results must also be considered, particularly as recent studies in rat bladder and uterine smooth muscle have reported that changes in oestrogen levels have effects on caveolin-1 expression and caveolae numbers (Zhu *et al.*, 2004; Turi *et al.*, 2001). However, in both these studies caveolin levels were examined following chronic oestrogen stimulation whereas a more recent study in

mice myometrium reported no significant changes in caveolin-1 levels at different stages of the oestrous cycle (Riley *et al.*, 2005). Additionally, results in these studies examining caveolin levels during pregnancy further suggest that *in vivo* caveolin expression in smooth muscle is not under strong oestrogenic control. Thus, it seems unlikely that the oestrous cycle could have had influences on experimental results reported here.

5.0 Summary and Conclusions

The results in this chapter indicate that a detergent-free isolation of caveolae/rafts is more effective in RMSA than that using the non-ionic detergent Triton X-100. However, whilst CD treatment significantly depleted cellular cholesterol, it had different effects on caveolae/raft markers suggesting that caveolae/rafts may not be fully disrupted.

CHAPTER 4

Localisation of NA and ET-1-stimulated PIP_2 Hydrolysis in Rat Mesenteric Small Arteries

1.0 Introduction

Caveolae/rafts are specialised plasma membrane microdomains and in VSM are implicated in PKC-mediated regulation of contractility and are suggested sites of calcium influx (Je *et al.*, 2004;Isshiki & Anderson, 2003;Taggart *et al.*, 2000). Recent work in cultured A431 cells has also reported caveolae/rafts as sites of agonist-stimulated PIP_2 turnover (Hope & Pike, 1996). In VSM, PLC-mediated PIP_2 hydrolysis is a primary event in agonist-induced contraction and localisation of PIP_2 hydrolysis within caveolae/rafts could be important for coupling of PI turnover to intracellular signalling pathways through localised production of the second messengers IP_3 and DAG. Accordingly, using [^{33}P]-phosphate radiolabelling and chromatography, the distribution of PIP_2 and involvement of caveolae/rafts in vasoconstrictor-stimulated PIP_2 hydrolysis was investigated.

Additionally, it is unclear which PLC isoforms are involved in vasoconstrictor-stimulated PIP_2 hydrolysis. Activity of the PLC-δ_1 isoform is calcium-dependent (Allen *et al.*, 1997), has previously been implicated in α_1-adrenergic signalling (Chen *et al.*, 1996) and is enhanced in hypertension (Kosugi *et al.*, 2003) suggesting a role in VSM contraction. Accordingly, using western blot analysis, the involvement of PLC-δ_1 in vasoconstrictor-stimulated PIP_2 hydrolysis in RMSA was investigated.

2.0 Methods

The protocols for the experiments detailed in this chapter are described in sections 2.1 – 2.4, 2.7 and 2.10 of chapter 2.

3.0 Results

3.1 Validation of phosphoinositide separation

To validate isolation of PIP_2 and PIP by TLC, phospholipid standards for PC, phosphatidylethanolamine (PEth), PI, phosphatidylserine (PS), PIP_2, PIP, and PA were spotted on an oxalate-coated TLC plate and processed for PI separation as described in chapter 2. Spots were visualised using I_2 vapour and outlined using ink. R_f values for lipid standards were calculated and used to determine corresponding spots for PIP and PIP_2 on autoradiographs of radiolabelled phospholipids (**Fig. 4.1**).

From the plate of known lipid standards, R_f values of 0.138 for PIP_2 and 0.269 for PIP were obtained when measured from the middle of the spots observed. From the autoradiograph of TLC separation of [^{33}P]-labelled phospholipids, R_f values of 0.143 for band 1 and 0.268 for band 2 were calculated when measured from the middle of the bands observed (**Table 4.1**).

3.2 PIP_2 hydrolysis in [^{33}P]-phosphate-labelled RMSA

3.2.1 Distribution of [^{33}P]-PIP_2

To investigate membrane distribution of PIP_2, membrane fractions were isolated from [^{33}P]-labelled RMSA and [^{33}P]-PIP_2 content analysed as described in chapter 2. In unstimulated RMSA, 52.9 ± 3.7% of [^{33}P]-PIP_2 was localised to

caveolae/raft fractions with 21.6 ± 4.2% in non-caveolae/rafts 1 and 25.5 ± 3.2% in non-caveolae/rafts 2 (n=5, **Fig. 4.2**).

3.2.2 Localisation of NA-stimulated [^{33}P]-PIP_2 hydrolysis

[^{33}P]-labelled RMSA were stimulated with NA (15μM), membrane fractions prepared and [^{33}P]-PIP_2 content analysed as described in chapter 2. Time points were chosen to correspond to the initial (20s) and sustained phases (5 min) of NA-stimulated contraction of RMSA (Ohanian *et al.*, 1990). NA induced a significant decrease in [^{33}P]-PIP_2 levels of caveolae/rafts at both 20s (54 ± 12%, P <0.01, n=4, **Fig. 4.3A closed bars**) and 5 min of stimulation (34 ± 10%, P <0.01, n=5, **Fig. 4.3A hatched bars**). However, no significant effect was observed on [^{33}P]-PIP_2 levels of the non-caveolae/raft fractions at either time point tested (P >0.5).

3.2.3 Localisation of ET-1-stimulated [^{33}P]-PIP_2 hydrolysis

[^{33}P]-labelled RMSA were stimulated with ET-1 (100nM), membrane fractions prepared and [^{33}P]-PIP_2 content analysed as described in chapter 2. Time points were chosen to correspond to the initial (20s) and sustained phases (10 min) of ET-1-stimulated RMSA contraction (Ohanian *et al.*, 1997). ET-1 induced a significant decrease in [^{33}P]-PIP_2 levels of caveolae/rafts at both 20s (26 ± 11%, P <0.05, n=5, **Fig. 4.3B closed bars**) and 10 min of stimulation (33 ± 6%, P <0.05, n=5, **Fig. 4.3B hatched bars**). However, no significant effect was observed on [^{33}P]-PIP_2 levels of the non-caveolae/raft fractions at either of the time points tested (P >0.5).

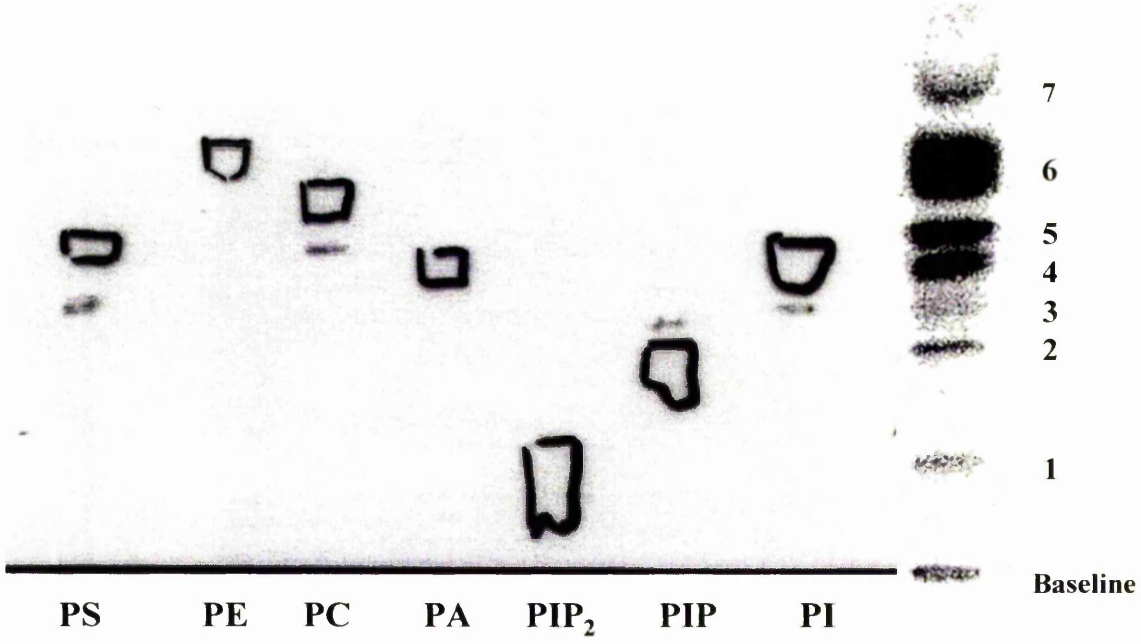


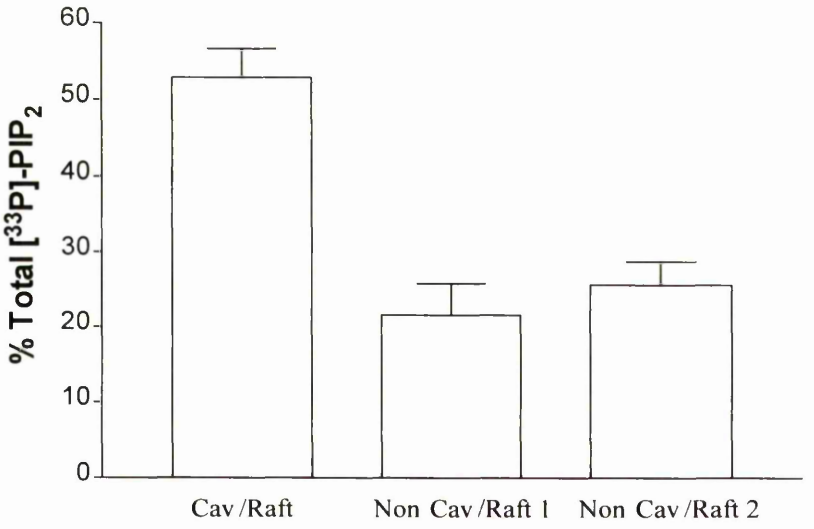
Fig. 4.1: Identification of PIP_2 and PIP using lipid standards. Lipid standards for phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI), PI-4-monophosphate (PIP), PI-4,5-bisphosphate (PIP_2) and phosphatidylcholine (PC) were processed for phosphoinositide separation as described in section 2.4.2 (E). Standard plate shown is representative of two experiments (left) and autoradiograph of endogenously [^{33}P]-labelled phospholipids from RMSA shown is representative of five experiments (right).

Standard	Rf Value (Front =145mm)	Band	Rf Values (Front = 160mm)
PIP_2	0.138	1	0.143
PIP	0.269	2	0.268
PI	0.372	3	0.313
PA	0.379	4	0.369
PS	0.393	5	0.400
PC	0.448	6	0.470
PE	0.510	7	0.494

Table 4.1: R_f Values of lipid standards and [^{33}P]-labelled phospholipids.

Results are calculated from Fig. 4.1

A



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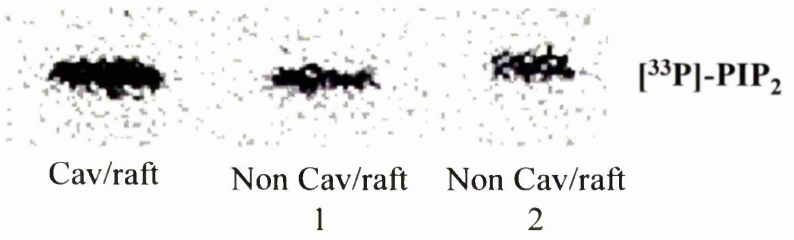
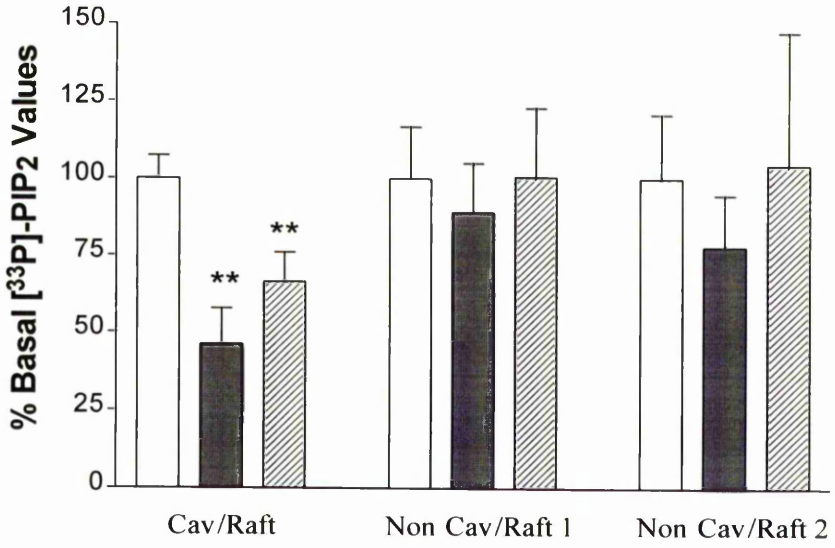


Fig. 4.2: Distribution of [^{33}P]-PIP$_2$ in unstimulated arteries. Caveolae/rafts were isolated from [^{33}P]-labelled RMSA and [^{33}P]-PIP$_2$ content analysed as described in Chapter 2. **A)** Results are expressed as mean ± SEM % total [^{33}P]-PIP$_2$ in fractions 2-5 (caveolae/raft), fractions 6-9 (non-caveolae/raft 1) and fractions 10-13 (non-caveolae/raft 2) **B)** Autoradiograph of [^{33}P]-PIP$_2$ representative of five experiments.

A



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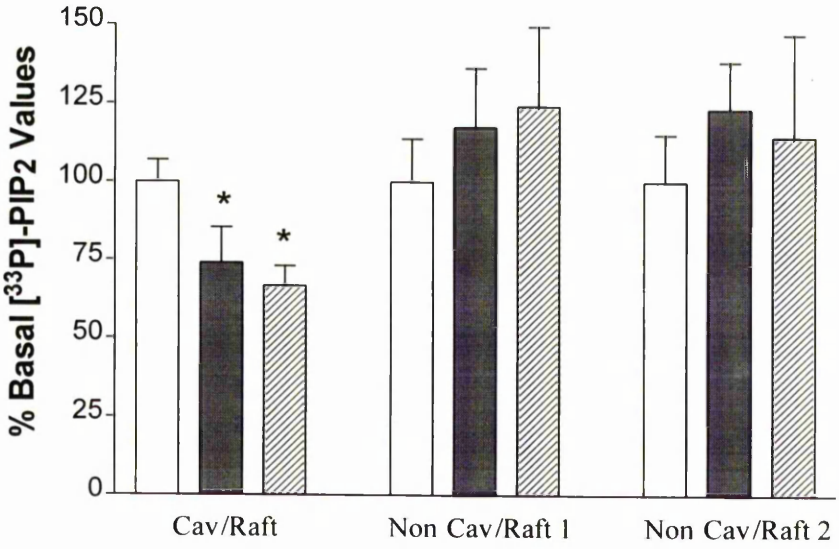


Fig. 4.3: Effect of NA and ET-1 on [^{33}P]-PIP$_2$ in caveolae/rafts. [^{33}P]-labelled RMSA were stimulated with NA (15μM), ET-1 (100nM) or dH_2O as vehicle, membrane fractions prepared and [^{33}P]-PIP$_2$ content analysed as described in chapter 2 **A)** Effect of NA at 20s (closed bars) and 5 min (hatched bars) on [^{33}P]-PIP$_2$ levels, data expressed as mean ± SEM % basal PIP$_2$ level in each of the individual fractions where basal PIP$_2$ = 100% (** P <0.01 compared to basal, ANOVA, n=4) **B)** Effect of ET-1 at 20s (closed bars) and 10 min (hatched bars) on [^{33}P]-PIP$_2$ levels, data expressed as mean ± SEM % basal PIP$_2$.level in each of the individual fractions where basal PIP$_2$ = 100% (* P <0.05 compared to basal, ANOVA, n=5)

3.3 Phospholipase C in rat mesenteric small arteries

3.3.1 Phospholipase C-δ_1

To confirm the presence of the PLC-δ_1 isoform, RMSA were homogenised in Na_2CO_3 and PLC-δ_1 content investigated by western blot as described in chapter 2 with 15 μg of total protein loaded. With anti-PLC-δ_1, two bands were visible in total RMSA extract: a strong band of approximately 84.5kD molecular weight and a fainter band (A) of 48.1 kD molecular weight as determined by use of a standard curve of molecular weight markers (**Fig. 4.4**).

3.3.2 Effect of NA on PLC-δ_1 localisation

To investigate the localisation of PLC-δ_1, RMSA were stimulated with NA (15μM) for 0, 20s, 2min and 5min, membrane fractions were prepared, pooled as caveolae/raft, non-caveolae/raft 1 and non-caveolae/raft 2 and PLC-δ_1 content analysed by western blot as described in chapter 2.

In unstimulated RMSA, 6.4 ± 1.1% of PLC-δ_1 was found in caveolae/raft domains with 22.5 ± 3.6% in non-caveolae/rafts 1 and 71.1 ± 3.4% in non-caveolae/rafts 2 (n=9, **Fig. 4.5**). NA stimulated a rapid (20s), transient increase in PLC-δ_1 levels of caveolae/rafts (2.04 ± 0.30-fold peak at 20s) with levels returning to basal by 5 minutes (n=4, **Fig. 4.6A, open bars**). Although PLC-δ_1 levels decreased in both non-caveolae/raft 1 and non-caveolae/raft 2 fractions, this was not statistically significant (P >0.1, n=4).

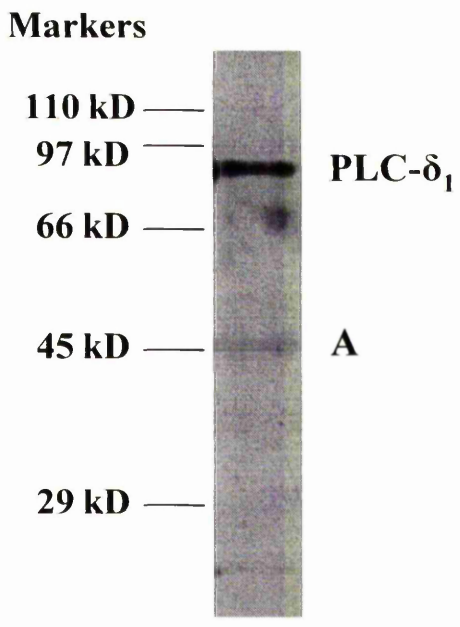


Fig. 4.4: PLC-δ_1 is expressed in RMSA. RMSA were homogenised in sodium carbonate and 15μg total protein extract was analysed for PLC-δ_1 content by immunoblot as described in chapter 2. The PLC-δ_1 immunoblot shown is representative of two experiments.

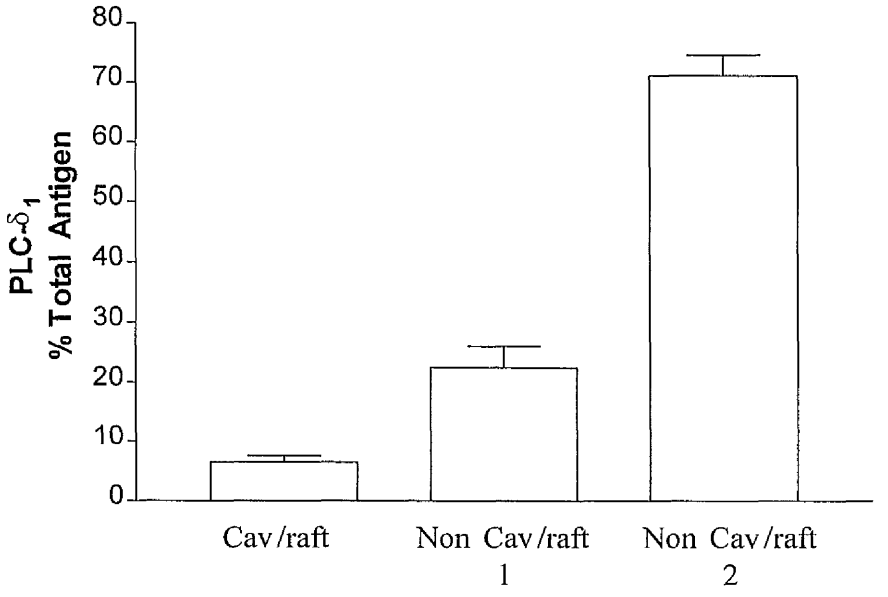
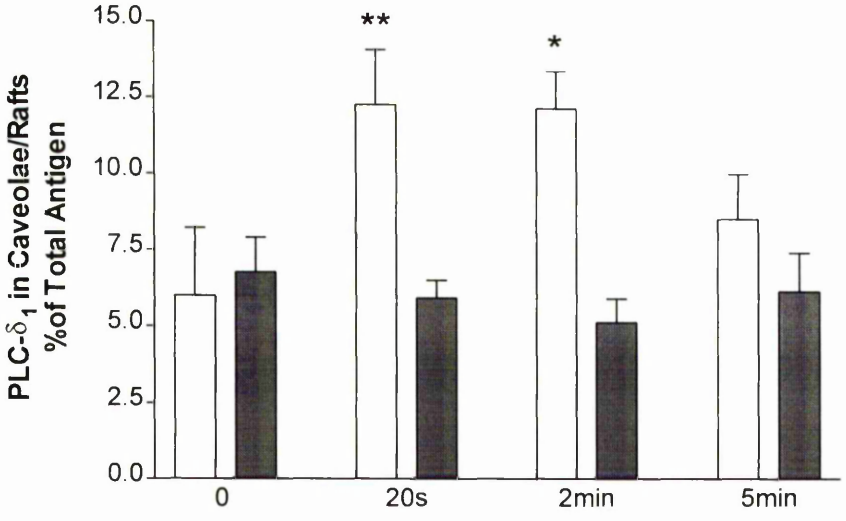


Fig. 4.5: Distribution of PLC-δ_1 in RMSA. Membrane fractions were prepared from unstimulated RMSA and analysed for PLC-δ_1 content by western blot as described in chapter 2. Results are expressed as mean ± SEM % PLC-δ_1 in fractions 2-5 (caveolae/raft), fractions 6-9 (non-caveolae/raft 1), and fractions 10-13 (non-caveolae/raft 2) and are the result of nine experiments.

A



B

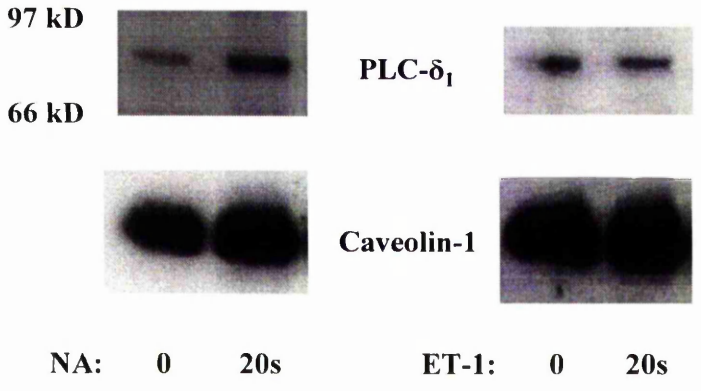


Fig. 4.6: NA and ET-1 have different effects on PLC-δ_1 levels in caveolae/rafts. RMSA were stimulated with NA (open bars) or ET-1 (closed bars), membrane fractions prepared and PLC-δ_1 content analysed as described in chapter 2. **A)** Densitometric data of the PLC-δ_1 signal in caveolae/rafts expressed as mean ± SEM % total PLC-δ_1 (* P <0.04; ** P <0.01, t-test, compared to basal, n=4). **B)** Representative immunoblots for NA (left) and ET-1 (right) are shown.

3.3.3 Effect of ET-1 on PLC-δ_1 localisation

RMSA were stimulated with ET-1 (100nM) for 0, 20s, 2min and 5min, membrane fractions were prepared, pooled as caveolae/raft, non-caveolae/raft 1and non-caveolae/raft 2 and PLC-δ_1 content analysed by western blot as described in chapter 2. ET-1 had no significant effect on PLC-δ_1 levels of caveolae/rafts at any of the time points investigated (P >0.05, n=5, **Fig. 4.6A closed bars**). No significant effect was observed on PLC-δ_1 levels in non-caveolae/raft fractions (P >0.2, n=5).

3.3.4 Effect of extracellular calcium removal on NA-stimulated PLC-δ_1 translocation to caveolae/rafts

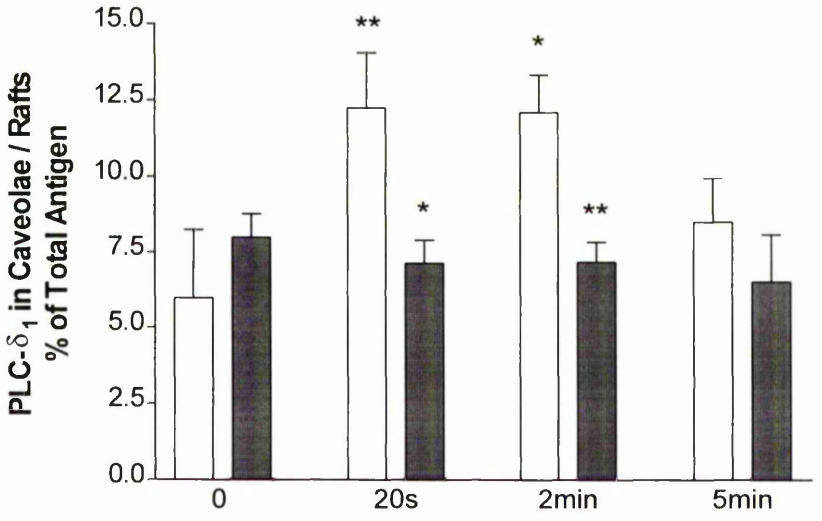
To determine if PLC-δ_1 translocation to caveolae/rafts was dependent on calcium influx, RMSA were incubated in calcium-free HEPES buffer containing 1mM EGTA for 10 min prior to stimulation with NA (15μM) for 0, 20s, 2min and 5min. Membrane fractions were prepared, pooled as caveolae/raft, non-caveolae/raft 1 and non-caveolae/raft 2 and PLC-δ_1 content analysed by western blot as described in chapter 2. Extracellular calcium removal prevented the NA-stimulated association of PLC-δ_1 with caveolae/rafts (**Fig. 4.7A closed bars**). At all time points investigated, PLC-δ_1 levels in caveolae/rafts were not significantly altered from basal levels (P >0.1, n=4).

3.4 Effect of extracellular calcium removal on PI turnover

3.4.1 Effect of extracellular calcium removal on NA-stimulated [^{33}P]-PIP_2 hydrolysis

If PLC-δ_1 were functionally involved in NA-stimulated PIP_2 hydrolysis in caveolae/rafts, preventing PLC-δ_1 translocation to these domains by extracellular

A



B

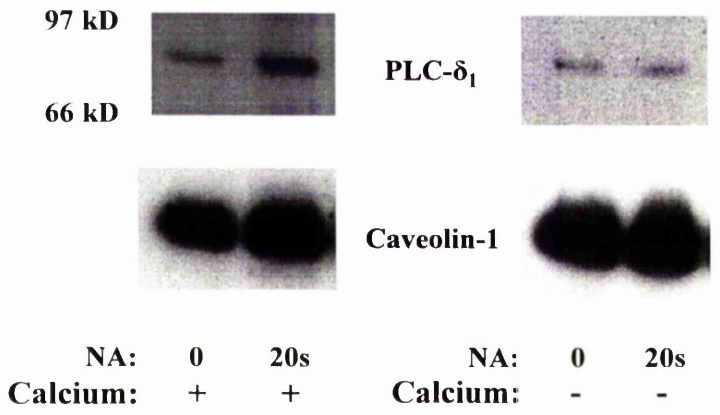


Fig. 4.7: NA-stimulated PLC-δ_1 translocation is extracellular calcium-dependent. RMSA were incubated in the presence (open bars) or absence (closed bars) of extracellular calcium prior to stimulation with NA (15μM), membrane fractions were prepared and PLC-δ_1 content analysed by immunoblot as described in chapter 2 **A)** Densitometric data of the PLC-δ_1 signal in caveolae/rafts expressed as mean ± SEM % total PLC-δ_1 (* P <0.04; ** P <0.01, t-test, NA to basal, and NA to calcium-free+NA, n=5) **B)** Representative PLC-δ_1 immunoblots for NA (left) and calcium-free (right) are shown.The control (NA) data shown is the same as that in **Fig. 4.6** (pg 116).

calcium removal would reduce NA-stimulated PIP_2 hydrolysis. To investigate this, [^{33}P]-labelled RMSA were incubated in calcium-free HEPES buffer containing 1mM EGTA for 10 min prior to stimulation with NA (15μM) or dH_2O (vehicle) for 20s, membrane fractions prepared and [^{33}P]-PIP_2 content analysed as described in chapter 2.

In unstimulated RMSA, extracellular calcium removal significantly increased [^{33}P]-PIP_2 levels of caveolae/rafts (1.49 ± 0.18-fold) and non-caveolae/rafts 1 (1.15 ± 0.24-fold, P <0.05, n=5). Similarly, in NA-stimulated RMSA, extracellular calcium removal increased caveolae/raft [^{33}P]-PIP_2 levels (2.49 ± 0.37-fold, P <0.01, n=5, **Fig. 4.8A**). However, NA-stimulated PIP_2 hydrolysis in caveolae/rafts was significantly reduced. The decrease in [^{33}P]-PIP_2 levels was reduced from 57 ± 7% to 27 ± 13% on removal of extracellular calcium (P <0.05, n=5, **Fig. 4.9A**).

3.4.2 Effect of extracellular calcium removal on ET-1-stimulated [^{33}P]-PIP_2 hydrolysis

As ET-1 did not induce PLC-δ_1 translocation to caveolae/rafts, extracellular calcium removal would have little effect on ET-1-stimulated PIP_2 hydrolysis. To investigate this, [^{33}P]-labelled RMSA were incubated in calcium-free HEPES buffer containing 1mM EGTA for 10 min prior to stimulation with ET-1 (100nM) or dH_2O (vehicle) for 20s, membrane fractions prepared and [^{33}P]-PIP_2 content analysed as described in chapter 2.

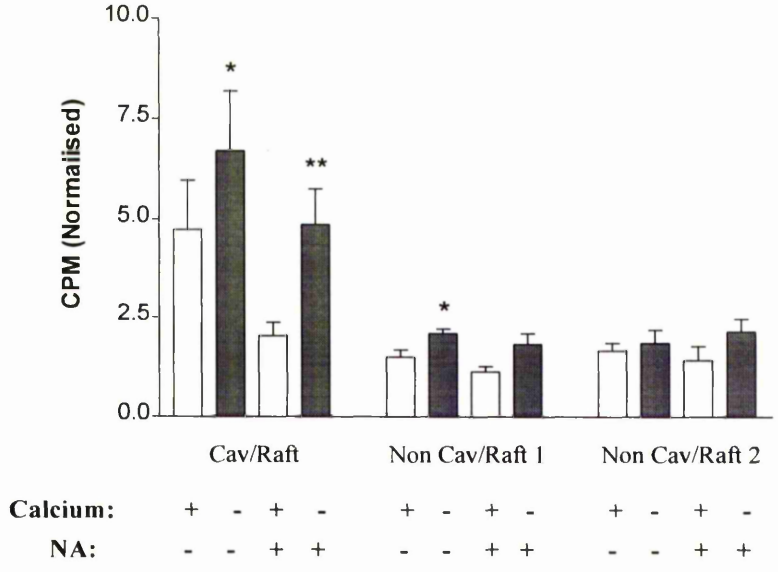
In ET-1-stimulated RMSA, extracellular calcium removal significantly increased [^{33}P]-PIP_2 levels of caveolae/rafts (1.68 ± 0.19-fold, P <0.01, n=3, **Fig. 4.8B**). However, [^{33}P]-PIP_2 hydrolysis was unaffected with levels decreasing by 58 ± 5% in controls compared to 51 ± 2% on extracellular calcium removal (P >0.15, n=3, **Fig. 4.9B**).

3.4.3 Effect of extracellular calcium removal on NA-stimulated [^{33}P]-PIP turnover

The differential effects of extracellular calcium removal on NA and ET-1-stimulated [^{33}P]-PIP_2 hydrolysis might be a result of an effect on PIP_2 production upstream of PLC. To investigate this, [^{33}P]-labelled RMSA were incubated in calcium-free HEPES buffer containing 1mM EGTA for 10 min prior to stimulation with NA (15μM) or dH_2O as vehicle for 20s, membrane fractions prepared and [^{33}P]-PIP content analysed as described in chapter 2.

Extracellular calcium removal significantly increased [^{33}P]-PIP levels of caveolae/rafts in both unstimulated (1.80 ± 0.39-fold, P >0.05, n=5) and NA-stimulated RMSA (1.65 ± 0.25-fold, P <0.05, n=5, **Fig. 4.10A**). However, extracellular calcium removal had no significant effect on NA-stimulated [^{33}P]-PIP turnover in caveolae/rafts. [^{33}P]-PIP levels increased 17.8 ± 14.3% in controls and 16.5 ± 17.7% on extracellular calcium removal (P >0.4, n=5, **Fig. 4.11A**).

A



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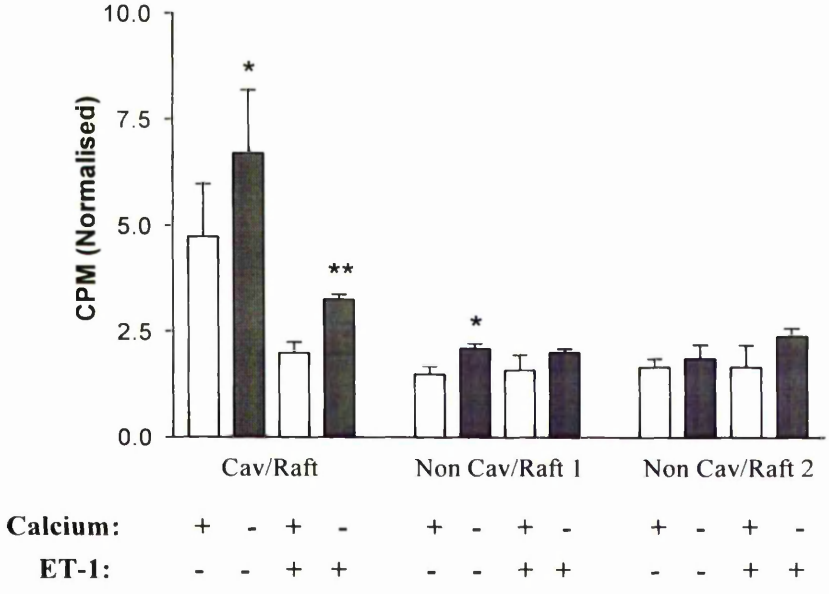


Fig. 4.8: Effect of extracellular calcium removal on [^{33}P]-PIP_2 levels of membrane fractions. [^{33}P]-labelled RMSA were incubated in the presence (open bars) or absence (closed bars) of extracellular calcium, stimulated with NA (15μM), ET-1 (100nM) or dH_2O (vehicle) for 20s, membrane fractions prepared and [^{33}P]-PIP_2 content analysed as described in chapter 2. **A)** NA: data are expressed as mean ± SEM counts per minute normalised for protein (n=5) **B)** ET-1: data are expressed as mean ± SEM counts per minute normalised for protein (n=3) (* P <0.05 ** P <0.01, t-test, calcium compared to calcium-free)

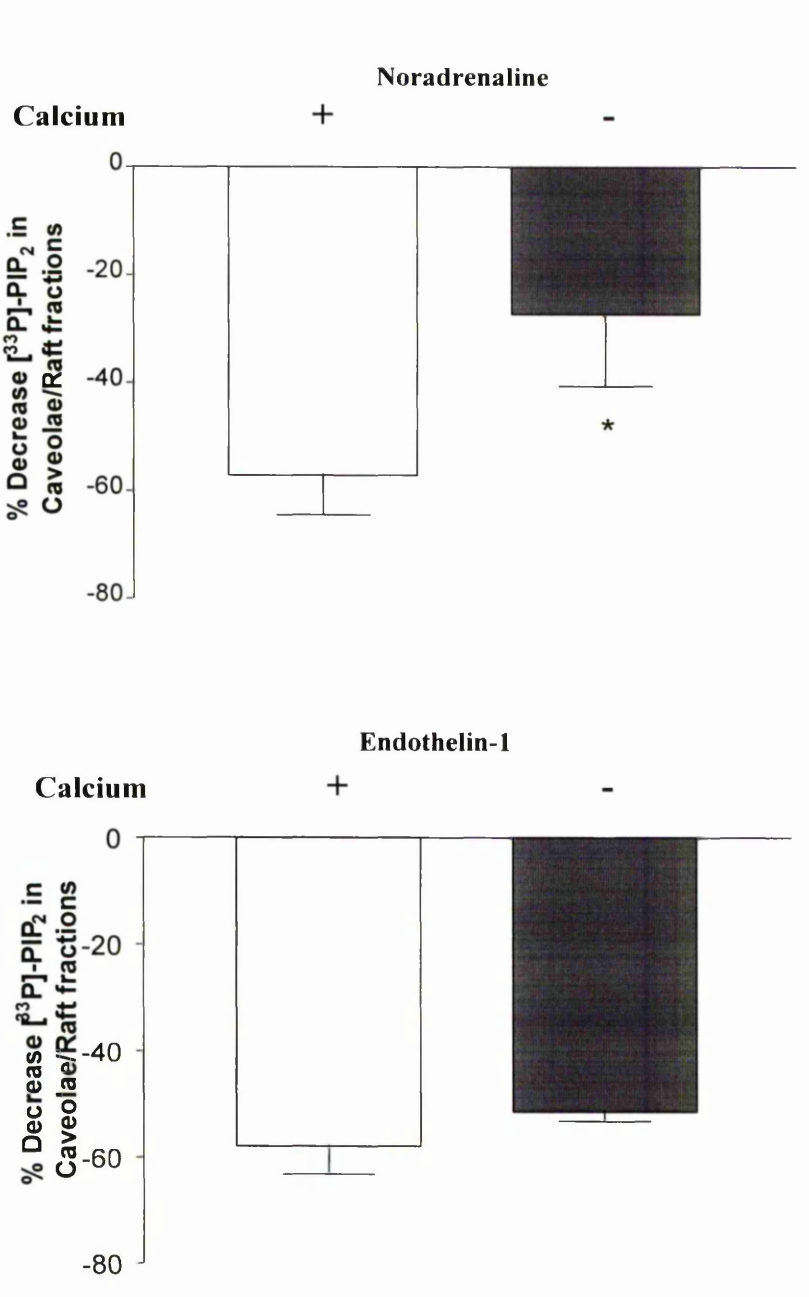
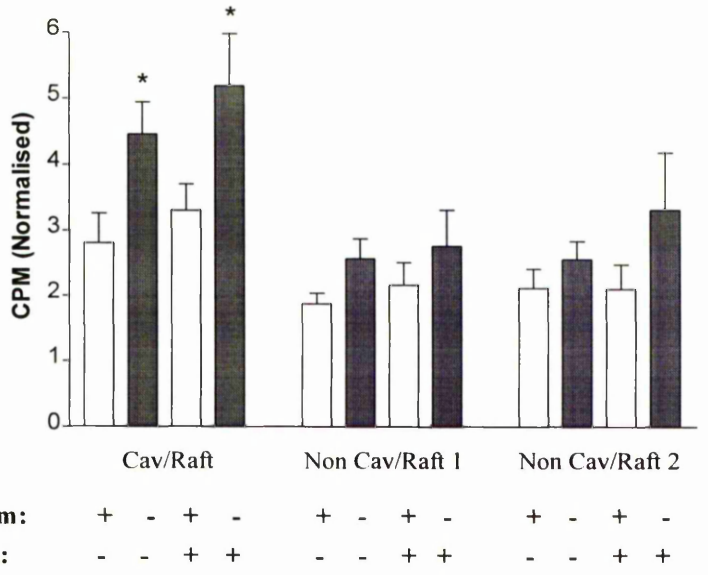


Fig. 4.9: Differential effect of extracellular calcium removal on NA- and ET-1-stimulated [^{33}P]-PIP$_2$ hydrolysis. [^{33}P]-labelled RMSA were incubated in the presence (open) or absence (closed bars) of extracellular calcium, stimulated with NA (15μM), ET-1 (100nM) or dH$_2$O (vehicle) for 20s, membrane fractions prepared and [^{33}P]-PIP$_2$ content analysed as described in chapter 2. **A)** NA: Data are expressed as mean ± SEM % decrease [^{33}P]-PIP$_2$ from basal levels of caveolae/rafts (n=5) **B)** ET-1: Data are expressed as mean ± SEM % decrease [^{33}P]-PIP$_2$ from basal levels of caveolae/rafts (n=3) (* P <0.05, t-test, calcium compared to calcium-free).

A



B

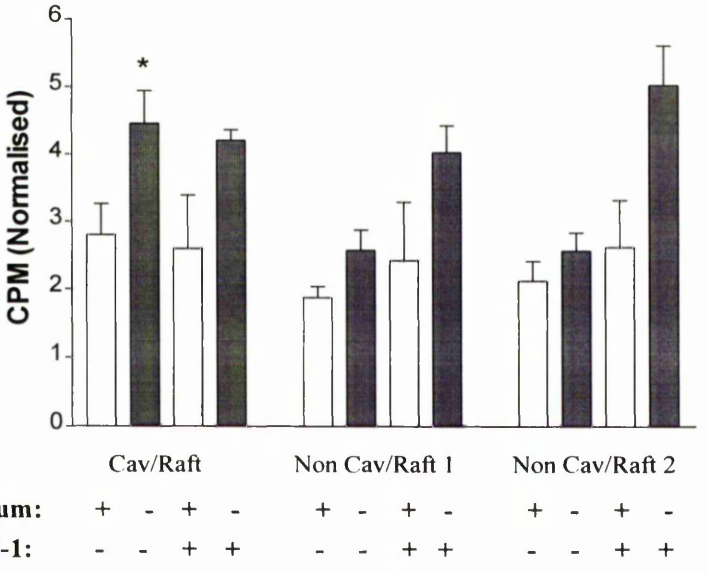
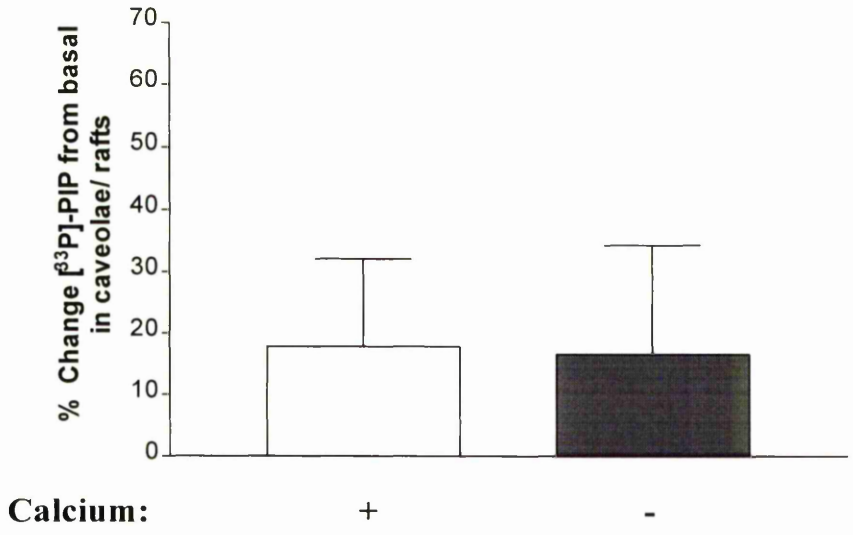


Fig. 4.10: Effect of extracellular calcium removal on [^{33}P]-PIP levels of membrane fractions. [^{33}P]-labelled RMSA were incubated in the presence (open bars) or absence (closed bars) of extracellular calcium, stimulated with NA (15μM), ET-1 (100nM) or dH_2O (vehicle) for 20s, membrane fractions prepared and [^{33}P]-PIP content analysed as described in chapter 2. **A)** NA: data expressed as mean ± SEM counts per minute normalised for protein (n=5) **B)** ET-1: data expressed as mean ± SEM counts per minute normalised for protein (n=3) (* P <0.05, t-test, calcium compared to calcium-free)

A



B

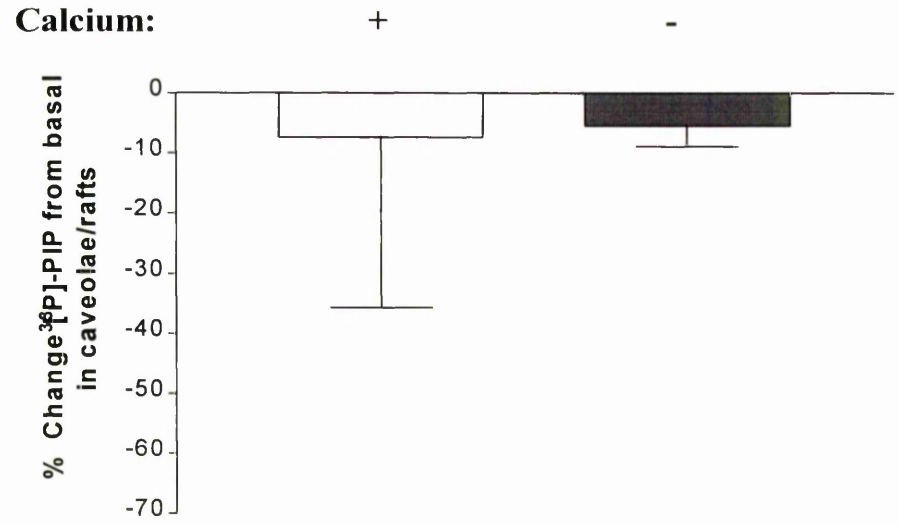


Fig. 4.11: Extracellular calcium removal has no effect on NA- or ET-1-stimulated [^{33}P]-PIP turnover. [^{33}P]-labelled RMSA were incubated in the presence (open) or absence (closed bars) of extracellular calcium, stimulated with NA (15μM), ET-1 (100nM) or dH_2O as vehicle for 20s, membrane fractions prepared and [^{33}P]-PIP content analysed as described in chapter 2. **A)** NA: Data are expressed as mean ± SEM % change [^{33}P]-PIP from basal levels of caveolae/rafts (n=5) **B)** ET-1: Data are expressed as mean ± SEM % change [^{33}P]-PIP from basal levels of caveolae/rafts (n=3).

3.4.4 Effect of extracellular calcium removal on ET-1-stimulated [^{33}P]-PIP turnover

[^{33}P]-labelled RMSA were incubated in calcium-free HEPES buffer containing 1mM EGTA for 10 min prior to stimulation with ET-1 (100nM) or dH_2O as vehicle for 20s, membrane fractions prepared and [^{33}P]-PIP content analysed as described in chapter 2. Whilst extracellular calcium removal increased [^{33}P]-PIP levels in caveolae/rafts of ET-1-stimulated RMSA (1.89 ± 0.46-fold), this was not statistically significant (P >0.05, n=3, **Fig. 4.10B**). Furthermore, no significant effect on ET-1-stimulated [^{33}P]-PIP turnover in caveolae/rafts was observed with levels decreasing 7.4 ± 28.3% in controls and 5.5 ± 3.4% on extracellular calcium removal (P >0.4, n=3, **Fig. 4.11B**).

3.5 Phospholipase C-γ_1

To investigate the presence of the PLC-γ_1 isoform, RMSA were homogenised in Na_2CO_3 and PLC-γ_1 content investigated by western blot as described in chapter 2 with 15 μg of total protein loaded. With anti-PLC-γ_1, two bands were visible in total RMSA extract: a band of approximately 102.6kD molecular weight (1) and a band of 74.1 kD molecular weight (2) as determined by use of a standard curve of molecular weight markers (**Fig. 4.12**).

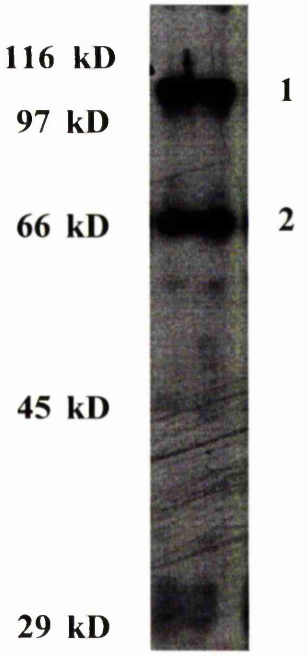


Fig. 4.12: Investigating the presence of PLC-γ_1 in RMSA. Unstimulated RMSA were homogenised in 0.5M Na_2CO_3 pH11 and PLC-γ_1 content analysed by immunoblot as described in chapter 2. 15µg protein was loaded. The PLC-γ_1 immunoblot shown is representative of three experiments

3.6 Localisation of cytoskeletal proteins

The presence of over 50% of [^{33}P]-PIP_2 in caveolae/rafts suggested they could be sites where PIP_2-mediated regulation of the cytoskeleton occurs. To investigate this, membrane fractions were prepared from unstimulated RMSA and protein content analysed for the cytoskeletal proteins paxillin, hic-5, spectrin and α-actinin by western blotting as described in chapter 2.

Anti-paxillin gave two bands (**Fig. 4.13**), an upper 70.6kD band and a lower 54.5 kD band. The lower band is likely to be the paxillin homologue hic-5, as the antibody is known to cross react with this protein (Matsuya *et al.*, 1998). Anti-hic-5 confirmed its presence in RMSA (**Fig. 4.14**) with a single 52.4kD band. Anti-spectrin gave a single 195.9kD band (**Fig. 4.15**). Anti-α-actinin (**Fig. 4.16**) gave a single 98.2kD band. Molecular weights were all calculated using a standard curve of molecular weight markers. Results indicated that paxillin, spectrin and α-actinin all localised to non-caveolae/raft fractions. In contrast, hic-5 had a broader distribution and was present in both caveolae/raft and non-caveolae/raft fractions.

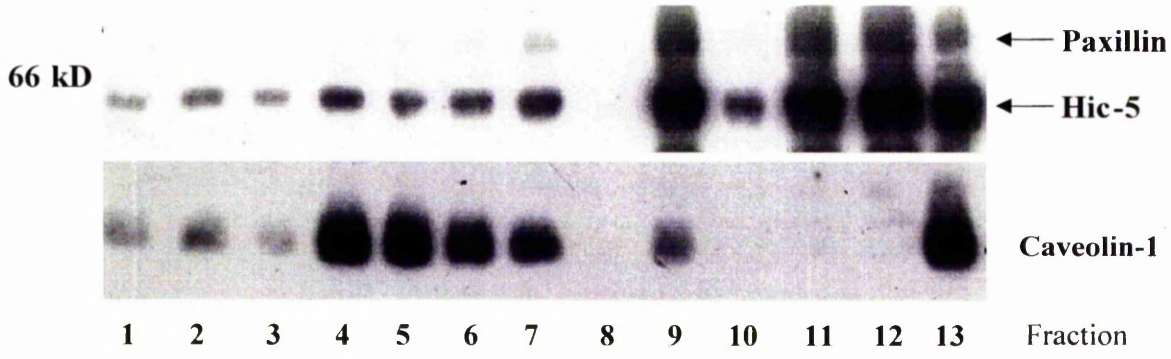


Fig. 4.13: Localisation of paxillin in membrane fractions. Caveolae/rafts were isolated from unstimulated RMSA and paxillin content analysed by immunoblot as described in chapter 2. The paxillin immunoblot shown is representative of three experiments with caveolin-1 distribution indicating caveolae/raft location.

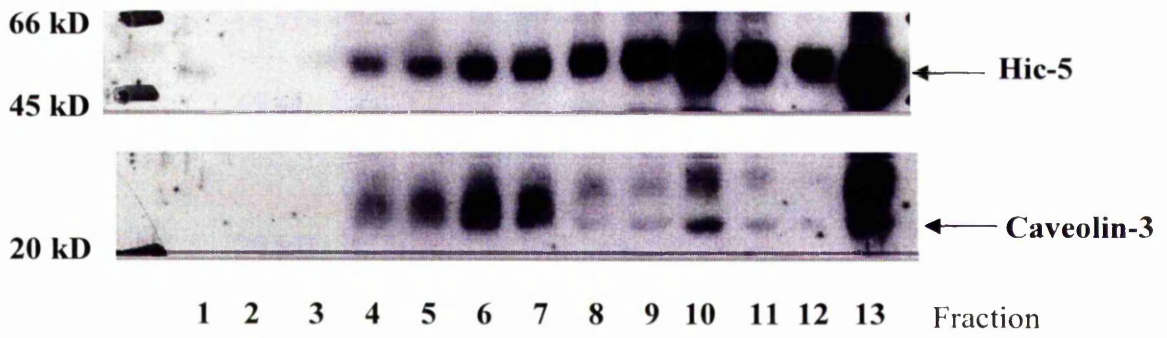


Fig. 4.14: Localisation of Hic-5 in membrane fractions. Caveolae/rafts were isolated from unstimulated RMSA and hic-5 content analysed by immunoblot as described in chapter 2. The hic-5 immunoblot shown is representative of three experiments with caveolin-3 distribution indicating caveolae/raft location.

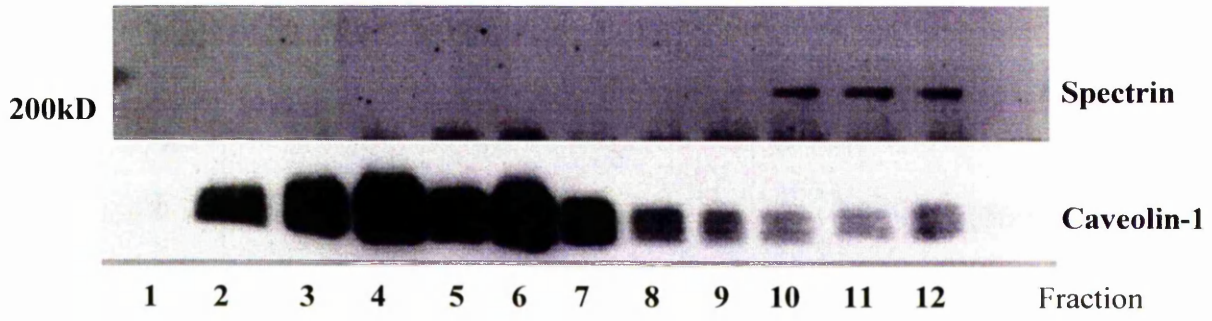


Fig. 4.15: Localisation of spectrin in membrane fractions. Caveolae/rafts were isolated from unstimulated RMSA and spectrin content analysed by immunoblot as described in chapter 2. The spectrin immunoblot shown is representative of three experiments with caveolin-1 distribution indicating caveolae/raft location.

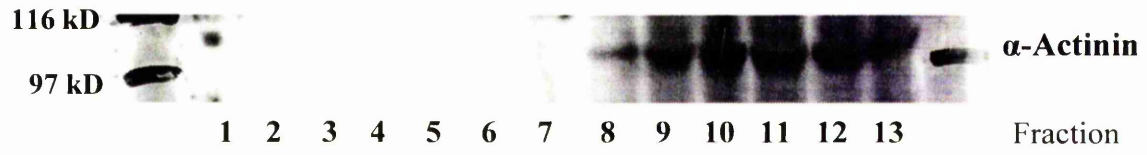


Fig. 4.16: Localisation of α-actinin in membrane fractions. Caveolae/rafts were isolated from unstimulated RMSA and α-actinin content analysed by immunoblot as described in chapter 2. The α-actinin immunoblot shown is representative of three experiments

4.0 Discussion

4.1 Isolation of PIP and PIP_2

The comparison of R_f values of ^{33}P-radiolabelled phospholipids to those of known lipid standards suggest that band 1 corresponds to [^{33}P]-PIP_2 and band 2 corresponds to [^{33}P]-PIP confirming their isolation from other ^{33}P-radiolabelled phospholipids in intact RMSA and in agreement with previous studies utilising this method (Wilson *et al.*, 1985;Gonzalez-Sastre & Folch-Pi, 1968).

4.2 Caveolae/rafts and PIP_2 turnover

In unstimulated RMSA, approximately 52% of [^{33}P]-PIP_2 was found in caveolae/raft domains (**Fig. 4.2**) agreeing with previous studies in cultured A431 cells (Waugh *et al.*, 1998;Hope & Pike, 1996). However, it is not clear if caveolae/rafts are PIP_2-enriched or if this reflects uniform PIP_2 distribution within the membrane as the proportion of plasma membrane comprising caveolae/rafts is not known. Previous work reporting that caveolae 'constitute a substantial proportion of the smooth muscle cell membrane' (Taggart, 2001) and are abundant in endothelial cells (Rizzo *et al.*, 2003) would favour a more uniform distribution of PIP_2. However, this is complicated by the suggested existence of additional PIP_2 pools within the plasma membrane inaccessible to radiolabelling (Yin & Janmey, 2003;Berridge, 1987). Thus, [^{33}P]-PIP_2 could reflect localisation of an agonist-sensitive PIP_2 pool rather than total PIP_2 distribution and consistent with this, caveolae/rafts were found to be the sole sites of [^{33}P]-PIP_2 hydrolysis in NA and ET-1-stimulated RMSA. This agrees with previous studies in agonist-stimulated T-cells, A431 and MDCK cells (Parmryd *et al.*, 2003;Hope & Pike, 1996;Pike & Casey, 1996) and correlates with the caveolae/raft localisation of the ET_A receptor in COS

cells (Chun *et al.*, 1994) and the α_1-adrenoreceptor in cardiomyocytes (Fujita *et al.*, 2001).

Both NA and ET-1 stimulated a significant decrease in [^{33}P]-PIP_2 levels of caveolae/rafts after 20s, consistent with rapid production of the downstream PIP_2 product IP_3 reported in response to these agonists in earlier studies (Ollerenshaw *et al.*, 1988;Resnik *et al.*, 1988). Results also indicate that, at the concentrations used, NA stimulated a larger decrease in PIP_2 than ET-1 and this could be a consequence of greater metabolism or slower production of PIP_2. Analysis of [^{33}P]-PIP levels (**Figs. 4.10 and 4.11**) found that NA and ET-1-stimulated PIP turnover was not significantly different. This suggests a greater NA-stimulated PIP_2 breakdown and, by extension, a greater NA-stimulated PLC activity in caveolae/rafts. This is consistent with the observed differential effects of NA and ET-1 on PLC-δ_1 as discussed later (**Fig. 4.6**).

With both agonists, [^{33}P]-PIP_2 levels in caveolae/rafts are still significantly decreased at later time points (NA 5 min, ET-1 10 min) suggesting sustained PIP_2 hydrolysis and, by extension, sustained IP_3 and DAG production. This is consistent with previous results in AngII-stimulated VSM cells reporting decreased PIP_2 levels at 5min (Griendling *et al.*, 1986). This is also supported by the observed increase in arachidonate-enriched DAG species in NA-stimulated RMSA at 5min (Ohanian *et al.*, 1990). However, these results are contrary to previous studies reporting a transient increase in IP_3 levels which implies short-term PIP_2 hydrolysis (Marsden *et al.*, 1989;Resnik *et al.*, 1988;Griendling *et al.*, 1986). One possible explanation for this discrepancy is that a transient increase in IP_3 could reflect increased IP_3

metabolism to inositol bisphosphate or phosphorylation to inositol-1,3,4,5-tetrakisphosphate (Pattni & Banting, 2004;Ollerenshaw *et al.*, 1988). An alternative explanation is that decreased [^{33}P]-PIP_2 levels at later time points do not reflect continued PIP_2 hydrolysis but are a result of decreased PIP_2 resynthesis. This could be due to decreased substrate availability for PIP_2-producing enzymes, particularly if enzyme activity is redirected towards PI and PIP as reported in AngII-stimulated VSM (Griendling *et al.*, 1986) and thrombin-stimulated platelets (Wilson *et al.*, 1985).

Another possibility is that [^{33}P]-PIP_2 levels may not reflect PLC-mediated PIP_2 hydrolysis alone as PIP_2 is also a substrate for PI 3-kinase (PI3K). Therefore, PI3K activity may also contribute to the decreased [^{33}P]-PIP_2 levels observed at the later time points for both NA and ET-1. Consistent with this, both PI3K and PI3K products have been localised to caveolae/rafts of agonist-stimulated platelets, T-cells and endothelial cells (Parmryd *et al.*, 2003;Bodin *et al.*, 2001;Liu *et al.*, 1997a) and both NA and ET-1 are reported to increase PI3K activity in VSM (Kawanabe *et al.*, 2004;Liu *et al.*, 2003;Walker *et al.*, 2001;Hu *et al.*, 1996). However, as PIP_3 was not isolated from other cellular phospholipids by the chromatographic separation used, this could not be investigated directly.

4.3 Consequences of localised PIP_2 hydrolysis

The localisation of PLC-mediated PIP_2 hydrolysis to caveolae/rafts could efficiently couple agonist-stimulated PI turnover to intracellular signalling pathways. IP_3, a product of PIP_2 hydrolysis, mobilises calcium by binding to its receptor on the sarcoplasmic reticulum (SR) (Ganitkevich *et al.*, 2002;Taggart, 2001) and the

sensitivity of these receptors is partly modulated by cytoplasmic calcium such that elevation of local calcium to 100 – 300 nM increases receptor sensitivity to IP_3 (Thrower *et al.*, 2001). In smooth muscle, caveolae are often found in close proximity to the SR (Moore *et al.*, 1993) and recently higher basal calcium was detected beneath caveolae relative to the plasma membrane (Isshiki *et al.*, 2002) suggesting that IP_3 receptors close to caveolae are sensitised and 'primed' for IP_3-induced calcium release (Isshiki & Anderson, 2003). Data in this chapter suggests that IP_3 would indeed be produced in caveolae/rafts, so facilitating calcium release from intracellular stores. Consistent with this, calcium waves originated in caveolin-rich membrane regions of endothelial cells stimulated by ATP and bradykinin, and were inhibited by a potent IP_3 receptor inhibitor (Isshiki *et al.*, 2002;Isshiki *et al.*, 1998).

IP_3 has also been implicated in calcium influx through capacitative calcium entry (CCE), the process by which depleted intracellular stores are refilled (Putney *et al.*, 2001). The IP_3 receptor is required for CCE in HEK cells (Ma *et al.*, 2000) and IP_3 was implicated in sustained VSM contraction as IP_3-mediated intracellular store depletion depolarised the membrane to allow calcium influx through voltage-dependent channels (McCarron *et al.*, 2002). Also, in smooth muscle, caveolae contain a plasma membrane IP_3 receptor-like protein (Fujimoto *et al.*, 1992) and caveolin-1 interacts and co-localises with TRPC1, TRPC3 and TRPC4, members of the transient receptor potential family of CCE channels (Bergdahl & Sward, 2004;Brazer *et al.*, 2003;Bergdahl *et al.*, 2003). Thus, locally produced IP_3 may bind to the IP_3 receptor-like protein of caveolae and initiate CCE through TRPC1. Consistent with this, caveolae were preferred sites of CCE in endothelial cells and

this was disrupted by cholesterol depletion (Isshiki *et al.*, 2002). Furthermore, cholesterol depletion of rat caudal artery reduced ET-1-stimulated CCE through delocalisation of TRPC1 and this correlated with decreased ET-1-stimulated contraction (Bergdahl *et al.*, 2003). In summary, these studies suggest that caveolae/rafts are important sites of agonist-stimulated calcium mobilisation and influx. As an increase in intracellular calcium is the prime modulator of VSM contraction (Ganitkevich *et al.*, 2002), the confinement of vasoconstrictor-stimulated PIP_2 hydrolysis to caveolae/rafts may be essential for rapid initiation of calcium mobilisation and development of agonist-induced contractile force.

DAG, the second product of PIP_2 hydrolysis, is the physiological activator of PKC (Kishimoto *et al.*, 1980) and localised production of DAG in caveolae/rafts, as previously shown in activated T-cells (Rouquette-Jazdanian *et al.*, 2002) and interleukin-stimulated fibroblasts (Liu & Anderson, 1995) could mediate activation of co-localised effectors. Consistent with this, previous studies report the presence of DAG-activated PKC isoforms in caveolae/rafts of unstimulated fibroblasts and on overexpression in COS-7 cells (Mineo *et al.*, 1998;Oka *et al.*, 1997). Furthermore, ET-1 stimulation of cardiomyocytes recruited the DAG-activated PKC-α and $-\varepsilon$ to caveolae although other regulatory factors are likely involved, as the DAG-activated PKC-δ was not recruited (Rybin *et al.*, 1999). Caveolae, through caveolin-1, have also been implicated in PKC regulation in cultured VSM cells as the loading of an inhibitory caveolin-1 peptide reduced PKC-dependent increases in contractility in response to α_1-adrenergic agonists (Je *et al.*, 2004) and prevented carbachol-stimulated redistribution of PKC to the membrane (Taggart *et al.*, 2000). As PKC has been implicated in calcium sensitisation and the contractile response in NA-

stimulated RMSA and ET-1-stimulated cultured VSM cells (Buus *et al.*, 1998;Ohanian *et al.*, 1996;Nishimura *et al.*, 1992) compartmentalisation of PIP_2 hydrolysis within caveolae/rafts may be an important step to facilitate activation of DAG-activated PKC isoforms and subsequent regulation of contractile force.

4.4 PLC-δ_1 in caveolae/rafts of RMSA

The localisation of PIP_2 hydrolysis to caveolae/rafts suggested the presence of active PLC isoforms within these domains. Immunoblot analysis of total RMSA extract with anti-PLC-δ_1 gave a visible band of approximately 84.1 kD molecular weight correlating with the reported molecular weight of PLC-δ_1 of 85 kD (Feng *et al.*, 1996) confirming its presence in RMSA in agreement with previous studies in rat tail artery and cultured VSM cells (LaBelle *et al.*, 2002;Ushio-Fakai *et al.*, 1998;Marrero *et al.*, 1994). The presence of a second band (approx. 48kD) could be a result of non-specific antibody binding or may reflect the presence of a truncated PLC-δ_1 product. Whilst this could be confirmed using blocking peptides to compete with the PLC-δ_1 antibody, this experiment was deemed unnecessary owing to the clarity of the primary 85 kD band corresponding to PLC-δ_1.

Data in this chapter indicates that NA but not ET-1 stimulated a rapid, transient translocation of PLC-δ_1 to caveolae/rafts. This suggests agonist-specific activation of PLC isoforms and is consistent with greater NA stimulation of PLC activity suggested by the greater NA-stimulated decrease in caveolae/raft [^{33}P]-PIP_2 levels at 20s (**Fig. 4.3**). This is also in agreement with a previous study in COS cells implicating PLC-δ_1 in α_1-adrenergic signalling (Chen *et al.*, 1996). The transient association of PLC-δ_1 with caveolae/rafts correlates with the difference in NA-

stimulated [^{33}P]-PIP_2 levels observed at 20s and 5 min. Thus, at 20s, PLC-δ_1 is present in caveolae/rafts and [^{33}P]-PIP_2 levels are lower than at 5min, when PLC-δ_1 has dissociated from caveolae/rafts. This suggests that PLC-δ_1 is functionally involved in PIP_2 hydrolysis in NA-stimulated RMSA.

The NA-stimulated translocation of PLC-δ_1 to caveolae/rafts was dependent on extracellular calcium. This corresponds with previous studies reporting calcium-dependent binding of PLC-δ_1 to *in vitro* liposomes (Pawelczyk & Matecki, 1999) and that bradykinin-stimulated translocation of overexpressed PLC-δ_1 is dependent on calcium influx (Kim *et al.*, 1999). Furthermore, agonist-stimulated translocation of PLC-δ_1 in cultured VSM cells is reported to depend on calcium influx (Murthy *et al.*, 2004). These results are also consistent with caveolae/rafts as preferred sites of CCE as reported in vascular endothelial cells (Isshiki *et al.*, 2002).

The functional importance of PLC-δ_1 in NA signalling is evident as extracellular calcium removal reduced PIP_2 hydrolysis in caveolae/rafts of NA-stimulated RMSA (**Fig. 4.9**). This is unlikely a consequence of altered PIP_2 production as NA-stimulated PIP turnover was unaffected (**Fig. 4.11**). As ET-1 had no effect on PLC-δ_1 distribution, this suggested a selective role for this isoform in NA signalling. This is further supported by extracellular calcium removal having no effect on ET-1-stimulated PIP_2 hydrolysis (**Fig. 4.9**) or PIP turnover (**Fig. 4.11**). However, the reason for this selectivity is unclear as both NA and ET-1 stimulate calcium influx in VSM (Nilsson *et al.*, 1994;Goto *et al.*, 1989) and this suggests other regulatory factors are involved. Multiple factors have been implicated in regulation of PLC-δ_1 activity. In expression studies, α_1-adrenoreceptors were shown to activate PLC-δ_1

through a high molecular weight G protein, G_h (Chen *et al.*, 1996). Therefore, differential regulation of PLC-δ_1 could reflect differential G_h-receptor coupling; for example, G_h is reported to couple to α_{1B}- and α_{1D}-adrenoreceptors but not the α_{1A}-adrenoreceptors. However, whilst *in vitro* regulation of PLC-δ_1 by G_h has been demonstrated, it is still unclear whether such regulation occurs *in vivo* (Nanda *et al.*, 2001;Murthy *et al.*, 1999).

It is also clear from results in this chapter that other PLC isoforms are involved in both NA and ET-1 responses as extracellular calcium removal did not abolish NA-stimulated [^{33}P]-PIP_2 hydrolysis completely and PLC-δ_1 did not appear to be activated by ET-1. A role for PLC-β isoforms in PIP_2 hydrolysis in RMSA is supported by previous work implicating PLC-β_2 in the NA response in rat-tail artery (LaBelle *et al.*, 2002) and also consistent with reports that PLC-β_1 and -β_3 were localised to caveolae/rafts of ventricular myocytes (Fujita *et al.*, 2001). Furthermore, PLC-β was detected in a multi-protein complex with caveolin-1 in human submandibular gland cells suggesting caveolae localisation (Lockwich *et al.*, 2001). Finally, PLC-γ_1 could also be involved in both NA and ET-1 responses as both agonists increase tyrosine phosphorylation in RMSA (Ohanian *et al.*, 1997;Ohanian *et al.*, 1990) and PLC-γ_1 was detected in caveolae/rafts of A431 cells (Jang *et al.*, 2001). However, experiments investigating the presence of PLC-γ_1 in RMSA by western blotting (**Fig. 4.12**) were not taken further owing to the presence of multiple bands that did not match the reported molecular weight of PLC-γ_1 (120-155 kD).

4.5 Effect of extracellular calcium removal on [^{33}P]-PIP_2 and [^{33}P]-PIP

Extracellular calcium removal significantly increased [^{33}P]-PIP_2 levels in caveolae/rafts of NA-stimulated RMSA consistent with the reduced [^{33}P]-PIP_2 hydrolysis observed. However, [^{33}P]-PIP_2 levels were also significantly increased in caveolae/rafts of unstimulated and ET-1-stimulated RMSA suggesting that calcium has other effects on the PI cycle. Consistent with this, a previous study in platelets reported calcium-dependent regulation of this pathway wherein decreased calcium enhanced turnover of the PI cycle (Lapetina *et al.*, 1981). As calcium influx is reported to be the major source of increased intracellular calcium in VSM (Ganitkevich *et al.*, 2002), this suggests that extracellular calcium removal would similarly enhance turnover of the PI cycle. As basal PIP_2 hydrolysis is low, this could account for the increased [^{33}P]-PIP_2 levels. Consistent with enhanced turnover of the PI cycle, [^{33}P]-PIP levels were also significantly increased in caveolae/rafts of unstimulated and NA-stimulated RMSA. This agrees with previous research reporting caveolae/rafts as sites of conversion of PI to PIP to PIP_2 (Waugh *et al.*, 2001). However, [^{33}P]-PIP levels were not significantly increased in ET-1-stimulated RMSA. Whilst this could be explained by agonist-specific activation of PIP kinases, there is no current evidence supporting this (Doughman *et al.*, 2003;Kanaho *et al.*, 2003). Furthermore, results indicated that NA and ET-1-stimulated [^{33}P]-PIP turnover were not significantly different in the presence or absence of extracellular calcium (**Fig. 4.11**) and so the reason for this difference is unclear.

4.6 PIP_2 and the cytoskeleton

In addition to being a source of second messengers, PIP_2 has also been implicated in regulation of the actin cytoskeleton (Yin & Janmey, 2003). The

cytoskeletal proteins α-actinin and spectrin directly bind PIP_2 with actin crosslinking activity of α-actinin enhanced by this and membrane association of spectrin determined by this interaction (Lemmon *et al.*, 2002;Sechi & Wehland, 2000). In contrast, paxillin and hic-5 are indirectly influenced by PIP_2, as membrane localisation of these proteins requires interaction with vinculin, which does require PIP_2 to bind to the membrane (Sechi & Wehland, 2000). As these proteins are all influenced by PIP_2, the localisation of [^{33}P]-PIP_2 to caveolae/rafts suggested that they may associate with these domains and this was investigated by immunoblotting. With anti-paxillin, the upper band matched the reported 68kD molecular weight of paxillin (Turner *et al.*, 1990) whilst the lower band is likely to be the paxillin homologue hic-5, as the antibody is known to cross react with this protein (Matsuya *et al.*, 1998). Anti-hic-5 confirmed its presence in RMSA with a single band corresponding to the 51-52kD molecular weight of hic-5 (Thomas *et al.*, 1999). Anti-spectrin gave a single band closely matching the reported 200-220kD molecular weight of spectrin (Prchal *et al.*, 1987) although the observed band was above the 200kD molecular weight marker (**Fig. 4.15**) suggesting a higher molecular weight than that calculated. Finally, anti-α-actinin (**Fig. 4.16**) gave a single band closely matching the 100kD molecular weight of α-actinin (Duhaiman & Bambury, 1984).

Of the four proteins, only hic-5 was associated with caveolae/rafts, although not exclusively, suggesting that these domains may regulate hic-5 function to some degree. However, further experiments with agonist-stimulation are needed to explore this in greater detail.

5.0 Summary and Conclusions

NA and ET-stimulated PIP_2 hydrolysis is confined to caveolae/rafts of intact RMSA and requires PLC-δ_1 in an extracellular calcium-dependent manner for NA but not for ET-1 indicating agonist-specific activation of PLC isoforms. The localisation of PIP_2 to caveolae/rafts could be important for efficient coupling of the second messengers IP_3 and DAG to co-localised effectors and downstream signalling cascades.

CHAPTER 5

Localisation of NA and ET-stimulated DGK Activity in Rat Mesenteric Small Arteries

1.0 Introduction

The localisation of PIP_2 hydrolysis to caveolae/rafts (**chapter 4**) suggested localised DAG production in these domains. However, previous work in RMSA reported that NA does not increase total DAG levels and ET-1 does not increase PI-derived DAG (Liu *et al.*, 1999a;Ohanian *et al.*, 1990). With NA, this is a result of increased membrane-associated DGK activity partly due to activation of DGKθ in a PI3K-dependent manner (Walker *et al.*, 2001;Ohanian & Heagerty, 1994) but the localisation of this activity is unknown. Furthermore, the effect of ET-1 on DGK activity in VSM is unknown. As DGK is a key enzyme in signalling transduction acting to terminate DAG signalling and initiate PA signalling, the presence of this activity in caveolae/rafts could be important for regulation of localised signalling cascades. Accordingly, using a mixed-micelle assay and cellular fractionation, the localisation of NA and ET-1-stimulated DGK activity in RMSA was investigated.

Previous work suggested that NA activates DGKθ by inducing association with PKB/Akt (Walker *et al.*, 2001) but the distribution of these proteins in VSM is unknown. Accordingly, using western blotting, the localisation of DGKθ and the PI3K target PKB/Akt was investigated in RMSA.

2.0 Methods

The protocols for the experiments detailed in this chapter are described in sections 2.1, 2.2, 2.3, 2.7, 2.9 and 2.10 of chapter 2.

3.0 Results

3.1 Effect of ET-1 on membrane-associated DGK activity

RMSA were stimulated with ET-1 (100nM) for a range of times from 0-10 min, membrane-associated DGK activity was extracted and assayed as described in chapter 2. ET-1 induced a rapid increase in membrane-associated DGK activity peaking at 2.02 ± 0.28-fold over basal activity after 5 min (P <0.03, n=5) and remaining significantly elevated after 10 min (P <0.02, n=5, **Fig. 5.1**).

3.2 Localisation of DGK activity

3.2.1 Resting distribution

To investigate localisation of DGK activity, caveolae/raft fractions were prepared from unstimulated RMSA, DGK activity was extracted and assayed as described in chapter 2. As protein content of membrane fractions was too low to be detected, data were normalised to total protein loaded on to sucrose gradients prior to isolation of membrane fractions. In unstimulated RMSA, 24 ± 2% of measurable DGK activity was localised to caveolae/raft domains with 30 ± 2% in non-caveolae/rafts 1 and 47 ± 4% in non-caveolae/rafts 2 (n=5, **Fig. 5.2 open bars**).

3.2.2 Effect of NA on caveolae/raft DGK activity

RMSA were stimulated with NA (15μM, 1min), caveolae/raft fractions prepared, DGK activity extracted and assayed as described in chapter 2. Stimulation time was chosen to reflect peak membrane-associated DGK activity in response to NA in RMSA (Ohanian & Heagerty, 1994). NA significantly increased DGK activity 1.59 ± 0.28-fold in caveolae/raft fractions (P >0.05, n=5) with no significant effect observed in non-caveolae/raft fractions (P >0.35, n=5, **Fig. 5.2 closed bars**).

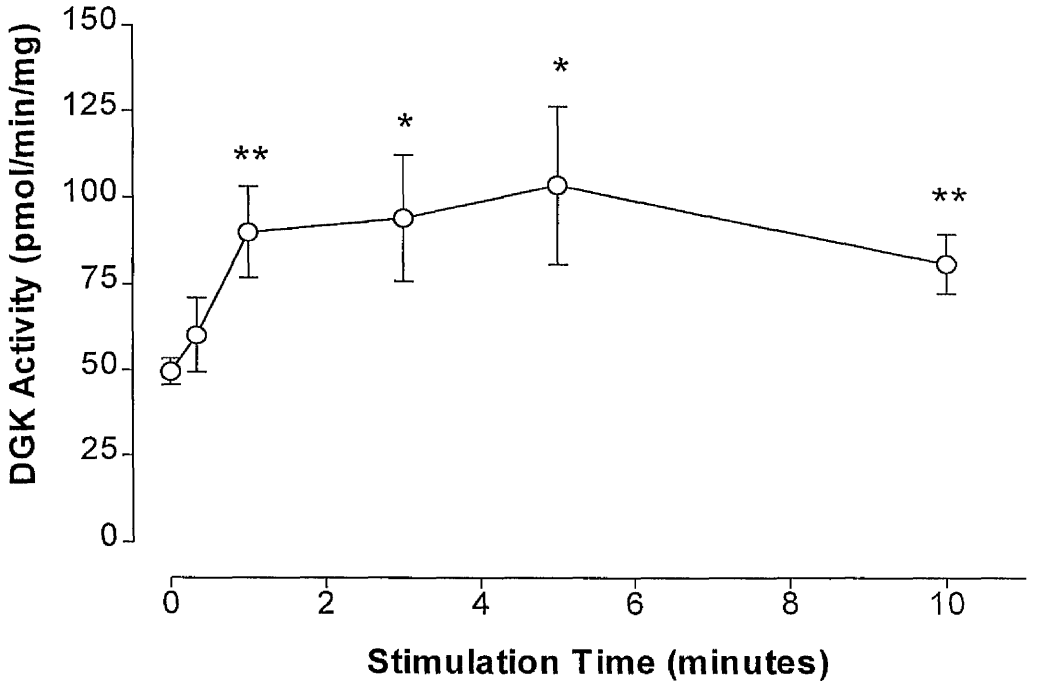


Fig. 5.1. ET-1 stimulation of membrane-associated DGK activity. RMSA were stimulated with ET-1 (100nM) for 0, 20s, 1, 3, 5 or 10 min, membrane-associated DGK activity extracted and assayed as described in chapter 2. Results are expressed as mean ± SEM pmol PA/min/mg protein. (* P <0.03, ** P <0.02, t-test, compared to basal, n=5)

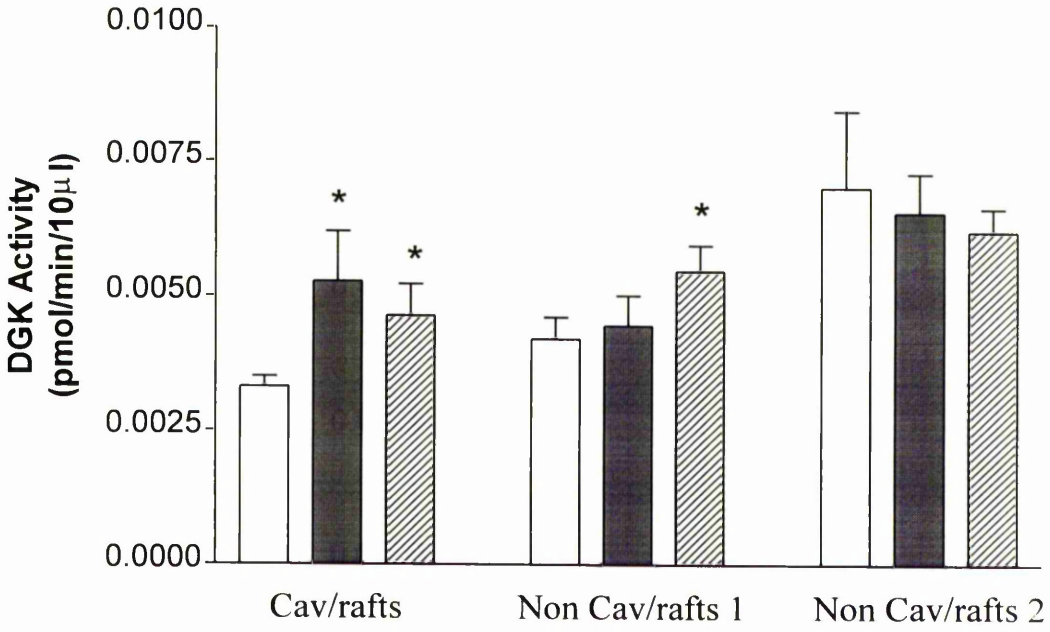


Fig. 5.2: Localisation of agonist-stimulated DGK activity in caveolae/raft fractions. RMSA were stimulated with NA (15µM, 1 min, closed bars), ET-1 (100nm, 5 min, hatched bars) or dH_2O (vehicle, open bars), membrane fractions prepared and pooled, and DGK activity extracted and assayed as described in chapter 2. Data are expressed as mean ± SEM pmol PA produced/min/10µl extract. (* P <0.05, t-test compared to basal, n=5).

3.2.3 Effect of ET-1 on caveolae/raft DGK activity

RMSA were stimulated with ET-1 (100nM, 5min), caveolae/raft fractions prepared, DGK activity extracted and assayed as described in chapter 2. Stimulation time was chosen to reflect peak membrane-associated DGK activity in response to ET-1 in RMSA (**section 3.1**). ET-1 significantly increased DGK activity 1.40 ± 0.18-fold in caveolae/raft fractions and 1.37 ± 0.12-fold in non-caveolae/rafts 1 (P <0.05, n=5) with no significant effect observed in non-caveolae/rafts 2 (P >0.30, n=5, **Fig. 5.2 hatched bars**).

3.2.4 Effect of sodium carbonate extraction on DGK activity

The use of high pH Na_2CO_3 for caveolae/raft isolation might adversely affect DGK activity. To investigate this, RMSA were stimulated with NA (15μM, 1min), ET-1 (100nM, 5min) or dH_2O (vehicle) and homogenised in 0.5M Na_2CO_3 pH 11, membrane-associated DGK activity extracted and assayed as described in chapter 2. Time points were chosen to reflect peak DGK activity in response to NA and ET-1 in RMSA (**section 3.1**) (Ohanian & Heagerty, 1994).

Extraction with high pH Na_2CO_3 dramatically reduced membrane-associated DGK activity (86.9 ± 3.7-fold). However, no significant effect was observed on the magnitude of NA and ET-1-stimulation of DGK activity (P >0.4, t-test, at least 4 experiments, **Fig. 5.3**). With Na_2CO_3, membrane-associated DGK activity was increased 1.80 ± 0.19- fold following NA stimulation (1.85 ± 0.19-fold in neutral extraction buffer) and 1.86 ± 0.28-fold following ET-1 stimulation (1.82 ± 0.32-fold in neutral extraction buffer).

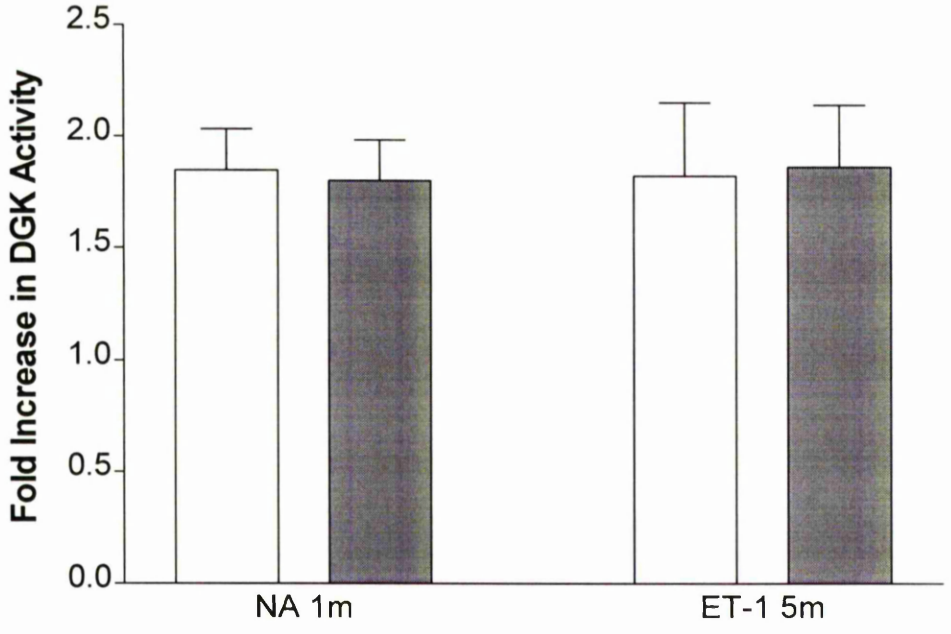


Fig. 5.3: Effect of sodium carbonate extraction on NA and ET stimulated DGK activity. RMSA were stimulated with NA (15μM, 1 min) or ET-1 (100nM, 5 min), membrane-associated DGK activity extracted with neutral homogenisation buffer (open bars) or 0.5M Na_2CO_3 pH 11 (closed bars) and assayed as described in chapter 2. Results are expressed as mean ± SEM fold-increase in DGK activity and are the result of at least four separate experiments.

3.3 Effect of extracellular calcium removal on NA and ET-1-stimulated DGK activity

To determine if calcium influx is required for NA and ET-1 stimulation of DGK activity, RMSA were incubated in calcium-free HEPES buffer containing 1mM EGTA for 10 min prior to stimulation with NA (15μM, 1min), ET-1 (100nM, 5min) or dH_2O (vehicle), membrane-associated DGK activity extracted and assayed as described in chapter 2. Time points were chosen to reflect peak DGK activity in response to NA and ET-1 in RMSA (**section 3.1**) (Ohanian & Heagerty, 1994). Extracellular calcium removal had no significant effect on basal DGK activity at either time point (P >0.2, at least four experiments). Similarly, no significant effect was observed on peak NA-stimulated DGK activity (P >0.2, n=5, **Fig. 5.4A**) or peak ET-1-stimulated DGK activity (P >0.4, n=5, **Fig. 5.4B**).

3.4 Effect of PI3K inhibition of DGK activity

To determine the effect of PI3K inhibition on DGK activity in RMSA, the PI3K inhibitor LY294002 was used. RMSA were preincubated in 10μM LY294002 prior to stimulation. This concentration of LY294002 was sufficient to inhibit NA-stimulated phosphorylation of the downstream PI3K target PKB/Akt in RMSA (Walker *et al.*, 2001).

3.4.1 NA-stimulated DGK activity

RMSA were incubated in 10μM LY294002 for 1 hour prior to stimulation with NA (15μM), membrane-associated DGK activity extracted and assayed as described in chapter 2. Stimulation time was chosen to reflect peak DGK activity in response to NA in RMSA (Ohanian & Heagerty, 1994). PI3K inhibition had no

significant effect on basal DGK activity (P >0.15, t-test) but significantly reduced NA-stimulation of DGK activity by 76.3 ± 3.5% (P <0.01, n=5, **Fig. 5.5**) although DGK activity was still significantly increased over basal (1.40 ± 0.14-fold, P <0.05, n=6).

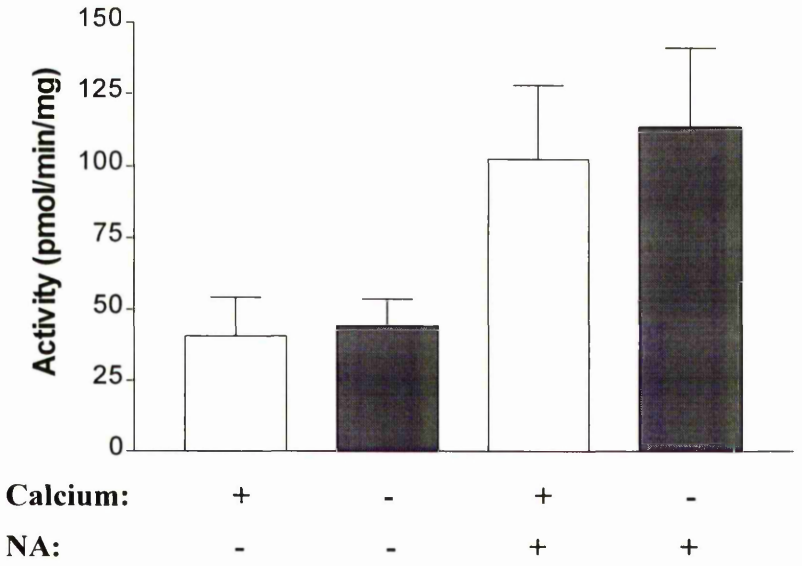
3.4.2 ET-1-stimulated DGK activity

To determine if ET-1-stimulated DGK activity is PI3K-dependent, RMSA were incubated in 10μM LY294002 for 1 hour prior to stimulation with NA (15μM), membrane-associated DGK activity extracted and assayed as described in chapter 2. To investigate if PI3K is required solely for early stimulation of DGK activity, stimulation times of both 1min (early) and 5min (peak ET-1 DGK activity) were investigated. PI3K inhibition had no significant effect on basal DGK activity at either time point (P >0.4, at least four experiments). Similarly, PI3K inhibition had no effect on ET-1-stimulated DGK activity at 1min (P >0.4, n=5, **Fig. 5.6A**) or at 5min (P >0.25, n=4, **Fig. 5.6B**).

3.4.3 Localisation of PI3K-dependent DGK activity

To determine localisation of the PI3K-dependent DGK activity, RMSA were incubated in 10μM LY294002 for 1 hour prior to stimulation with NA (15μM, 1min), membrane fractions prepared, DGK activity extracted and assayed as described in chapter 2. Stimulation time was chosen to reflect peak DGK activity in response to NA in RMSA (Ohanian & Heagerty, 1994). PI3K inhibition significantly reduced NA-stimulated DGK activity in caveolae/rafts (P <0.05, n=4) with no significant effect on DGK activity in non-caveolae/raft fractions (P >0.15, n=4, **Fig. 5.7 hatched bars**).

A



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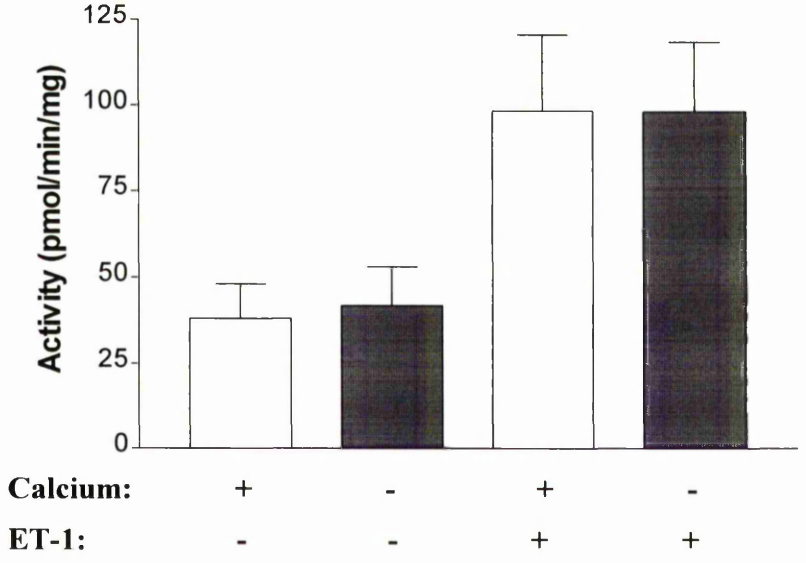


Fig. 5.4. Effect of extracellular calcium removal on membrane-associated DGK activity. RMSA were incubated in the presence (open bars) or absence (closed bars) of extracellular calcium for 10 min prior to stimulation with **A)** NA (15μM, 1 min) **B)** ET-1 (100nM, 5 min), membrane-associated DGK activity extracted and assayed as described in chapter 2. Results are expressed as mean ± SEM pmol PA/min/mg protein and are the result of at least four separate experiments.

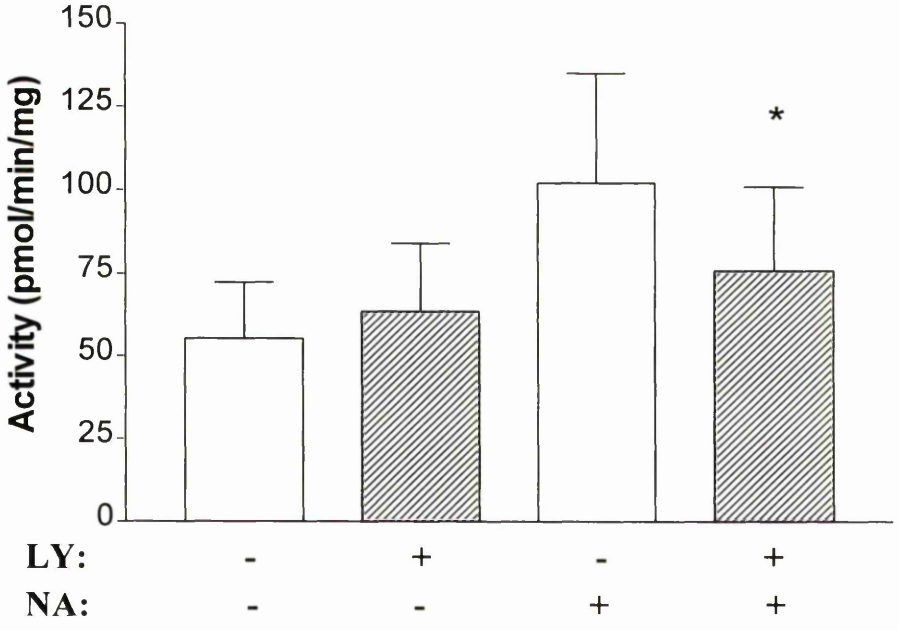
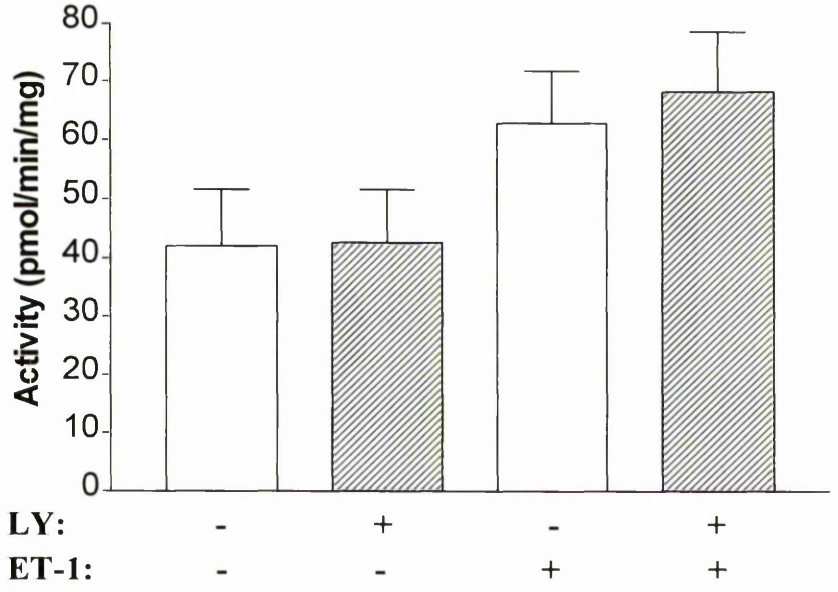


Fig. 5.5. Effect of PI3K inhibition on NA-stimulated DGK activity. RMSA were incubated in the presence (hatched bars) or absence (open bars) of 10μM LY294002 for 1 hour prior to stimulation with NA (15μM, 1 min), membrane-associated DGK activity extracted and assayed as described in chapter 2. Results are expressed as mean ± SEM pmol PA/min/mg protein.

(* P <0.02, t-test, NA compared to LY+NA, n=6).

A



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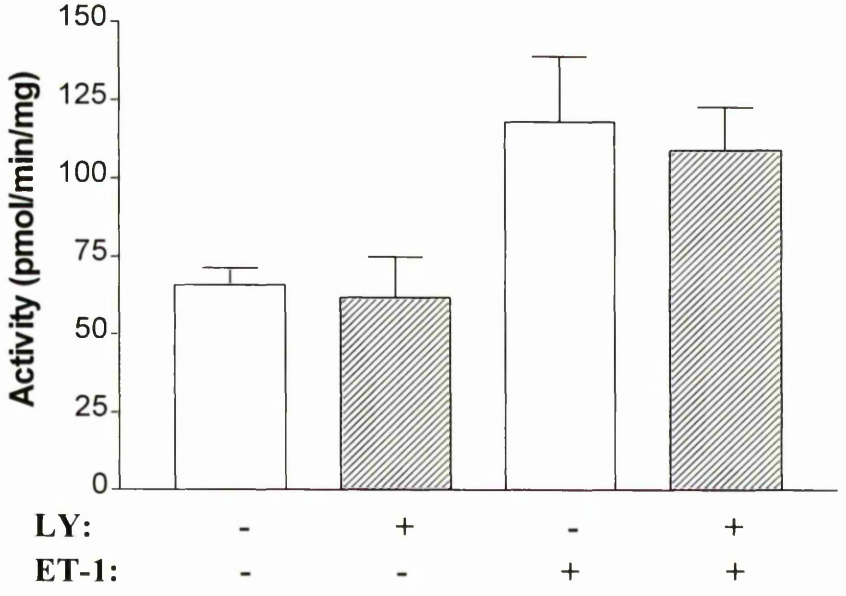


Fig. 5.6. Effect of PI3K inhibition on ET-1-stimulated DGK activity. RMSA were incubated in the presence (hatched bars) or absence (open bars) of 10μM LY294002 for 1 hour prior to stimulation with ET-1 (100nM) for **A)** 1 min or **B)** 5 min, membrane-associated DGK activity extracted and assayed as described in chapter 2. Results are expressed as mean ± SEM pmol PA/min/mg protein and are the result of at least four separate experiments.

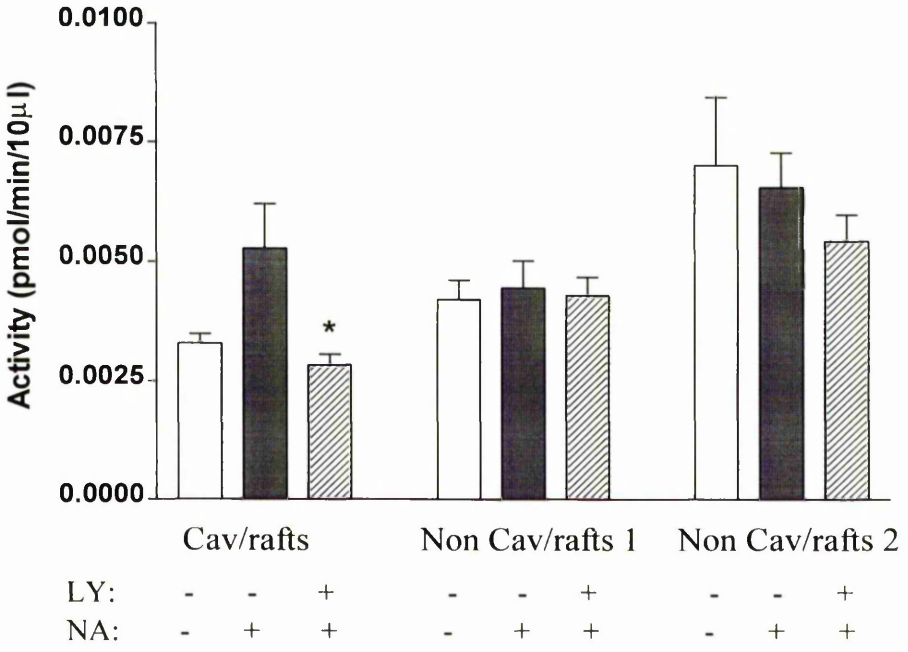


Fig. 5.7: Localisation of PI3K-dependent DGK activity RMSA were incubated in the presence (hatched bars) or absence (open bars) of 10µM LY294002 for 1 hour prior to stimulation with dH_2O (vehicle, open bars) or NA (15µM, 1min), caveolae/rafts fractions prepared and pooled, and DGK activity extracted and assayed as described in chapter 2. Results are expressed as mean ± SEM pmol PA/min/10µl extract (* P <0.05, t-test, NA compared to LY+NA, at least four experiments).

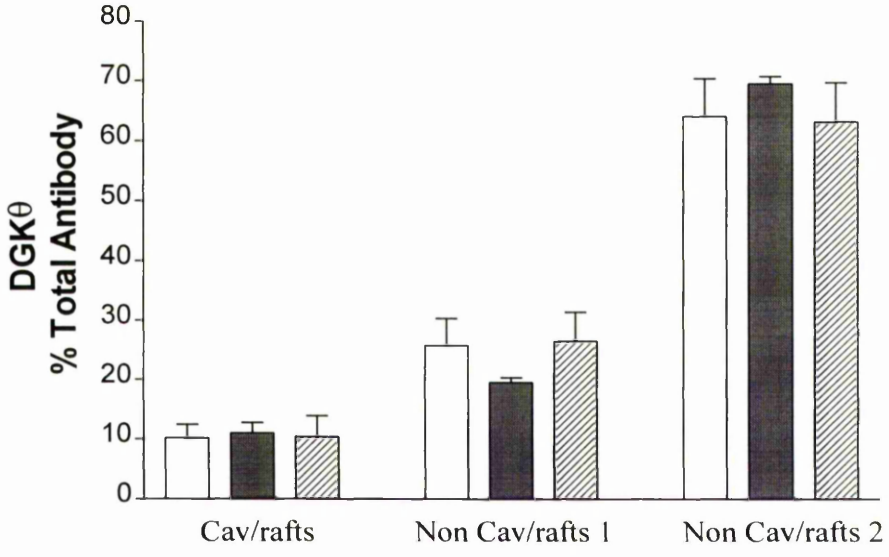
3.5 DGKθ distribution in RMSA

To determine localisation of the DGKθ isoform, RMSA were stimulated with NA (15μM, 1min), ET-1 (100nM, 5min) or dH_2O (vehicle), membrane fractions prepared and pooled, and DGKθ content analysed by immunoblot as described in chapter 2. Time points were chosen to reflect peak DGK activity in response to these agonists in RMSA (**section 3.1**) (Ohanian & Heagerty, 1994). In unstimulated arteries, 10 ± 2% of DGKθ localised to caveolae/raft fractions with 26 ± 5% in non-caveolae/rafts 1 and 64 ± 6% in non-caveolae/rafts 2 (**Fig. 5.8 open bars**). NA stimulation had no significant effect on DGKθ levels in caveolae/raft levels (11 ± 2%, P >0.4, n=4, **Fig. 5.8 closed bars**). Similarly, ET-1 stimulation had no significant effect on DGKθ levels in caveolae/rafts (10 ± 4%, P >0.4, n=4, **Fig. 5.8 hatched bars**). Also, neither NA nor ET-1 had a significant effect on DGKθ levels in either of the non-caveolae/raft fractions (P >0.1, t-test, n=4).

3.6 PKB/Akt distribution in RMSA

To investigate localisation of PKB/Akt, RMSA were stimulated with NA (15μM, 1min), ET-1 (100nM, 5min) or dH_2O (vehicle), membrane fractions prepared and pooled, and PKB/Akt content analysed by immunoblot as described in chapter 2. Time points were chosen to reflect peak DGK activity in response to these agonists in RMSA (**section 3.1**) (Ohanian & Heagerty, 1994).

A



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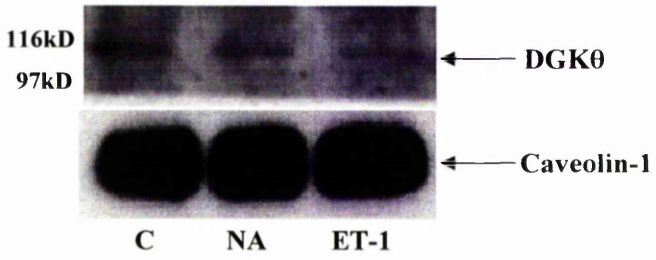
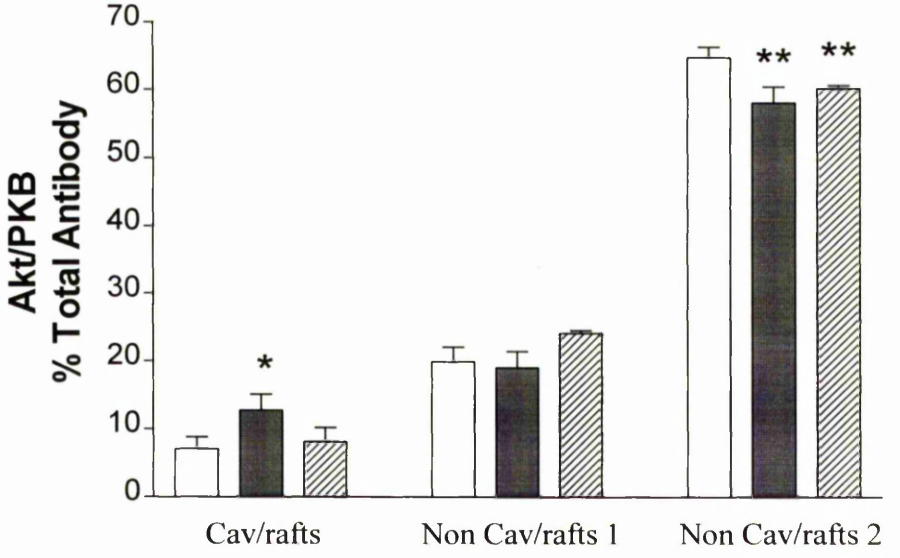


Fig. 5.8: Distribution of DGKθ in membrane fractions. RMSA were stimulated with NA (15μM, 1 min, closed bars), ET-1 (100nM, 5 min, hatched bars) or dH_2O (vehicle, open bars), membrane fractions prepared and pooled, and DGKθ content analysed by immunoblot as described in chapter 2. **A)** Densitometric data of the DGKθ signal in caveolae/rafts expressed as mean ± SEM % total DGKθ (n=4). **B)** Representative DGKθ immunoblot shown with caveolin-1 indicating caveolae enrichment in the membrane fractions.

A



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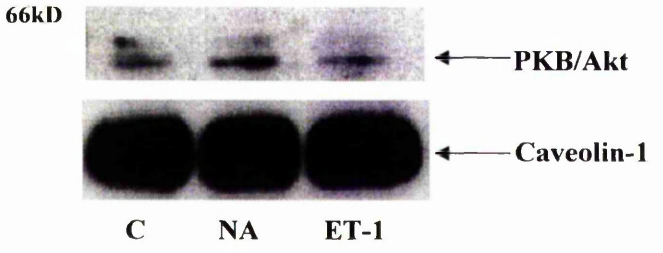


Fig. 5.9: Localisation of PKB/Akt in membrane fractions. RMSA were stimulated with NA (15μM, 1 min, closed bars), ET-1 (100nM, 5 min, hatched bars) or dH_2O (vehicle, open bars), membrane fractions prepared and pooled, and PKB/Akt content analysed by immunoblot as described in chapter 2. **A)** Densitometric data of the PKB/Akt signal in caveolae/rafts expressed as mean ± SEM % total PKB/Akt (*P <0.05 ** P <0.02, t-test, compared to basal, n=4). **B)** Representative PKB/Akt blot shown with caveolin-1 indicating caveolae enrichment in the membrane fractions.

In unstimulated arteries, 7.0 ± 1.7% of PKB/Akt localised to caveolae/raft fractions with 19.9 ± 2.2% in non-caveolae/rafts 1 and 64.8 ± 1.6% in non-caveolae/rafts 2 (**Fig. 5.9 open bars**). NA stimulation significantly increased PKB/Akt levels of caveolae/rafts to 12.7 ± 2.4% (P <0.05, n=4) and significantly reduced PKB/Akt levels in non-caveolae/rafts 2 to 58.2 ± 2.3% (P <0.03, n=4, **Fig. 5.9 closed bars**). By contrast, ET-1 stimulation had no significant effect on PKB/Akt levels in caveolae/rafts (8.1 ± 2.1%, P >0.3, n=4) but did significantly reduce PKB/Akt levels of non-caveolae/rafts 2 to 60.0 ± 0.5% (P <0.03, n=4, **Fig. 5.9 hatched bars**).

4.0 Discussion

4.1 ET-1 stimulation of DGK activity

In RMSA, ET-1 stimulated a rapid (1 min) increase in membrane-associated DGK activity peaking at 5 min and remaining elevated for up to 10 minutes. As far as is known, this is the first investigation of the effects of ET-1 on DGK activity and correlates with a previous study in RMSA reporting that PI-derived DAG does not accumulate in response to ET-1 (Liu *et al.*, 1999a). This suggests that DGK is a major pathway of metabolism for PI-derived DAG in RMSA, consistent with previous results in fibroblasts where PI-derived DAG was preferentially metabolised by DGK (Florin-Christensen *et al.*, 1992). However, this is contrary to results in cultured VSM cells were DAG lipase was the predominant metabolic pathway for both endogenous and exogenous DAG (Chuang & Severson, 1998;Chuang *et al.*, 1990) although this could reflect differences between cultured cells and intact tissue. Furthermore, the latter experiments were in unstimulated conditions, which could also account for the observed differences.

The effect of ET-1 on membrane-associated DGK activity contrasts to that of NA previously reported, which stimulates a more rapid (20s) transient increase in DGK activity peaking at 1 min and returning to basal by 3 min (Ohanian & Heagerty, 1994). Although DGK activity was stimulated to a comparable magnitude, the temporal differences in NA and ET-1-stimulated DGK activity suggest agonist-specific regulation of DGK activity. Differences in DGK activation were previously reported between NA and AngII (Ohanian & Heagerty, 1994) and the further differences between these agonists and ET-1 lends support to the theory that differential regulation of DGK is important in the specificity of contractile response in RMSA (Ohanian & Ohanian, 2001;Ohanian & Heagerty, 1994;Ohanian *et al.*, 1993). Such agonist-specific regulation of DGK could have important consequences for downstream signalling cascades by having distinct effects on the levels of the two lipid second messengers DAG and PA.

4.2 Localisation of DGK activity

In unstimulated RMSA, approximately 24% of measured DGK activity was localised to caveolae/rafts, which suggests that they are not major sites of basal DGK activity. However, owing to the small amounts of starting material used, the protein content of these fractions was too low to be measured by Bradford assay although attempts were made. As previous studies in cultured cells have reported that protein content of the caveolae/raft fractions are significantly lower than either non-caveolae/raft fractions (Song *et al.*, 1996;Smart *et al.*, 1995;Lisanti *et al.*, 1994b) this would consequently suggest that caveolae/rafts contain relatively high DGK activity in unstimulated RMSA. This disagrees with a previous study in MDCK cells localising DGK activity to non-caveolae/raft fractions (Hope & Pike, 1996), which

could reflect tissue-specific differences, or differences between cultured cells and intact tissues. Additionally, the previous study utilised detergent separation of caveolae/rafts, which could have adversely affected protein composition of the isolated caveolae/rafts (Foster *et al.*, 2003).

Both NA and ET-1 stimulated DGK activity within caveolae/rafts, consistent with these domains as sites of localised DAG production as suggested by results in chapter 3. Whilst the use of high pH Na_2CO_3 extraction did significantly reduce DGK activity, both NA and ET-1 stimulated comparable rises in DGK activity when extracted with either high pH Na_2CO_3 or neutral homogenisation buffer. This suggests that agonist-stimulation of DGK isoforms remains intact and this is an accurate reflection of the membrane localisation of agonist-stimulated DGK activity. ET-1 also increased DGK activity in non-caveolae/raft domains. However, it cannot be determined from these results if the non-caveolae/raft DGK activity is at the plasma membrane or the intracellular organelles as DGKs have also been localised to the nucleus (Luo *et al.*, 2004;Ohanian & Ohanian, 2001). Furthermore, it is not clear if this is due to activation of a spatially distinct DGK pathway or represents translocation of an active DGK isoform from caveolae/rafts to non-caveolae/rafts. Results in chapter 3 suggesting localised DAG production solely in caveolae/rafts would favour the latter theory as previous research reported DGK activation solely at sites of receptor-stimulated DAG production (Van der Bend *et al.*, 1994). However, it is also possible that DAG substrate may diffuse away from the site of production.

The localisation of DGK activity to caveolae/rafts would have functional consequences for local signalling cascades. As discussed in chapter 3, a number of

studies have reported the presence of DAG-activated classical and novel PKC isoforms within caveolae/rafts and the co-localisation of DGK activity would function to antagonise this activation. Such antagonism of DAG signalling would be agonist-specific as evidenced by the different temporal effects of NA and ET-1 on DGK activity. Thus, with NA, this would be transient as DGK levels return to basal and PI-DAG is allowed to accumulate as previously reported in RMSA (Ohanian & Heagerty, 1994;Ohanian *et al.*, 1990). In contrast, with ET-1, this antagonism would be sustained, as DGK activity remains significantly elevated for up to 10 minutes. The spatial differences in DGK activation by NA and ET-1 may also play a role in signalling specificity by allowing the differential regulation of compartmentalised signalling cascades localised to caveolae/rafts and non-caveolae/rafts.

In addition to terminating DAG signalling, activation of DGK results in production of the second messenger PA and could activate PA-regulated signalling cascades – this will be discussed further in chapter 5.

4.3 Extracellular calcium removal on DGK activity

Of the nine cloned DGK isoforms, the type 1 DGK subtype (α, β, γ) are characterised by the presence of calcium-binding EF-hand motifs with studies indicating calcium binding to these motifs (Yamada *et al.*, 1997). Furthermore, the presence of a domain homologous to that of the calcium sensor recoverin in these isoforms has been reported. This domain acts in concert with the EF-hands to function as an autoinhibitory domain (Jiang *et al.*, 2000a). These studies suggest calcium regulation of the type 1 DGK isoforms and this has been shown to occur *in vitro* with DGK-α (Sakane *et al.*, 1991). Results in this chapter indicate that neither

NA- nor ET-1-stimulated DGK activity was affected by extracellular calcium removal suggesting that the type 1 DGK isoforms are not involved in the NA and ET-1 response in RMSA. This is contrary to results in NA-stimulated rat aorta (Nobe *et al.*, 2002) and activated T-lymphocytes (Sanjuan *et al.*, 2001) where the type 1 DGK inhibitors R59949 and R59022 (Jiang *et al.*, 2000b) inhibited agonist-stimulated DGK activity. This could reflect tissue-specific differences. Another possibility is that the inhibitors used in the latter studies had non-specific inhibitory effects. Alternatively, calcium released from intracellular stores may be sufficient for activation of these DGK isoforms in RMSA. However, studies have suggested that this contributes very little to the NA and ET-1-stimulated increase in cellular calcium in VSM (Nilsson *et al.*, 1994;Goto *et al.*, 1989). Finally, it is possible that the type 1 DGK isoforms are not regulated by calcium *in vivo*.

4.4 PI3K inhibition on DGK activity

Inhibition of PI3K significantly reduced NA-stimulated DGK activity. This is consistent with the PI3K-dependent activation of DGKθ in NA-stimulated RMSA as previously reported (Walker *et al.*, 2001). However, PI3K inhibition had no effect on peak ET-1-stimulated DGK activity suggesting that DGKθ is not involved in the ET-1 response. Furthermore, PI3K inhibition had no effect on DGK activity following a 1min ET-1 stimulation indicating that DGKθ is not activated early and transiently as with NA. As both NA and ET-1 are reported to activate PI3K in VSM (Miao *et al.*, 2002; Hu *et al.*, 1996), this suggests that ET-1 may not induce translocation of DGKθ to the plasma membrane, as has been previously shown with AngII in RMSA, whereas NA can (Walker *et al.*, 2001). Alternatively, PI3K-independent activation pathways for this isoform may exist.

4.5 DGKθ and PKB/Akt Distribution

Further experiments indicated that the PI3K-dependent DGK activity stimulated by NA localised to caveolae/raft domains suggesting the presence of DGKθ. Western blotting with anti-DGKθ gave a single band of approximately 107.9 kD molecular weight as calculated from a standard curve and correlating with the reported 110kD molecular weight of this isoform (Houssa *et al.*, 1997). Results confirmed the presence of DGKθ in caveolae/rafts. However, neither NA nor ET-1 induced a significant translocation of DGKθ to these domains suggesting that NA activates DGKθ pre-localised to caveolae/rafts. This is consistent with previous studies reporting localisation of PI3K and PI3K products to caveolae/rafts of platelets (Bodin *et al.*, 2001), T-cells (Parmryd *et al.*, 2003) and Vero cells (Peres *et al.*, 2003) and also agrees with a previous study in NA-stimulated RMSA indicating that activation but not translocation of DGKθ was PI3K-dependent (Walker *et al.*, 2001).

Although PI3K inhibition did not completely abolish membrane-associated DGK activity in response to NA, the sole NA-stimulated DGK activity localised to caveolae/rafts was completely inhibited. One possible reason for this discrepancy could be due to experimental difficulties in obtaining the complete non-caveolae/raft membrane pellet resulting in loss of DGK activity in the non-caveolae/raft 2 fractions. Alternatively, the low levels of DGK activity following high pH sodium carbonate extraction may have made it more difficult for a smaller increase in non-caveolae/raft DGK activity to be observed.

Western blotting with anti-PKB/Akt gave a band of approximately 58.3kD molecular weight as calculated by use of a standard curve and correlating with the reported

PKB/Akt molecular weight of 56kD (Jones *et al.*, 1991). The downstream PI3K target PKB/Akt was also present in caveolae/raft domains in agreement with a previous study in CD3-activated T-cells (Bauer *et al.*, 2003) and results indicated that NA stimulated a significant translocation of PKB/Akt to caveolae/rafts with a coincident decrease in PKB/Akt levels of non-caveolae/raft domains. This correlates with previous results in RMSA showing that NA increased PKB/Akt-associated DGK activity (Walker *et al.*, 2001) and suggests an NA-induced association of PKB/Akt occurring in caveolae/rafts. Whilst ET-1 did not significantly increase PKB/Akt in caveolae/raft domains, a significant decrease of PKB/Akt was observed in the non-caveolae/raft 2 fractions. However, this could be explained by ET-1 inducing a more general redistribution of PKB/Akt rather than the specific caveolae/raft translocation stimulated by NA. These results suggest that the agonist-specific activation of DGKθ could be due to the differential translocation of PKB/Akt to caveolae/rafts. Thus, NA induces association of PKB/Akt and DGKθ but ET-1 does not. However, as previous studies have shown that both NA and ET-1 can activate PKB/Akt in VSM (Liu *et al.*, 2003;Walker *et al.*, 2001), this suggests the involvement of other regulatory factors.

4.6 Involvement of other DGK isoforms

It is also clear that other DGK isoforms are involved in both NA and ET-1 responses, as PI3K inhibition did not completely abolish NA-stimulated DGK activity and ET-1-stimulated DGK activity is PI3K-independent. However, with limited information as to which DGK isoforms are present in RMSA, it is unclear which could be involved in the NA and ET-1 responses. Currently, nine mammalian DGK isoforms have been cloned and are separated into five types according to their

domain structure, substrate specificity and regulation (reviewed in Luo *et al.*, 2004;Van Blitterswijk & Houssa, 2002). In RMSA, DGKθ and –ζ have been detected by immunoblotting and messenger RNA for DGK-β has also been found (Ohanian & Ohanian, 2001) although it is possible that other isoforms are expressed. For example, investigations of DGK isoform tissue distribution indicated that DGK-δ, -ε and -η are all expressed in muscle tissue and could conceivably also be present in VSM (Sakane *et al.*, 1996;Klauck *et al.*, 1996;Tang *et al.*, 1996a). However, the current lack of DGK antibodies makes it difficult to resolve this issue.

The lack of effect of extracellular calcium removal suggests that the type 1 DGK isoforms are not involved, in this case DGK-β whose messenger RNA has been found in RMSA. DGK-ζ, detected in RMSA by immunoblot, has been implicated in regulation of DAG levels in the nucleus (Topham *et al.*, 1998) but also translocates to the plasma membrane in carbachol-stimulated T-cells (Santos *et al.*, 2002) and so could potentially contribute to agonist-stimulated DGK activity. Consistent with this, DGK-ζ associates with PKC-α (Luo *et al.*, 2003a), localised to caveolae/rafts of fibroblasts and COS-7 cells (Mineo *et al.*, 1998). DGK- ζ (Oka *et al.*, 1997) also co-immunoprecipitates with PLC-β_1 and –γ_1 (Luo *et al.*, 2004). As PLC-β_1 has been found in a complex with caveolin-1 in human submandibular gland cells (Lockwich *et al.*, 2001) and PLC-γ_1 was localised to caveolae/rafts of A431 cells (Jang *et al.*, 2001), this further suggests that DGK-ζ could also localise to these domains. The accumulation of PC-DAG but not PI-DAG in ET-1-stimulated RMSA (Liu *et al.*, 1999a) would implicate the DGK-ε isoform in the ET-1 response. This is a predominantly membrane bound DGK with specificity for PI-derived DAG species (Walsh *et al.*, 1994) and is thought to play an important role in PI turnover. Thus, the

association of this DGK with caveolae/rafts would be consistent with the localisation of PIP_2 hydrolysis (**chapter 4**) and production to these domains (Waugh *et al.*, 2001). However, although expressed in muscle tissue (Tang *et al.*, 1996a), the presence of DGK-ε has not been investigated in VSM or RMSA. Thus, further research into the DGK isoforms present in RMSA is needed to resolve this.

5.0 Summary and Conclusions

Caveolae/rafts are sites of NA and ET-1-stimulated DGK activity consistent with these domains as sole sites of NA and ET-1-stimulated PIP_2 hydrolysis and, by extension, DAG production. DGK activation by NA and ET-1 occurs with distinct temporal and spatial profiles with differential PI3K dependence of DGK activity suggesting agonist-specific activation of isoforms. NA-stimulated PI3K-dependent DGK activity localised solely to caveolae/rafts consistent with the presence of DGKθ and the NA-stimulated translocation of PKB/Akt to caveolae/rafts. The localisation of DGK activity to caveolae/rafts could be important for localised signalling through regulating the levels of the second messengers DAG and PA.

CHAPTER 6

Localisation of NA and ET-1-stimulated PA Production in Rat Mesenteric Small Arteries

1.0 Introduction

Previous studies in VSM have reported that accumulation of PA correlates with the contractile response and have proposed a role for this lipid second messenger in regulation of contraction (Jones *et al.*, 1993;Ohanian *et al.*, 1990). The localisation of PIP_2 hydrolysis and DGK activity to caveolae/rafts (**chapters 4 and 5**) suggested that PA production would also localise to these domains. Furthermore, the agonist-specific differences in NA and ET-1-stimulated PIP_2 hydrolysis and DGK activity suggested differential PA production in response to these two agonists. Accordingly, using [^{33}P]-radiolabelling, and chromatography, the localisation of NA and ET-1-stimulated PI- and DGK-derived PA production was investigated in RMSA.

A number of putative *in vitro* targets for PA have been identified (**chapter 1**) and the localisation of PA production to caveolae/rafts could be important for signalling through the activation of co-localised effectors. Accordingly, using western blotting, the localisation of the *in vitro* PA targets Raf-1, MYPT1 and PP1c was investigated.

2.0 Methods

The protocols for the experiments detailed in this chapter are described in sections 2.1-2.7 and 2.10 of chapter 2.

3.0 Results

3.1 Total [^{33}P]-PA production in RMSA

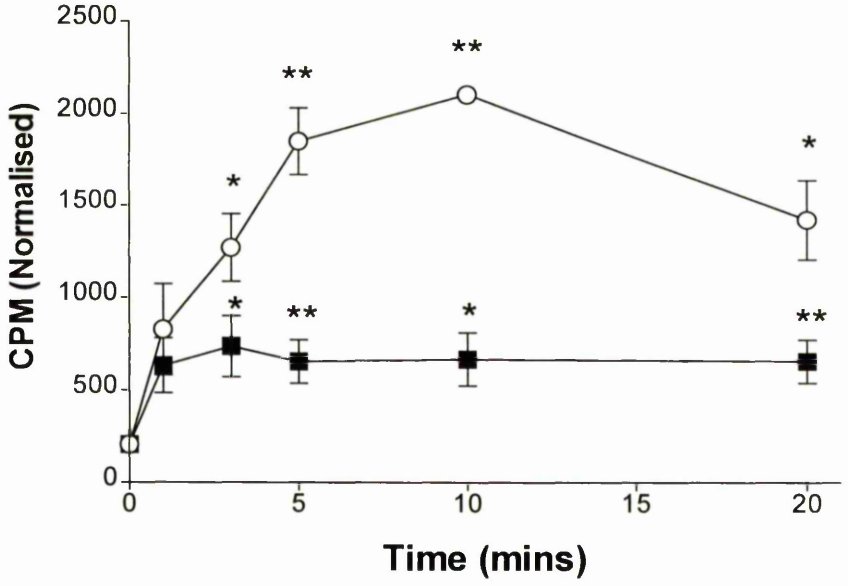
3.1.1 Effect of NA and ET-1 on total [^{33}P]-PA levels

[^{33}P]-labelled RMSA were stimulated with NA (15μM) or ET-1 (100nM) for a range of times from 0-20 min and total [^{33}P]-PA content was analysed as described in chapter 2. NA stimulated a rapid increase in [^{33}P]-PA levels peaking at 3.59 ± 0.80-fold over basal after 3 minutes (P <0.02, n=5) and remaining elevated up to 20 min (P <0.01, n=5, **Fig. 6.1 closed squares**). ET-1 stimulated a rapid increase in [^{33}P]-PA levels peaking 10.27 ± 0.08-fold over basal after 10 min (P <0.001, n=3) with levels declining by 20 min although still significantly higher than basal (P <0.03, n=3, **Fig. 6.1 open circles**).

3.1.2 Effect of butan-1-ol on total [^{33}P]-PA levels

To confirm that PLD does not contribute to [^{33}P]-PA production, [^{33}P]-labelled RMSA were preincubated in 2% butan-1-ol for 10 min prior to stimulation with NA (15 μM, 5min) or ET-1 (100nM, 10min) and total [^{33}P]-PA content analysed as described in chapter 2. Time points were chosen to reflect when [^{33}P]-PA levels were close to a peak. Preincubation with butan-1-ol had no significant effect on basal [^{33}P]-PA levels (P >0.3, at least three experiments). Similarly, no effect was observed on NA and ET-1-stimulated [^{33}P]-PA production (P >0.1, at least three experiments, **Fig. 6.2**).

A



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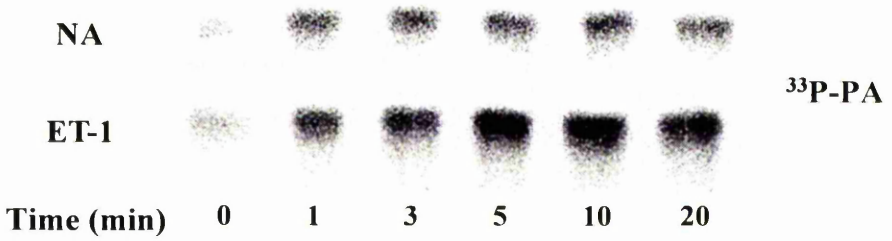


Fig. 6.1: Effect of NA and ET-1 on total [^{33}P]-PA levels. [^{33}P]-labelled RMSA were stimulated with NA (15μM) or ET-1 (100nM) over a range of times and total [^{33}P]-PA levels analysed as described in chapter 2. **A)** NA and ET-1 on total [^{33}P]-PA, Results are expressed as mean ± SEM counts per minute normalised for protein content (* P <0.05, ** P <0.01, t-test, compared to basal, at least three experiments) **B)** Representative [^{33}P]-PA autoradiograph for NA and ET-1

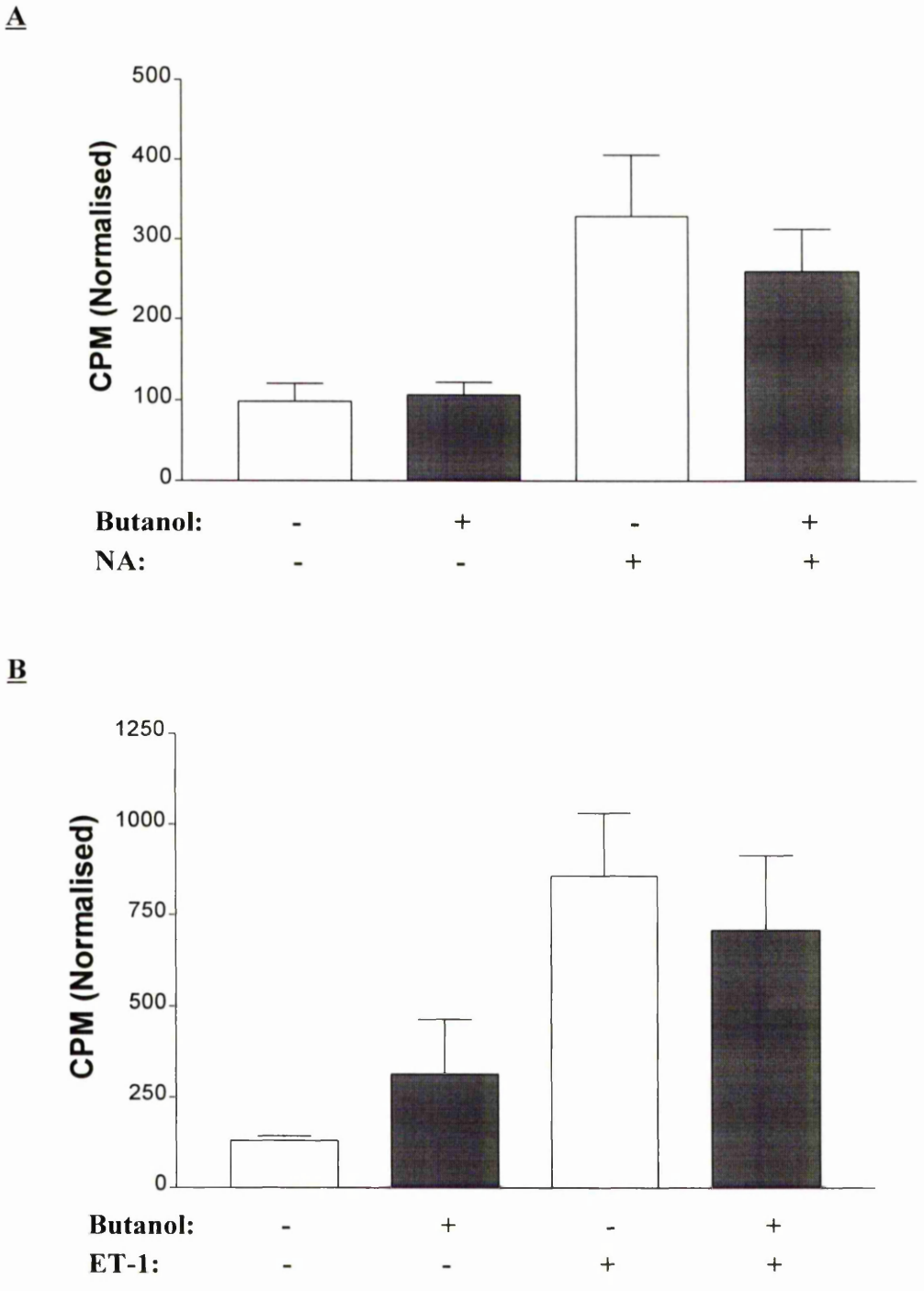


Fig. 6.2: Effect of butan-1-ol on total [^{33}P]-PA levels. [^{33}P]-labelled RMSA were incubated in the presence (closed bars) or absence (open bars) of 2% butan-1-ol for 10 min prior to stimulation with dH_2O (vehicle), **A)** NA (15μM, 5min) or **B)** ET-1 (100nM, 10min) and total [^{33}P]-PA levels analysed as described in chapter 2. Results are expressed as mean ± SEM counts per minute normalised for protein content and are from at least three separate experiments.

3.1.3 Effect of PC-PLC inhibition on total [^{33}P]-PA levels

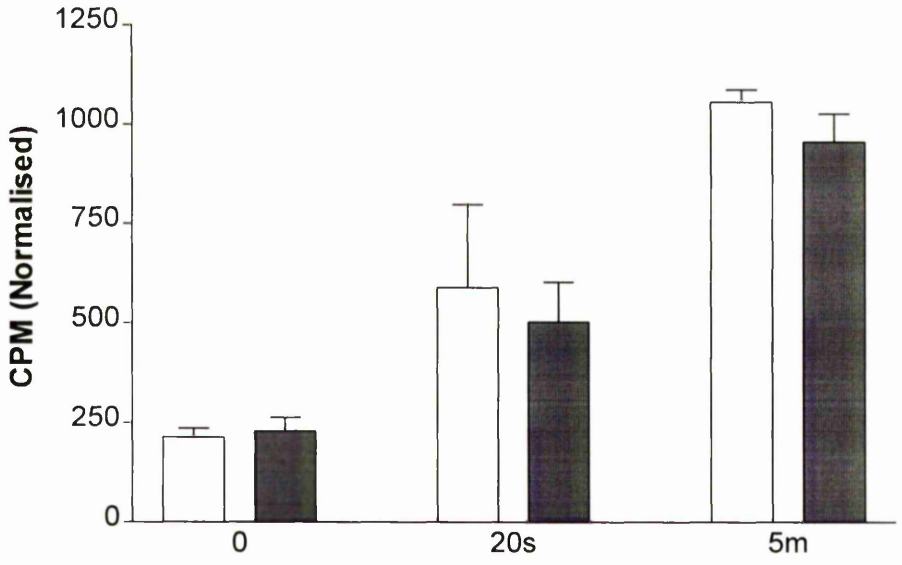
To determine if PC-PLC is involved in [^{33}P]-PA production, [^{33}P]-labelled RMSA were preincubated in 2.5μg/ml D609 for 1 hour prior to stimulation with NA (15μM), ET-1 (100nM) or dH_2O (vehicle) and total [^{33}P]-PA levels analysed as described in chapter 2. Time points were chosen to correspond to the initial (20s, NA and ET-1) and sustained phases (NA: 5 min, ET-1: 10 min) of RMSA contraction in response to these agonists (Ohanian *et al.*, 1997;Ohanian *et al.*, 1990). Inhibition of PC-PLC with D609 had no significant effect on basal [^{33}P]-PA levels (P >0.15, n=3). Similarly, no significant effect on NA and ET-1-stimulated [^{33}P]-PA production was observed at either time point investigated (P > 0.1, n=3, **Fig. 6.3**).

3.2 Localisation of [^{33}P]-PA production in RMSA

3.2.1 [^{33}P]-PA distribution

To determine resting distribution of [^{33}P]-PA, membrane fractions were prepared from [^{33}P]-labelled RMSA and [^{33}P]-PA content analysed as described in chapter 2. In unstimulated RMSA, 40.1 ± 2.4% of [^{33}P]-PA was localised to caveolae/rafts (fractions 2-5) with 26.2 ± 1.0% in non-caveolae/rafts 1 (fractions 6-9) and 25.1 ± 2.8% in non-caveolae/rafts 2 (fractions 10-13) (**Figs. 6.4 and 6.5, open circles**)

A



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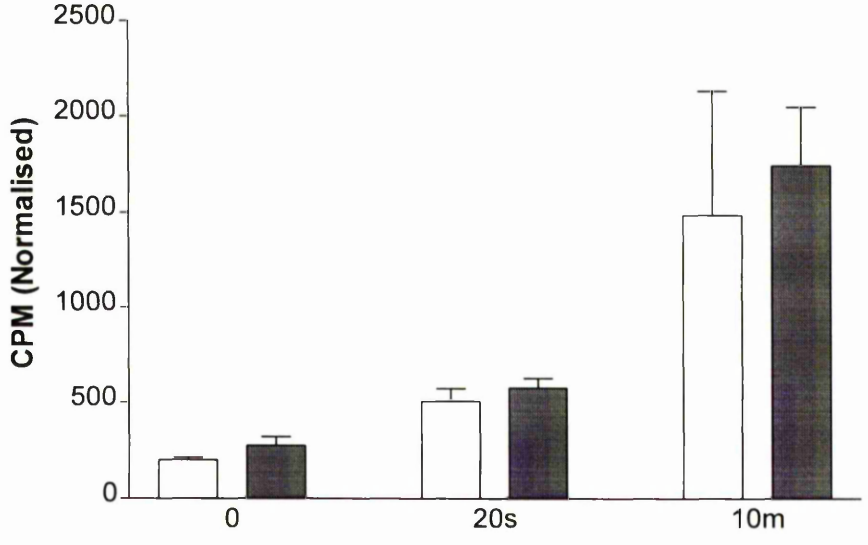


Fig. 6.3: Effect of D609 on total [^{33}P]-PA levels. [^{33}P]-labelled RMSA were incubated in the presence (closed bars) and absence (open bars) of 2.5μg/ml D609 for 1 hour prior to stimulation with dH_2O (vehicle), **A)** NA (15μM) or **B)** ET-1 (100nM) and total [^{33}P]-PA levels analysed as described in chapter 2. Results are expressed as mean ± SEM counts per minute normalised for protein content (n=3).

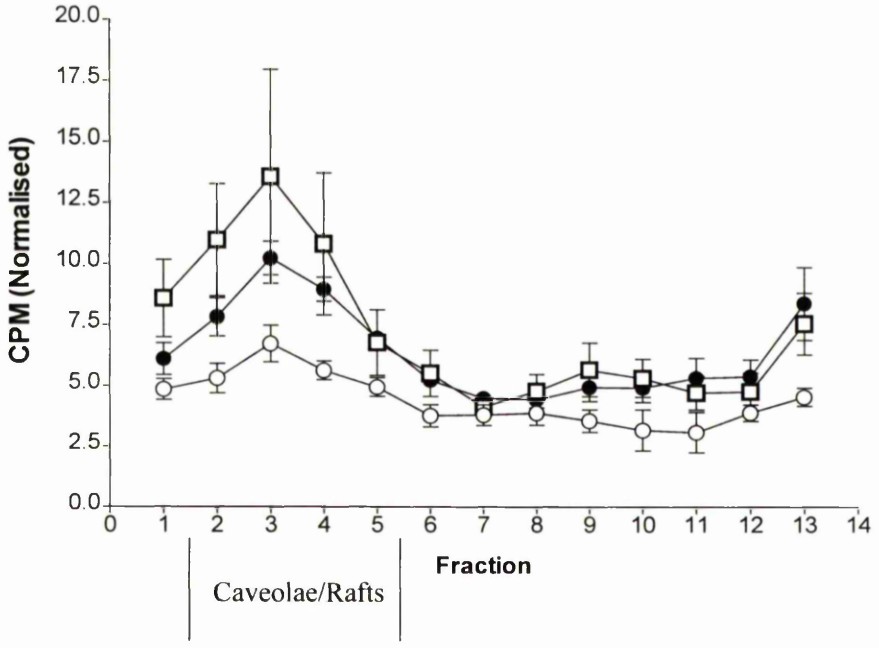
3.2.2 NA-stimulated [^{33}P]-PA production

To determine localisation of agonist-stimulated [^{33}P]-PA production, [^{33}P]-labelled RMSA were stimulated with NA (15μM), membrane fractions prepared and [^{33}P]-PA content analysed as described in chapter 2. Time points were chosen to correspond to the initial (20s) and sustained phases (5 min) of RMSA contraction to this agonist (Ohanian *et al.*, 1990). Analysis by ANOVA indicated that NA-stimulated [^{33}P]-PA levels were significantly increased at both 20s and 5 min (P <0.001, n=5, **Fig. 6.4A**). Further analysis found that at 20s, NA increased levels by a similar magnitude in both caveolae/rafts (1.50 ± 0.04-fold) and non-caveolae/rafts (1.45 ± 0.08-fold, P >0.25, n=5) whilst at 5min, levels increased significantly higher in caveolae/rafts (1.84 ± 0.16-fold) than in non-caveolae/rafts (1.43 ± 0.08-fold, P <0.05, n=5 **Fig. 6.4B**).

3.2.3 ET-1-stimulated [^{33}P]-PA production

[^{33}P]-labelled RMSA were stimulated with ET-1 (100nM), membrane fractions prepared and [^{33}P]-PA content of individual fractions analysed as described in chapter 2. Time points were chosen to correspond to the initial (20s) and sustained phases (10 min) of RMSA contraction to this agonist (Ohanian *et al.*, 1997). Analysis by ANOVA indicated that ET-1-stimulated [^{33}P]-PA levels were significantly increased at both 20s and 10 min (P <0.02, at least four experiments, **Fig. 6.5A**). Further analysis found that at 20s, ET-1 increased levels significantly higher in caveolae/rafts (1.53 ± 0.12-fold) than in non-caveolae/rafts (1.22 ± 0.07-fold, P <0.05, n=6) whilst at 10 min, levels were increased by a similar magnitude in caveolae/rafts (2.02 ± 0.22-fold) and non-caveolae/rafts (2.27 ± 0.24-fold, P >0.25, n=4, **Fig. 6.5B**).

A



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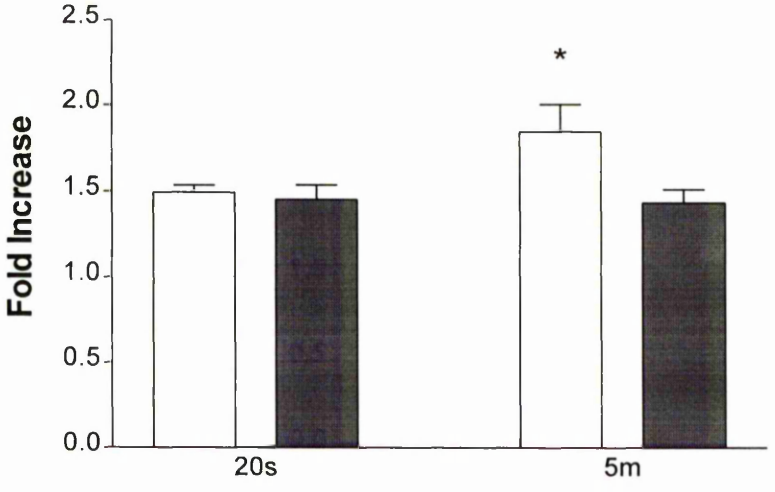
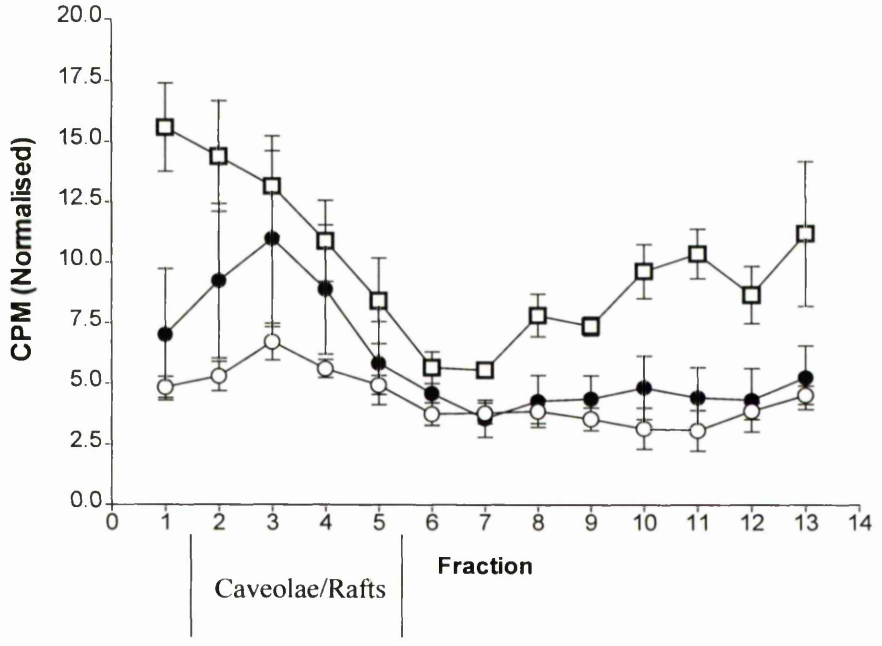


Fig. 6.4: Effect of NA on [^{33}P]-PA levels of membrane fractions. [^{33}P]-labelled RMSA were stimulated with dH_2O (vehicle, open circles) or NA (15μM) for 20s (closed circles) or 5 min (open squares), membrane fractions prepared and [^{33}P]-PA content analysed as described in chapter 2. **A)** Results are expressed as mean ± SEM counts per minute normalised for total protein loaded on to sucrose gradients (at least four experiments) **B)** Mean ± SEM fold increase in [^{33}P]-PA from basal in caveolae/rafts (open bars) and non-caveolae/rafts (closed bars) (* P <0.05, t-test, compared to non-caveolae/raft, at least four experiments).

A



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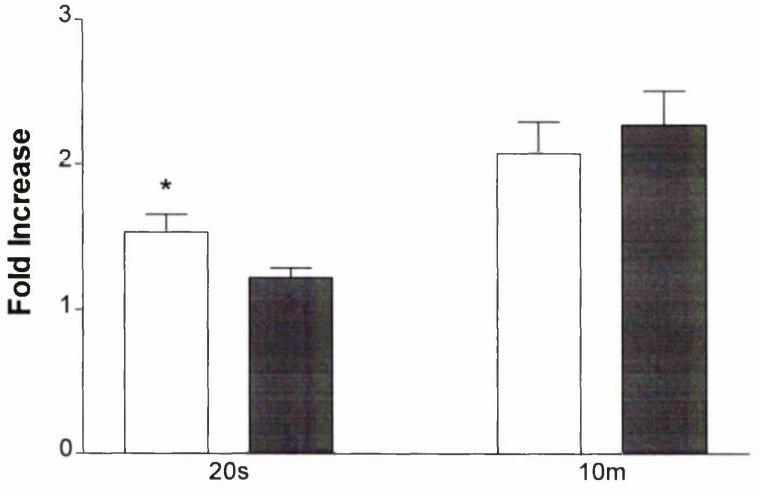


Fig. 6.5: Effect of ET-1 on [^{33}P]-PA levels of membrane fractions. [^{33}P]-labelled RMSA were stimulated with dH_2O (vehicle, open circles) or ET-1 (100nM) for 20s (closed circles) or 10 min (open squares), membrane fractions prepared and [^{33}P]-PA content analysed as described in chapter 2. **A)** Results are expressed as mean ± SEM counts per minute normalised for total protein loaded on to sucrose gradients (at least four experiments) **B)** Mean ± SEM fold increase in [^{33}P]-PA from basal in caveolae/rafts (open bars) and non-caveolae/rafts (closed bars) (* P <0.05, t-test, compared to non-caveolae/raft, at least four experiments).

3.3 Receptor subtypes mediating [^{33}P]-PA production

3.3.1 Effect of an α_1-adrenoreceptor agonist and antagonist

To determine if α_1-adrenoreceptors mediated NA-stimulated [^{33}P]-PA production, [^{33}P]-labelled RMSA were stimulated with PE (15μM, 5min) or incubated with 10μM prazosin for 1 hour prior to stimulation with NA (15μM, 5 min), membrane fractions were prepared and [^{33}P]-PA content analysed as described in chapter 2. In the isolated perfused mesentery of the rat, incubation for one hour with 30nM prazosin was sufficient to inhibit 1μM NA-stimulated vasoconstriction (Williams & Clarke, 1995). However, as 15μM NA was used in these experiments, 10μM prazosin was used to ensure sufficient antagonist was present.

Analysis by ANOVA indicated that the PE-stimulated [^{33}P]-PA profile was significantly higher than the NA-stimulated [^{33}P]-PA profile (P <0.05, n=4, **Fig. 6.6A**) although Bonferroni post-ANOVA analysis indicated that [^{33}P]-PA levels in individual fractions were not significantly different. Analysis by ANOVA indicated that prazosin significantly reduced NA-stimulated [^{33}P]-PA levels (P <0.001, n=4, **Fig. 6.6B**).

3.3.2 Effect of an ET_A receptor antagonist

To determine if the ET_A receptor mediated ET-1-stimulated [^{33}P]-PA production, [^{33}P]-labelled RMSA were incubated with 10μM BQ-123 for 1 hour prior to stimulation with ET-1 (100nM, 10min), membrane fractions were prepared and [^{33}P]-PA content analysed as described in chapter 2. Incubation for one hour with 10μM BQ-123 inhibited ET-1-stimulated IP_3 formation in cultured rat VSM cells (Eguchi *et al.*, 1992) and cardiomyocytes (Clerk *et al.*, 1994). Analysis by

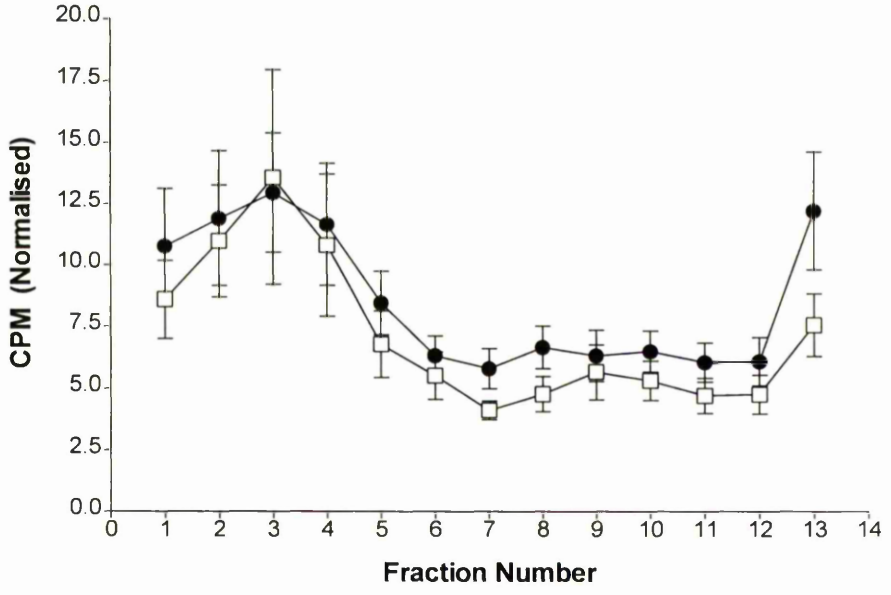
ANOVA indicated that BQ-123 significantly reduced ET-1-stimulated [^{33}P]-PA levels (P <0.001, n=4, **Fig. 6.7**).

3.4 Effect of extracellular calcium removal on [^{33}P]-PA production

3.4.1 NA-stimulated [^{33}P]-PA levels in caveolae/rafts

[^{33}P]-labelled RMSA were incubated in calcium-free HEPES buffer containing 1mM EGTA for 10 min prior to stimulation with dH_2O (vehicle) or NA (15μM), membrane fractions prepared and [^{33}P]-PA content analysed as described in chapter 2. A time point of 2 min was chosen to ensure a discernible effect on PA levels could be observed. Extracellular calcium removal had no significant effect on basal [^{33}P]-PA levels in any fractions (P >0.1, n=6). However, NA-stimulated [^{33}P]-PA levels were significantly reduced in caveolae/rafts (65 ± 9%, P <0.03, n=6), non-caveolae/rafts 1 (63 ± 13% P <0.02, n=6) and non-caveolae/rafts 2 (68 ± 7%, P <0.01, n=6, **Fig. 6.8A**).

A



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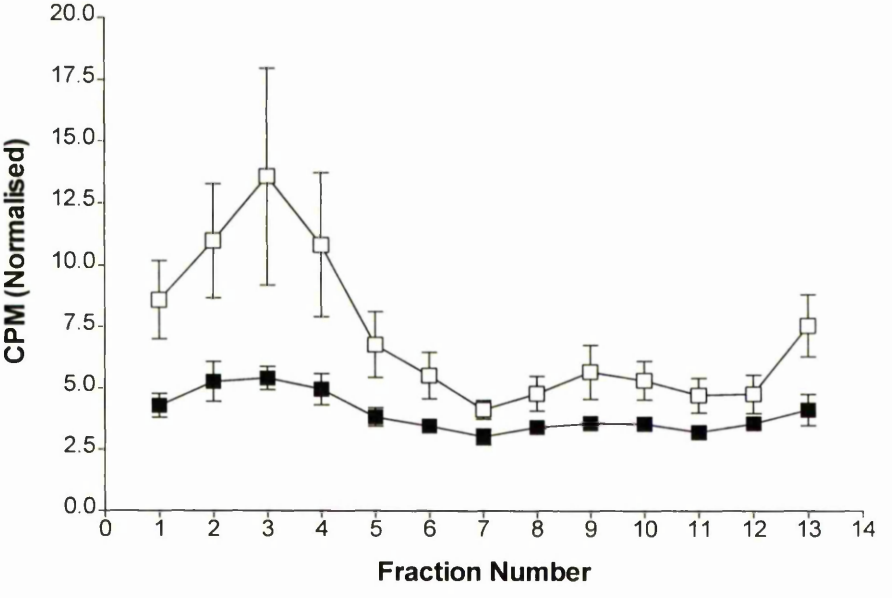


Fig. 6.6: NA-stimulated [^{33}P]-PA is mediated by α_1-adrenoreceptors. [^{33}P]-labelled RMSA were stimulated with **A)** PE (15μM, 5min, closed circles) or NA (15μM, 5min, open squares) or **B)** incubated in the presence (closed squares) or absence (open squares) of 10μM prazosin for 1 hour prior to stimulation with NA (15μM, 5min), membrane fractions prepared and [^{33}P]-PA content analysed as described in chapter 2. Results are expressed as mean ± SEM counts per minute normalised for total protein loaded on to sucrose gradients and are from at least four separate experiments.

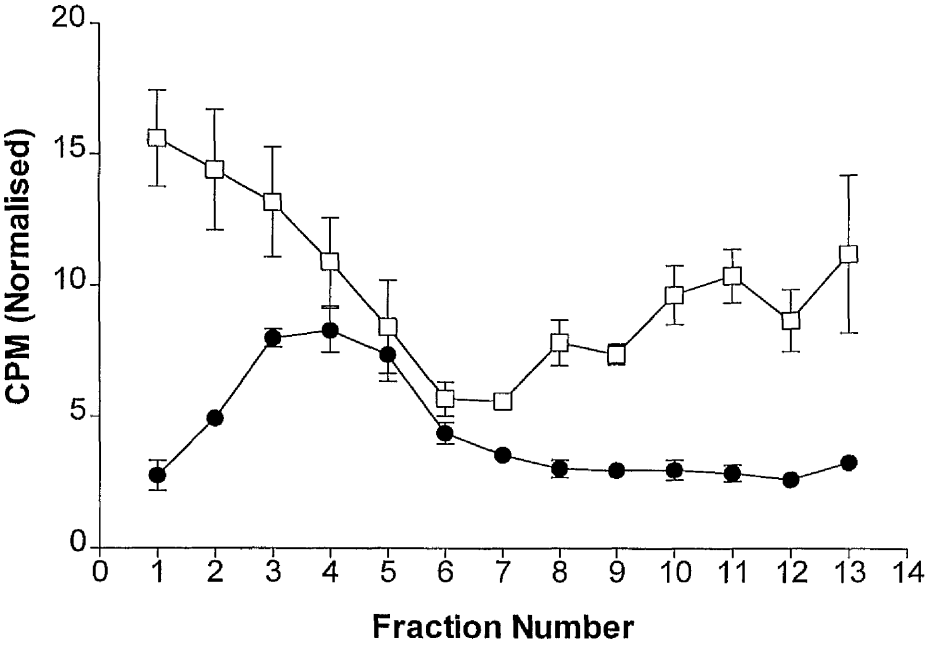


Fig. 6.7: ET-1-stimulated [^{33}P]-PA is mediated by the ET_A receptor subtype.

[^{33}P]-labelled RMSA were incubated in the presence (closed circles) or absence (open squares) of 10μM BQ-123 for 1 hour prior to stimulation with ET-1 (100nM, 10 min), membrane fractions prepared and [^{33}P]-PA content analysed as described in chapter 2. Results are expressed as mean ± SEM counts per minute normalised for total protein loaded on to sucrose gradients and are from at least four separate experiments.

3.4.2 ET-1-stimulated [^{33}P]-PA levels in caveolae/rafts

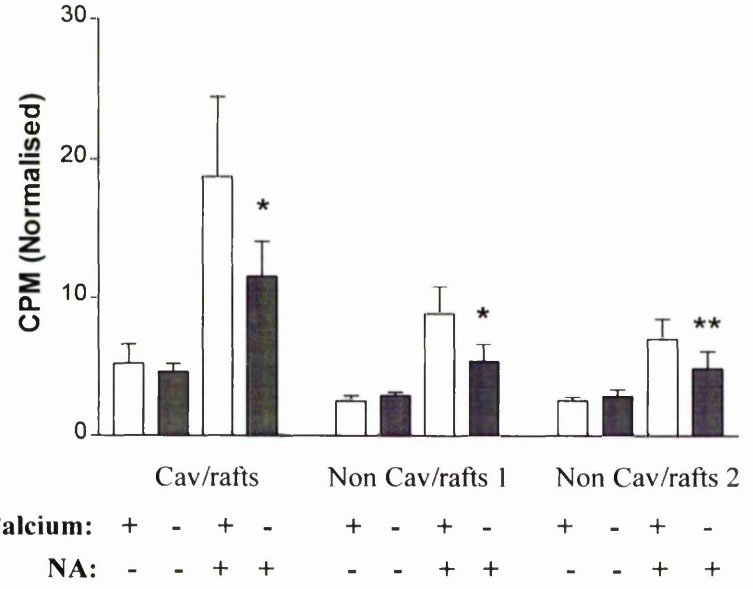
[^{33}P]-labelled RMSA were incubated in calcium-free HEPES buffer containing 1mM EGTA for 10 min prior to stimulation with dH_2O (vehicle) or ET-1 (100nM), membrane fractions prepared and [^{33}P]-PA content analysed as described in chapter 2. A time point of 2 min was chosen to ensure a discernible effect on PA levels could be observed. Extracellular calcium removal had no significant effect on basal [^{33}P]-PA levels in any fractions (P >0.1, n=5). With ET-1, [^{33}P]-PA levels in caveolae/raft fractions were significantly reduced (P <0.04, 57 ± 12%, n=5). However, although levels in non-caveolae/rafts were reduced, this was not statistically significant (P >0.05, n=6, **Fig. 6.8B**).

3.5 Effect of PI3K inhibition on [^{33}P]-PA production

3.5.1 NA-stimulated [^{33}P]-PA levels in caveolae/rafts

[^{33}P]-labelled RMSA were incubated in 10μM LY294002 for 1 hour prior to stimulation with NA (15μM, 1 min) or dH_2O (vehicle), membrane fractions prepared and [^{33}P]-PA content analysed as described in chapter 2. The time point was chosen to correspond to peak DGK activity in response to NA in RMSA (Ohanian & Heagerty, 1994). PI3K inhibition had no significant effect on basal [^{33}P]-PA levels in any fraction (P >0.15, n=3) but significantly reduced NA-stimulated [^{33}P]-PA levels in caveolae/rafts (77 ± 9%, P <0.04, n=5) and non-caveolae/rafts 2 (71 ± 11%, P <0.04, n=5, **Fig. 6.9A**).

A



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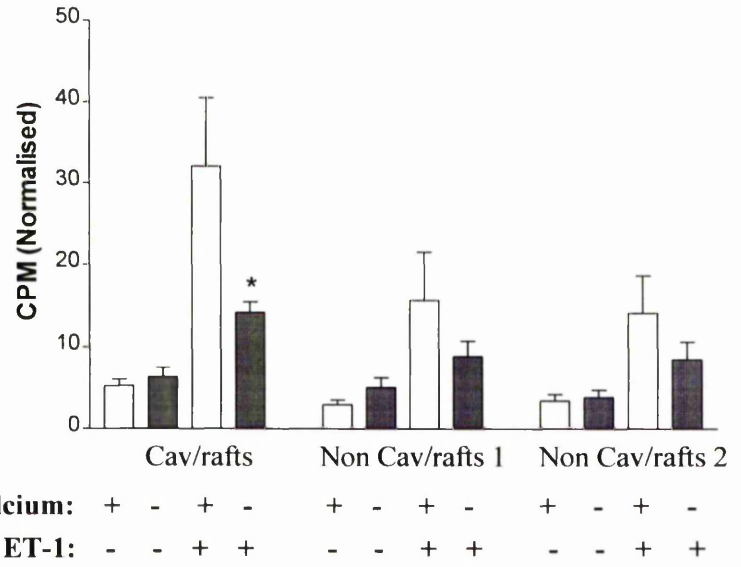
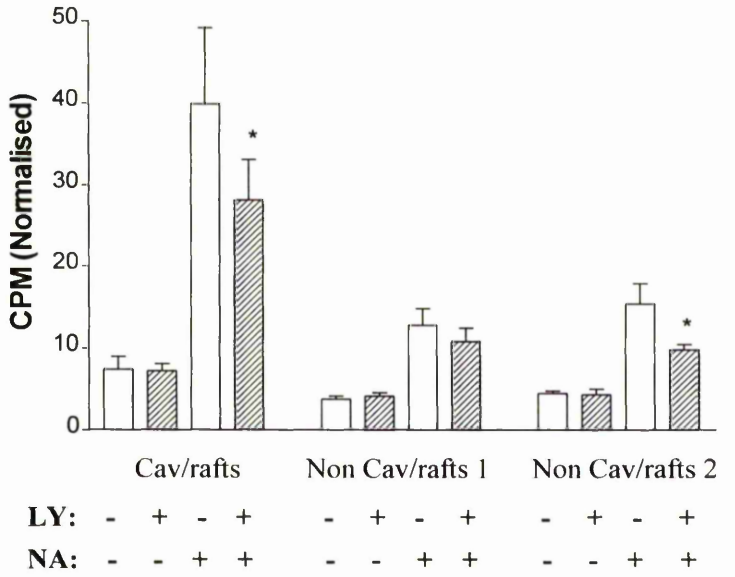


Fig. 6.8: Effect of extracellular calcium removal on [^{33}P]-PA levels of caveolae/rafts. [^{33}P]-labelled RMSA were incubated in the presence (open bars) or absence (closed bars) of extracellular calcium for 10 mins prior to stimulation with **A)** NA (15μM) or **B)** ET-1 (100nM), membrane fractions prepared and [^{33}P]-PA content analysed as described in chapter 2. Results are expressed as mean ± SEM counts per minute normalised for total protein loaded on to sucrose gradients. (* P <0.04, ** P <0.01, t-test, calcium compared to calcium free, at least five experiments).

A



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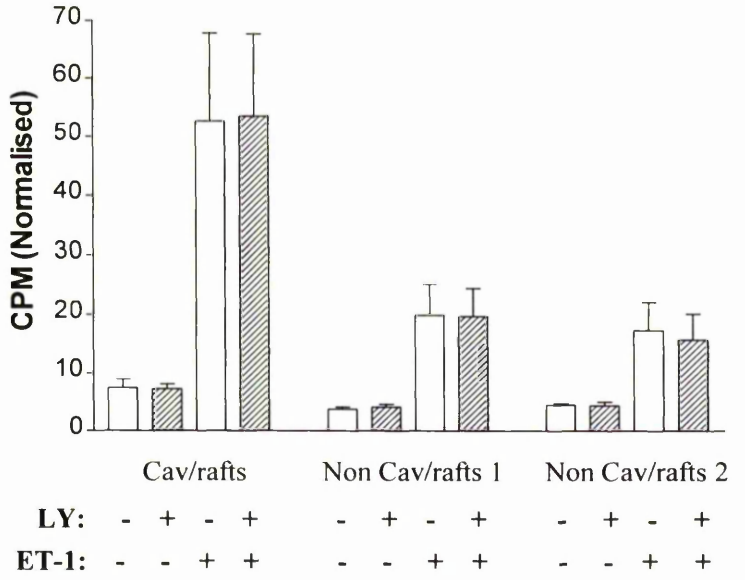


Fig. 6.9: Effect of PI3K inhibition on [^{33}P]-PA levels of caveolae/rafts. [^{33}P]-labelled RMSA were incubated in the presence (hatched bars) or absence (open bars) of 10μM LY294002 for 1 hour prior to stimulation with **A)** NA (15μM, 1 min) or **B)** ET-1 (100nM, 5 min), membrane fractions prepared and [^{33}P]-PA content analysed as described in chapter 2. Results are expressed as mean ± SEM counts per minute normalised for total protein loaded on to sucrose gradients (* P <0.05, t-test, NA compared to LY+NA, at least five experiments).

3.5.2 ET-1-stimulated [^{33}P]-PA levels in caveolae/rafts

[^{33}P]-labelled RMSA were incubated in 10μM LY294002 for 1 hour prior to stimulation with ET-1 (100nM, 5 min) or dH_2O (vehicle), membrane fractions prepared and [^{33}P]-PA content analysed as described in chapter 2. The time point was chosen to correspond to peak DGK activity in response to ET-1 in RMSA (**chapter 5**). PI3K inhibition had no significant effect on basal [^{33}P]-PA levels in any fraction (P >0.15, n=3). Similarly, no significant effect on ET-1-stimulated [^{33}P]-PA levels in either caveolae/raft or non-caveolae/raft fractions was observed (P >0.15, n=5, **Fig. 6.9B**).

3.6 Functional consequences of localised [^{33}P]-PA production

Localisation of PA production to caveolae/rafts could facilitate translocation of downstream PA targets to these domains. To investigate this, RMSA were stimulated with NA (15μM, 5 min) or dH_2O (vehicle), membrane fractions prepared and analysed for content of the *in vitro* PA targets MYPT1, Raf-1 and PP1c (Jones & Hannun, 2002;Rizzo *et al.*, 2000;Ito *et al.*, 1997) by immunoblot as described in chapter 2. In unstimulated arteries, Raf-1, MYPT-1 and PP1c all localised to non-caveolae/raft fractions. Following NA stimulation, all three proteins remained localised to non-caveolae/raft fractions (**Fig. 6.10**).

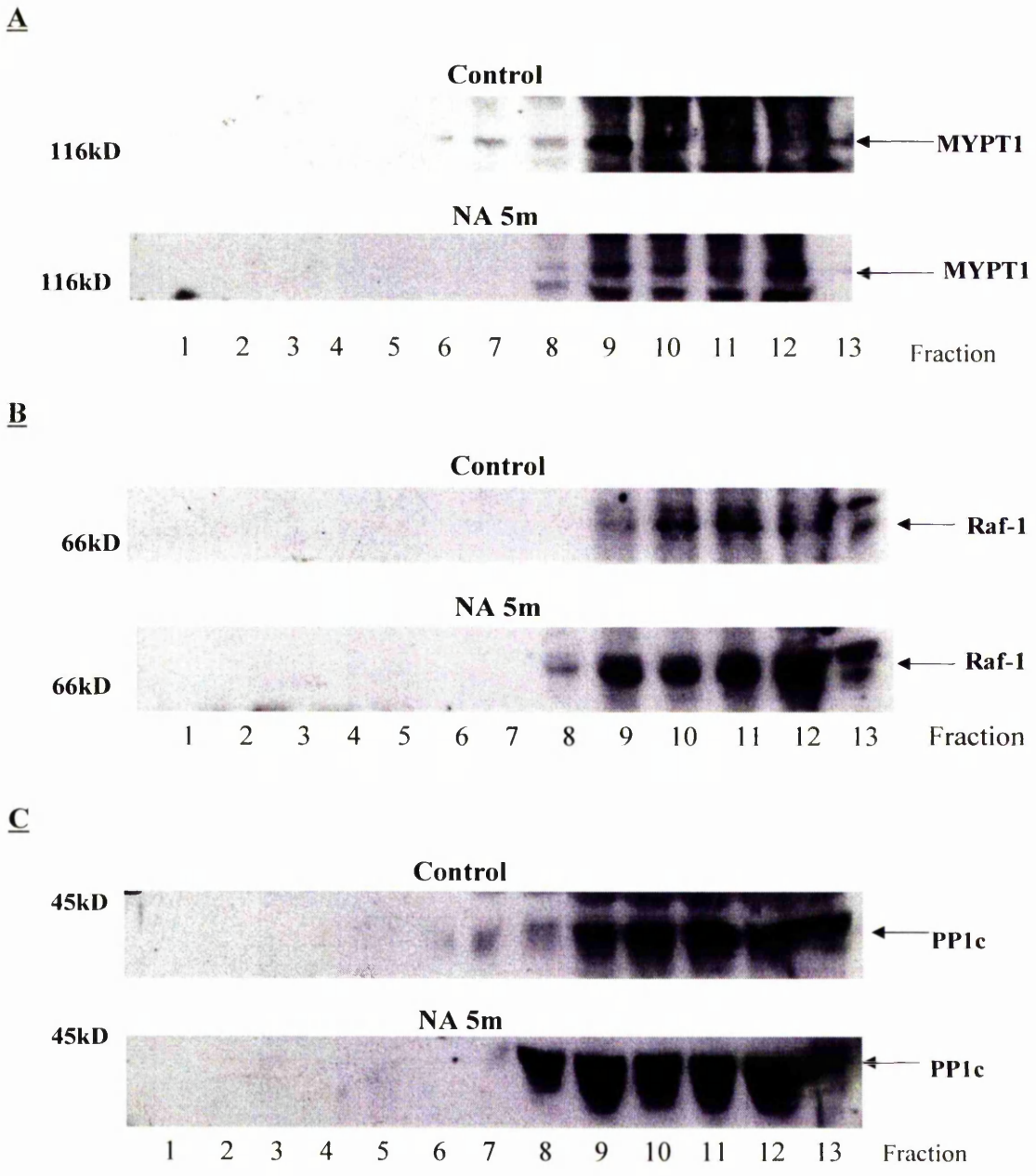


Fig. 6.10: Localisation of PA targets in RMSA. RMSA were stimulated with dH_2O (vehicle) or NA (15μM, 5 min), membrane fractions prepared and protein content analysed by immunoblot for **A)** MYPT1 **B)** Raf-1 and **C)** PP1c as described in chapter 2. Immunoblots shown for unstimulated RMSA (top) and following 5min NA stimulation (bottom) are representative of three experiments.

4.0 Discussion

4.1 NA and ET-1 on total [^{33}P]-PA production

Both NA and ET-1 stimulated an increase in total [^{33}P]-PA levels in agreement with previous studies in RMSA (Ohanian *et al.*, 1990) and rat cardiomyocytes (Ye *et al.*, 1994) and consistent with the proposed role for this second messenger in regulation of VSM contraction (Jones *et al.*, 1993;Ohanian *et al.*, 1990). However, the two agonists had markedly different effects. NA stimulated a rapid increase to peak [^{33}P]-PA levels at 3 minutes that remained elevated for up to 20 minutes whilst ET-1 induced a slower increase to peak PA levels at 10 minutes, that by 20 minutes levels had begun to fall. The lack of effect of butan-1-ol and D609 on [^{33}P]-PA levels by either agonist confirmed that [^{33}P]-PA is derived from the DGK pathway and is of PI origin. The observed effects of NA and ET-1 on total [^{33}P]-PA levels correlate with the differential stimulation of DGK activity by each agonist (**chapter 5**) (Ohanian & Heagerty, 1994) – the rapid, transient increase in NA-stimulated DGK activity giving rise to the rapid and sustained [^{33}P]-PA levels whilst the slower and sustained DGK activity for ET-1 matches the gradual and substantially greater increase in [^{33}P]-PA levels seen with this agonist. As [^{33}P]-PA is solely PI-derived, these results also indicate prolonged hydrolysis of PIs as suggested by results in chapter 3. The sustained elevation of NA-stimulated [^{33}P]-PA levels once DGK activity has returned to basal suggests there may also be some inhibition of [^{33}P]-PA metabolism. As the major fate of PI-derived PA is to feed back into the PI cycle (Hodgkin *et al.*, 1998), this suggests that modulation of CDP-DAG synthase (CDS) activity downstream of PA also occurs, presumably for both NA and ET-1. Alternatively, this could be due to the involvement of constituitively active substrate-driven DGK isoforms in the NA response as, for example, DGK-ε is reported to

localise predominantly to the plasma membrane, and has specificity for arachidonate-enriched DAG (Walsh *et al.*, 1994). The involvement of such isoforms could also account for the sustained PA levels observed.

4.2 Localisation of [^{33}P]-PA production.

The broad distribution of [^{33}P]-PA reflects the distribution of [^{33}P]-PIP_2 and DGK activity as described in chapters 3 and 4. Agonist stimulation increases [^{33}P]-PA levels in caveolae/rafts consist with localisation of [^{33}P]-PIP_2 hydrolysis and stimulation of DGK activity within these domains. However, with both NA and ET-1, there is also an increase in [^{33}P]-PA in non-caveolae/raft domains. As there is no detectable [^{33}P]-PIP_2 hydrolysis in these fractions, this must reflect diffusion of DAG substrate or [^{33}P]-PA away from the site of production. Of the two agonists, ET-1 appeared to stimulate a greater increase in [^{33}P]-PA in non-caveolae/raft fractions during the sustained phase of the response. As ET-1 increases DGK activity in these domains but NA does not, this suggests that it is the DAG substrate diffusing away from the caveolae/raft fractions. This is consistent with the current view of lipid rafts as dynamically interacting with the non-raft environment (Subczynski & Kusumi, 2003;Anderson & Jacobson, 2002) and with reports that DAG has a high rate of lateral diffusion in membranes (Prieto *et al.*, 1994).

4.3 Receptor subtypes mediating PA production

The effect of PE on [^{33}P]-PA levels closely matched that of NA with [^{33}P]-PA accumulating in caveolae/raft fractions over non-caveolae/raft fractions, which suggests that α_1-adrenoreceptors mediate [^{33}P]-PA production. This was further supported by the inhibition of NA-stimulated [^{33}P]-PA production by the α_1-

adrenoreceptor antagonist prazosin and is in agreement with previous studies in rat heart (Kurz *et al.*, 1999) and rabbit ventricular myocytes (Ye *et al.*, 1994). Similarly, the inhibition of ET-1-stimulated [^{33}P]-PA production by the ET_A receptor antagonist BQ-123 indicates the involvement of this receptor subtype in this process consistent with previous research in rat myometrium (Dokhac *et al.*, 1995). As previous studies in VSM have reported that the α_1-adrenoreceptors and the ET_A subtype are the major receptor types involved in NA and ET-1-induced vasoconstriction, this further supports the theory that accumulation of PA plays a role in contractile signalling in VSM (Ohanian *et al.*, 1993;Jones *et al.*, 1993;Ohanian *et al.*, 1990).

4.4 The effect of extracellular calcium removal on [^{33}P]-PA levels

Although it had no effect on basal [^{33}P]-PA levels, the removal of extracellular calcium had differential effects on NA and ET-1-stimulated [^{33}P]-PA levels causing a significant decrease across all membrane fractions in NA-stimulated RMSA whilst causing a significant decrease in [^{33}P]-PA levels solely in caveolae/rafts of ET-1-stimulated RMSA. In previous chapters, extracellular calcium removal was found to significantly reduce NA but not ET-1-stimulated PIP_2 hydrolysis (**chapter 4**) and had no effect on NA and ET-1-stimulated DGK activity (**chapter 5**). Therefore, a decrease in NA-stimulated [^{33}P]-PA levels could be predicted as a consequence of reduced DAG availability for DGK. However, [^{33}P]-PA levels were decreased in response to both NA and ET-1 which suggests that extracellular calcium removal causes enhanced PA metabolism. This is consistent with a previous study reporting that flux of PA to PI through the enzyme CDS is inhibited by calcium influx and enhanced by calcium removal (Lapetina *et al.*, 1981). Furthermore, this supports the theory that regulation of CDS activity contributes to

PA accumulation for both NA and ET-1 as discussed above. However, it is also clear that extracellular calcium removal had a greater effect on NA-stimulated [^{33}P]-PA levels reducing levels across all membrane fractions. This is consistent with the reduced [^{33}P]-PIP_2 hydrolysis caused by extracellular calcium removal in NA- but not ET-1-stimulated RMSA with subsequent reduced DAG levels providing less substrate for DGK-mediated phosphorylation to PA.

4.5 PI3K inhibition on [^{33}P]-PA levels

PI3K inhibition significantly reduced NA- but not ET-1-stimulated [^{33}P]-PA levels consistent with the observed effects of PI3K inhibition on NA and ET-1-stimulated DGK activity (**chapter 5**), further supporting a role for DGKθ in NA-stimulated PI signalling. The reduction of NA-stimulated [^{33}P]-PA production in caveolae/rafts is consistent with the localisation of the PI3K-dependent DGK activity to these domains. However, [^{33}P]-PA levels were also reduced in non-caveolae/raft 2 fractions. The reasons for this are not clear, as DGK activity in these fractions was not significantly reduced by PI3K inhibition. This could reflect reduced PA availability for the PI cycle, thus causing a subsequent decrease in [^{33}P]-PA levels in intracellular membranes. However, as the non-caveolae/raft pellet contains other membranes besides the ER, this cannot be verified directly in these experiments.

4.6 Functional consequences of localised [^{33}P]-PA production

The localisation of PA production within caveolae/rafts could be important for efficient coupling of agonist-stimulated PI turnover to intracellular signalling pathways through the activation of co-localised effectors. Accordingly, the localisation of three *in vitro* PA targets – Raf-1, MYPT1 and PP1c (Jones & Hannun,

2002;Rizzo *et al.*, 2000;Ito *et al.*, 1997) was investigated in unstimulated RMSA and after 5min NA stimulation, when [^{33}P]-PA levels are significantly raised in caveolae/rafts.

The use of anti-MYPT1 gave a band of approximately 125.6 kD correlating with the 130kDa molecular weight of MYPT1 previously reported (Shirazi *et al.*, 1994). Immunoblotting with anti-Raf-1 gave a band of approximately 81.3 kD in molecular weight comparing with the 74 kD previously reported for Raf-1 (Schultz *et al.*, 1988) and confirming its presence in RMSA. Finally, anti-PP1c gave a single band of approximately 36.4 kD closely matching the 38kD molecular weight of PP1c (Shirazi *et al.*, 1994). Thus, all three proteins could be detected by immunoblot in RMSA. However, results indicated that all three proteins localised to non-caveolae/raft domains in unstimulated RMSA and, furthermore, remained in the non-caveolae/raft domains following a 5min NA stimulation suggesting that localisation of [^{33}P]-PA production to caveolae/rafts does not induce translocation of these proteins.

With Raf-1, this is inconsistent with recent studies reporting that PA, rather than Ras, is essential for translocation of Raf-1 to the membrane (Andresen *et al.*, 2002;Rizzo *et al.*, 2000) and subsequent activation of the Ras-Raf-1-Mek-ERK cascade with other studies reporting localisation of Raf-1 and the ERK cascade to caveolae/rafts of fibroblasts (Liu *et al.*, 1997b), cardiomyocytes (De Luca *et al.*, 2000), mesangial cells (Hua *et al.*, 2003) and aortic endothelial cells (Rizzo *et al.*, 1998). This could reflect tissue-specific differences, or differences between cultured cells and intact tissue. However, as PA did also increase in non-caveolae/rafts, this may have been

sufficient to recruit Raf-1 to non-caveolae/raft membranes. Alternatively, as a single 5min time point was investigated in the current study, the possibility of an earlier translocation of Raf-1 to caveolae/rafts cannot be ruled out, particularly as peak NA-stimulated ERK activation in RMSA was previously reported to occur after 2.5 minutes (Ward *et al.*, 2001). Finally, it is also possible that this process requires PC-derived PA rather than the PI-derived PA measured here.

With MYPT1 and PP1c, although these proteins were previously reported to be targeted to the membrane in ferret aortic VSM cells (Shin *et al.*, 2002), there is no *in vivo* evidence indicating a role for PA in this process. Furthermore, both proteins have only been shown to bind PA *in vitro* resulting in inhibition of activity (Jones & Hannun, 2002;Ito *et al.*, 1997). Thus, although the single 5min time point investigated here could equally have overlooked an earlier translocation of these proteins to caveolae/rafts in response to localised PA production, it is also possible that these proteins are not direct *in vivo* PA targets.

Of the numerous *in vitro* PA targets identified (**table 1.1**), a number have been localised to caveolae/rafts including dynamin (Oh *et al.*, 1998), PKC isoforms (Oka *et al.*, 1997), PLC isoforms (Jang *et al.*, 2001;Lockwich *et al.*, 2001), and PI-4-P 5-kinase (Waugh *et al.*, 2001). Therefore, it is possible that production of PA within caveolae/rafts may serve to regulate the activity of these various targets; for example, the localised production of PA is reported to be the trigger for dynamin-mediated membrane fission (Burger *et al.*, 2000) and so, in this context, localised PA production could facilitate dynamin-mediated endocytosis of caveolae (Henley *et al.*, 1998). Alternatively, it is also possible that the PI and DGK-derived PA measured in

this thesis is a metabolic by-product and that the major function of DGK activation reported in chapter 4 is to ensure that DAG levels are reduced. This would be consistent with a recent theory that the PC-derived PA species are the important second messengers (Hodgkin *et al.*, 1998). However, as cellular targets have been identified demonstrating a greater response to PI-derived PA over PC-derived PA such as mTOR (Avila-Flores *et al.*, 2005) this suggests that specific targets for this species do exist. Considerable research in identifying such downstream effectors of PI-derived PA is required before the functional consequences of the PA production in caveolae/rafts discussed in this chapter can be elucidated.

5.0 Summary and Conclusions

Caveolae/rafts are major sites of PI- and DGK-derived PA production consistent with these domains as sites of agonist-induced PIP_2 hydrolysis and DGK activity as discussed in chapters 3 and 4. The localisation of PA production to caveolae/rafts may be important for signal transduction through the activation of co-localised effectors.

CHAPTER 7

Effect of Cholesterol Depletion on NA and ET-1-stimulated Phosphoinositide Signalling

1.0 Introduction

Cholesterol depletion has been used to implicate caveolae/rafts in a number of cellular processes (Schlegl & Lisanti, 2001;Fielding & Fielding, 2001;Kabouridis *et al.*, 2000) and, in VSM, is reported to disrupt agonist-induced contraction (Je *et al.*, 2004;Dreja *et al.*, 2002). Previous unpublished work in RMSA found that cholesterol depletion increased the contractile response to NA (**Fig. 7.1**) and the localisation of NA and ET-1-stimulated PI signalling to caveolae/rafts (**chapters 4-6**) suggested that disruption of these domains could affect vasoconstrictor-induced signalling through the PI pathway. Accordingly, using CD treatment the effect of cholesterol depletion on NA and ET-1-stimulated PI signalling in intact RMSA was investigated.

2.0 Methods

The protocols for the experiments detailed in this chapter are described in sections 2.1-2.4 and 2.8-2.10 of chapter 2.

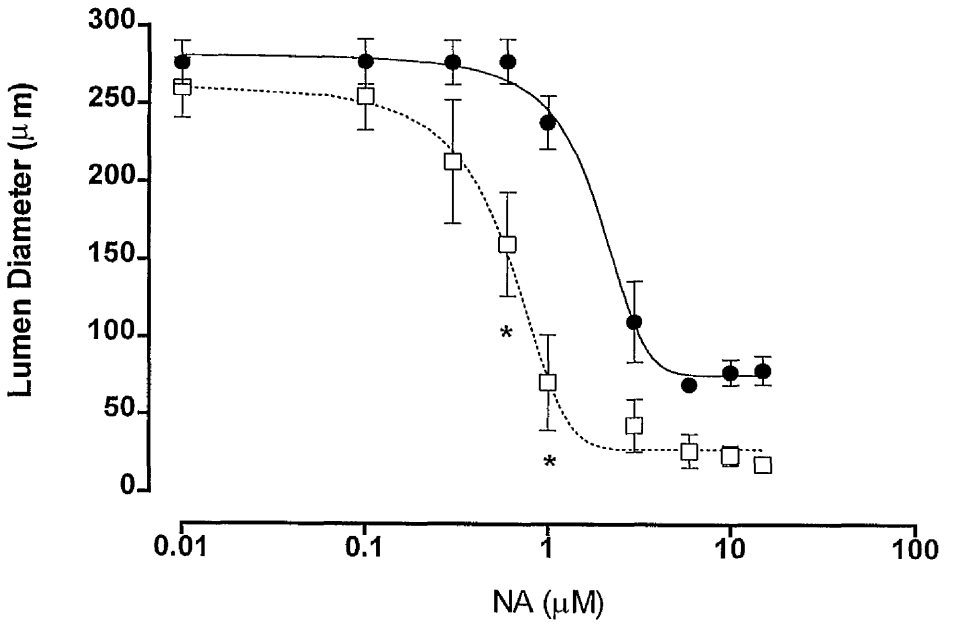


Fig. 7.1: The effect of cholesterol depletion on NA-stimulated RMSA contraction. This is a previously unpublished observation (Alder and Ohanian). Data are expressed as mean ± SEM lumen diameter. RMSA were stimulated with NA in the absence (closed circles, solid line) and presence (open squares, dashed line) of 2% CD. (* P <0.05, ANOVA and Bonferroni post-test, CD compared to basal, n=4).

3.0 Results

3.1 Effect of cholesterol depletion on PI levels

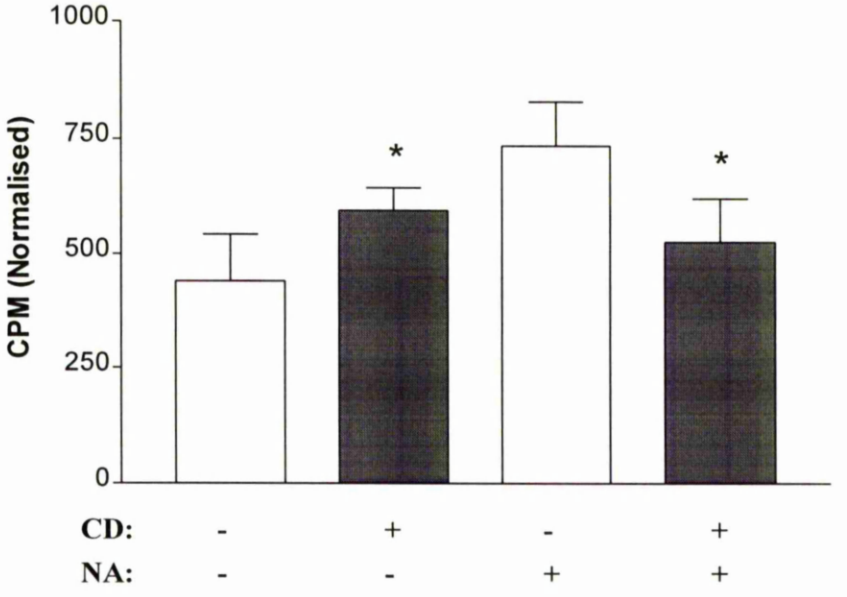
3.1.1 NA-stimulated [^{33}P]-PIP_2 and [^{33}P]-PIP levels

[^{33}P]-labelled RMSA were incubated in 2% CD for 1 hour prior to stimulation with NA (15μM, 5 min) or dH_2O (vehicle). Total [^{33}P]-PIP_2 and [^{33}P]-PIP levels were analysed as described in chapter 2. In unstimulated RMSA, cholesterol depletion significantly increased [^{33}P]-PIP_2 levels by 1.59 ± 0.29-fold (P <0.05, n=5, **Fig. 7.2A**). However, although [^{33}P]-PIP levels were also increased (1.43 ± 0.27-fold), this was not significant (P >0.1, n=5, **Fig. 7.2B**). By contrast, with NA stimulation, cholesterol depletion significantly decreased [^{33}P]-PIP_2 levels to 71 ± 8% of non-depleted samples (P <0.02, n=5, **Fig. 7.2A**) whilst [^{33}P]-PIP levels were significantly decreased to 65 ± 6% of non-depleted samples (P <0.01, n=5, **Fig. 7.2B**).

3.1.2 ET-1-stimulated [^{33}P]-PIP_2 and [^{33}P]-PIP levels

[^{33}P]-labelled RMSA were incubated in 2% CD for 1 hour prior to stimulation with ET-1 (100nM, 10 min) or dH_2O (vehicle). Total [^{33}P]-PIP_2 and [^{33}P]-PIP levels were analysed as described in chapter 2. In unstimulated RMSA, cholesterol depletion significantly increased [^{33}P]-PIP_2 levels by 1.18 ± 0.07-fold (P <0.05, n=5, **Fig. 7.3A**). However, although [^{33}P]-PIP levels were also increased (1.28 ± 0.16-fold), this was not significant (P >0.2, n=5, **Fig. 7.3B**). With ET-1 stimulation, [^{33}P]-PIP_2 levels were reduced to 79 ± 11% of non-depleted samples but this was not significant (P >0.05, n=5, **Fig. 7.3A**). Similarly, [^{33}P]-PIP levels were reduced to 92 ± 15% of non-depleted samples but this was not significant (P >0.15, n=5, **Fig. 7.3B**).

A



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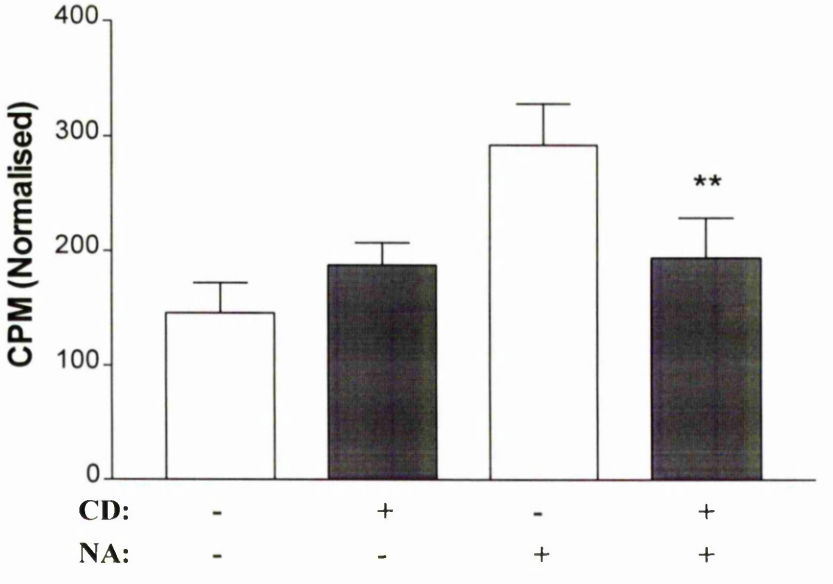
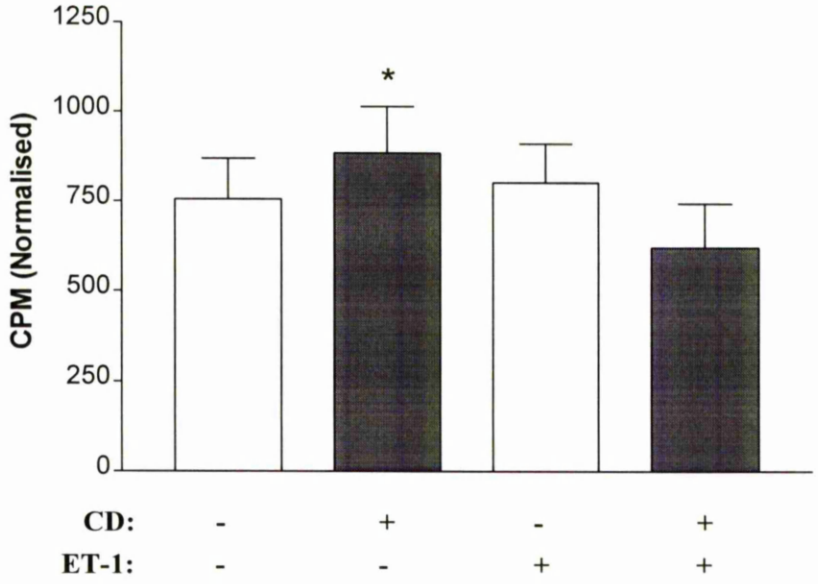


Fig. 7.2: Effect of cholesterol depletion on NA-stimulated PI levels. [^{33}P]-labelled RMSA were incubated in the presence (closed bars) or absence of 2% CD (open bars) for 1 hour prior to stimulation with dH_2O (vehicle) or NA (15μM, 5 min), and total **A)** [^{33}P]-PIP_2 and **B)** [^{33}P]-PIP content analysed as described in chapter 2. Results are expressed as mean ± SEM counts per minute normalised for protein content (* P <0.05; ** P <0.01, t-test, CD compared to basal, n=5).

A



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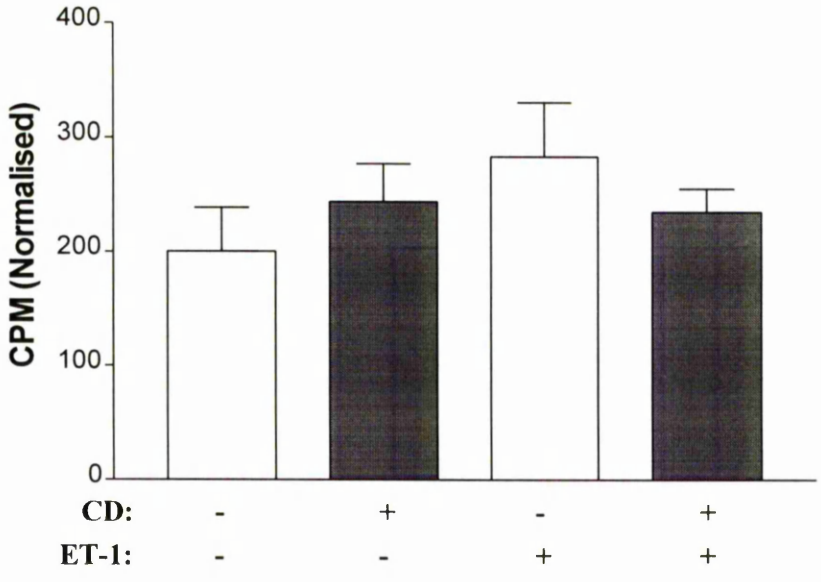


Fig. 7.3: Effect of cholesterol depletion on ET-1-stimulated PI levels. [^{33}P]-labelled RMSA were incubated in the presence (closed bars) or absence of 2% CD (open bars) for 1 hour prior to stimulation with dH_2O (vehicle) or ET-1 (100nM, 10 min), and total A) [^{33}P]-PIP_2 and B) [^{33}P]-PIP content analysed as described in chapter 2. Results are expressed as mean ± SEM counts per minute normalised for protein content (* P <0.05, t-test, CD compared to basal, n=5).

3.2 Effect of cholesterol depletion on membrane-associated DGK activity

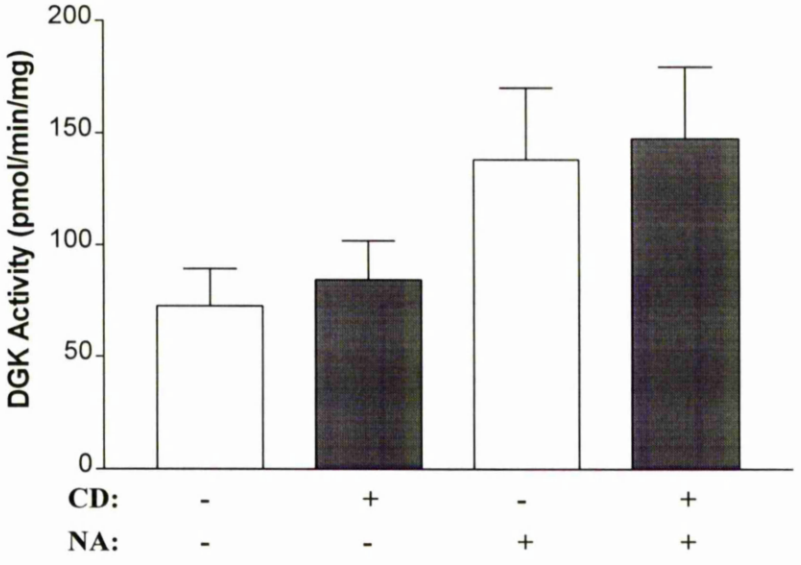
To determine if DGK activity is affected by cholesterol depletion, RMSA were incubated in 2% CD for 1 hour prior to stimulation with NA (15μM, 1 min), ET-1 (100nM, 5 min) or dH_2O (vehicle) and membrane-associated DGK activity extracted and assayed as described in chapter 2. Time points were chosen to reflect peak DGK activity by NA and ET-1 (**chapter 5**) (Ohanian & Heagerty, 1994). In unstimulated RMSA, cholesterol depletion had no significant effect on membrane-associated DGK activity at either time point tested (P >0.15, at least three experiments, **Fig. 7.4**). Similarly, both NA- and ET-1-stimulated membrane-associated DGK activity were unaffected by cholesterol depletion (P >0.30, at least three experiments, **Fig. 7.4**).

3.3 Effect of cholesterol depletion of [^{33}P]-PA levels

3.3.1 NA-stimulated total [^{33}P]-PA levels

[^{33}P]-labelled RMSA were incubated in 2% CD for 1 hour prior to stimulation with NA (15μM, 5 min) or dH_2O (vehicle) and total [^{33}P]-PA levels analysed as described in chapter 2. In unstimulated RMSA, cholesterol depletion significantly increased [^{33}P]-PA levels by 2.05 ± 0.36-fold (P < 0.03, n=6). Similarly, cholesterol depletion significantly increased NA-stimulated [^{33}P]-PA levels by 1.47 ± 0.16-fold (P <0.02, n=6, **Fig. 7.5A**).

A



B

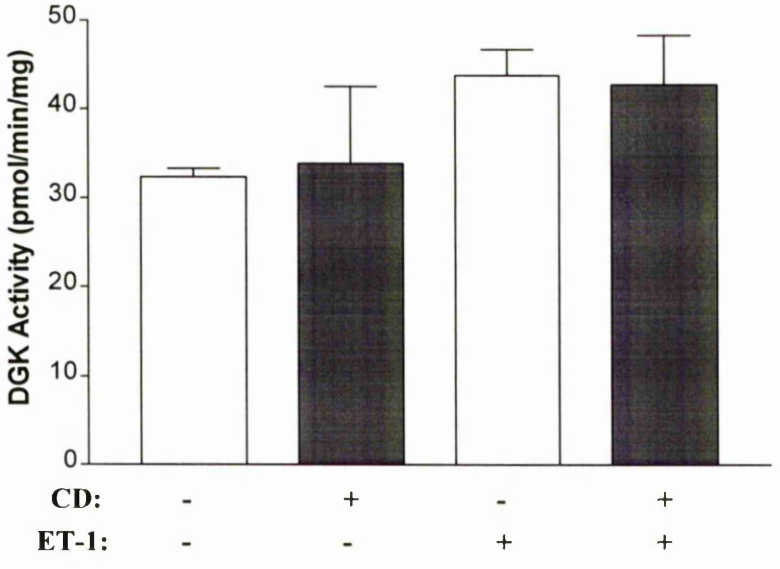


Fig. 7.4: Effect of cholesterol depletion on membrane-associated DGK activity. RMSA were incubated in the presence (closed bars) or absence of 2% CD (open bars) for 1 hour prior to stimulation with dH_2O (vehicle), **A)** NA (15μM, 1 min) or **B)** ET-1 (100nM, 5 min), membrane-associated DGK activity extracted and assayed as described in chapter 2. Results are expressed as mean ± pmol PA/min/mg protein and are from at least three separate experiments.

3.3.2 NA-stimulated [^{33}P]-PA levels in caveolae/rafts

To determine where in the membrane [^{33}P]-PA levels are increased following cholesterol depletion, [^{33}P]-labelled RMSA were incubated with 2% CD for 1 hour prior to stimulation with NA (15μM, 5 min), membrane fractions prepared and [^{33}P]-PA levels analysed as described in chapter 2. Analysis by ANOVA indicated that the NA-stimulated [^{33}P]-PA profile was significantly increased following cholesterol depletion (P <0.02, n=5) although Bonferroni post-test analysis indicated that [^{33}P]-PA levels were not significantly increased in any individual fraction (P >0.05, n=5, **Fig. 7.5B**).

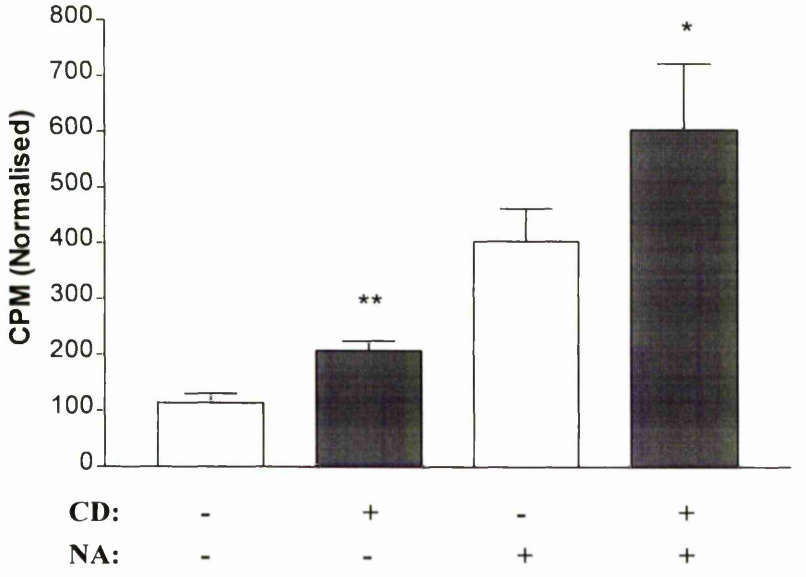
3.3.3 ET-1-stimulated total [^{33}P]-PA levels

[^{33}P]-labelled RMSA were incubated in 2% CD for 1 hour prior to stimulation with ET-1 (100nM, 10 min) or dH_2O (vehicle) and total [^{33}P]-PA levels analysed as described in chapter 2. In unstimulated RMSA, cholesterol depletion significantly increased [^{33}P]-PA levels by 1.79 ± 0.09-fold (P < 0.03, n=3). Similarly, cholesterol depletion significantly increased ET-1-stimulated [^{33}P]-PA levels by 1.61 ± 0.03-fold (P <0.04, n=3, **Fig. 7.6A**).

3.3.4 ET-1-stimulated [^{33}P]-PA levels in caveolae/rafts

[^{33}P]-labelled RMSA were incubated with 2% CD for 1 hour prior to stimulation with ET-1 (100nM, 10 min), membrane fractions prepared and [^{33}P]-PA levels analysed as described in chapter 2. Analysis by ANOVA indicated that the ET-1-stimulated [^{33}P]-PA profile was significantly increased following cholesterol depletion (P <0.01, n=3). Furthermore, Bonferroni post-test analysis indicated that [^{33}P]-PA levels were significantly increased specifically in fractions 2,5 and 13

A



B

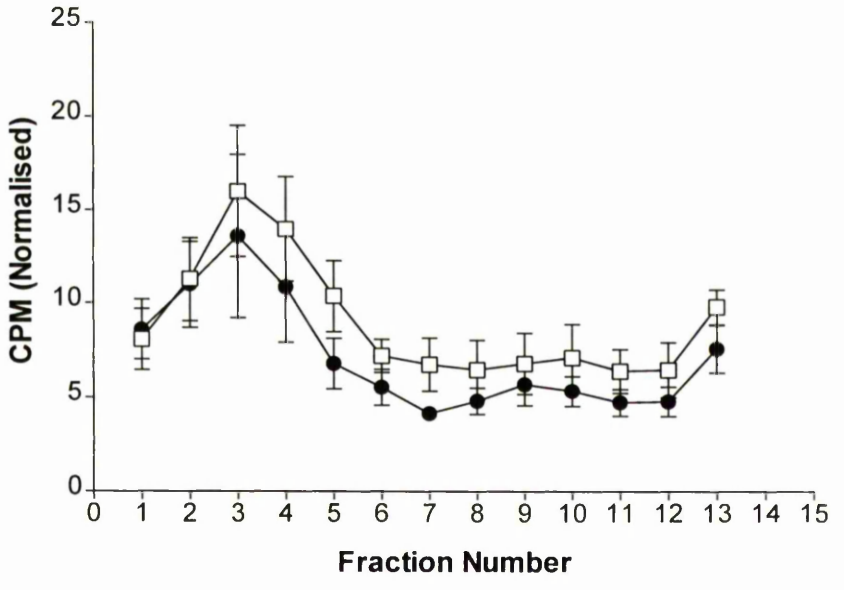
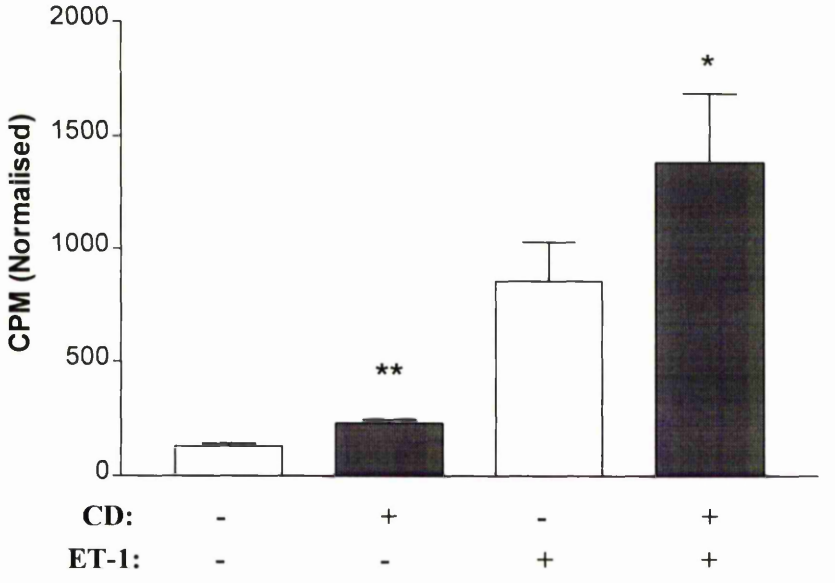


Fig. 7.5: Effect of cholesterol depletion on NA-stimulated PA levels. [^{33}P]-labelled RMSA were incubated in the presence (closed bars, open squares) or absence of 2% CD (open bars, closed circles) for 1 hour prior to stimulation with dH_2O (vehicle) or NA (15μM, 5 min) and **A)** total [^{33}P]-PA content analysed **B)** membrane fractions prepared and [^{33}P]-PA content analysed as described in chapter 2. Results are expressed as mean ± SEM counts per minute normalised for total protein content (* P <0.05; ** P <0.01, t-test, CD compared to basal, at least three experiments)

A



B

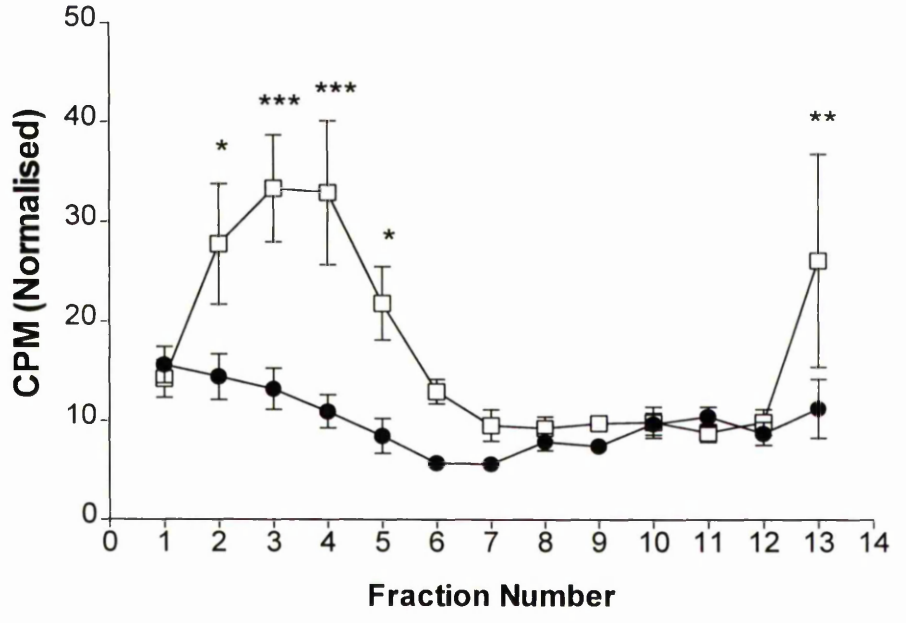


Fig. 7.6: Effect of cholesterol depletion on ET-1-stimulated PA levels. [^{33}P]-labelled RMSA were incubated in the presence (closed bars, open squares) or absence of 2% CD (open bars, closed circles) for 1 hour prior to stimulation with dH_2O (vehicle) or ET-1 (100nM, 10 min) and A) total [^{33}P]-PA content analysed B) membrane fractions prepared and [^{33}P]-PA content analysed as described in chapter 2. Results are expressed as mean ± SEM counts per minute normalised for total protein content (* P <0.05; ** P <0.01; *** P <0.001, t-test, CD compared to basal, at least three experiments)

(P >0.05, n=3) and very significantly increased in fractions 3 and 4 (P <0.001, **Fig. 7.6B**).

4.0 Discussion

4.1 Effect of CD on [^{33}P]-PIP_2 levels

In unstimulated RMSA, CD treatment significantly increased [^{33}P]-PIP_2 levels. As basal PIP_2 metabolism would be low, this suggests enhanced PIP_2 production. This is supported by a recent *in vitro* study reporting that CD enhances activity of recombinant PIP kinases (Davis *et al.*, 2004). However, [^{33}P]-PIP levels were not significantly decreased following cholesterol depletion suggesting that this may not be the case *in vivo* although this could be explained by a similarly enhanced turnover of PI to [^{33}P]-PIP. As PI is not isolated by the chromatographic separation used, this could not be verified directly.

In NA-stimulated RMSA, cholesterol depletion significantly reduced both [^{33}P]-PIP_2 levels and [^{33}P]-PIP levels suggesting an enhanced metabolism of PIP_2. Consistent with this, CD treatment also significantly reduced [^{33}P]-PIP levels in NA-stimulated RMSA. By contrast, cholesterol depletion had no effect on ET-1-stimulated [^{33}P]-PIP_2 or [^{33}P]-PIP levels suggesting that caveolae/rafts may be less important for ET-1-stimulated PI signalling and consistent with a greater NA-induced hydrolysis of [^{33}P]-PIP_2 as suggested by results in chapter 3. The enhanced metabolism of [^{33}P]-PIP_2 in NA-stimulated RMSA suggested is contrary to previous studies in A431 cells (Pike & Miller, 1998) and in agonist-stimulated T-cells - where CD treatment disrupted arachidonate-DAG production (Rouquette-Jazdanian *et al.*, 2002).

However, this could be a result of tissue-specific differences or reflect differences between intact tissue and cultured cells.

The increased PIP_2 hydrolysis could be a result of increased substrate availability through enhanced activity of PIP kinases. However, as basal PIP levels were unaffected whereas PIP_2 levels were increased, it is unclear if PIP kinase activity is affected by CD or cholesterol depletion in RMSA. Alternatively, cholesterol depletion could activate PLC-γ_1 through ligand-independent activation of growth factor receptors as was reported recently in COS cells (Chen & Resh, 2004) although if this was a general effect, it would be expected to occur in both NA and ET-1-stimulated RMSA. Another possibility is that increased basal PIP_2 levels could have effects such as on PLC-δ_1 whose activity is markedly increased by PIP_2 (Lomasney *et al.*, 1996). As PLC-δ_1 is involved in NA but not ET-1 stimulated PIP_2 hydrolysis (**chapter 4**), this could also account for the different effects of cholesterol depletion on [^{33}P]-PIP_2 levels in NA and ET-1-stimulated RMSA. Another possibility is that [^{33}P]-PIP_2 is delocalised across the membrane allowing easier access for active metabolising enzymes although this theory is contrary to an early study in agonist-stimulated A431 cells wherein delocalisation of PIP_2 away from caveolae/rafts inhibited PIP_2 hydrolysis (Pike & Miller, 1998). Furthermore, as caveolae/rafts may be intact in cholesterol-depleted RMSA, delocalisation of [^{33}P]-PIP_2 might not occur. However, this was not examined in the current study.

Surprisingly, NA stimulated a significant increase in total [^{33}P]-PIP_2 and [^{33}P]-PIP levels in non-treated RMSA, contrary to the results in chapters 3 indicating decreased [^{33}P]-PIP_2 in caveolae/rafts of NA-stimulated RMSA at the same time point. The

reasons for this discrepancy are unclear although it could be a result of less efficient lipid extraction from the caveolae/raft membranes following treatment with sodium carbonate. Alternatively, the difficulties in obtaining the total non-caveolae/raft membrane pellet may have led to an underestimation of [^{33}P]-PIP_2 levels in non-caveolae/raft fractions that could account for the observed difference.

4.2 Effect on membrane-associated DGK activity

Depletion of cellular cholesterol had no significant effect on basal, NA- or ET-1-stimulated membrane-associated DGK activity. As enhanced PIP_2 hydrolysis would lead to increased DAG production, this suggests that DAG levels do not regulate NA or ET-1-stimulated DGK activity *per se* in RMSA consistent with other research in fibroblasts and T-cells (Van der Bend *et al.*, 1994) and WRK-1 cells (Monaco *et al.*, 2002)

4.3 Effect on [^{33}P]-PA levels

Cholesterol depletion significantly increased basal, NA and ET-1-stimulated [^{33}P]-PA levels. Whilst this disagrees with previous studies in thrombin-stimulated platelets where PA production was disrupted by CD depletion (Bodin *et al.*, 2001), this could reflect tissue-specific differences. Alternatively, as caveolae/rafts might be intact in cholesterol-depleted RMSA, this could also account for the differences.

As NA and ET-1-stimulated DGK activity was unaffected, this suggests that downstream metabolism of [^{33}P]-PA has been reduced. As the primary fate of PI-derived PA is conversion to CDP-DAG by the enzyme CDP-DAG synthase (CDS) (Heacock & Agranoff, 1997), this suggests inhibition of CDS activity. However, this

enzyme is localised to the ER (Heacock & Agranoff, 1997) whereas cholesterol extracted by CD is from the extracellular leaflet of the plasma membrane suggesting otherwise (Lai, 2003;Ohno-Rekila *et al.*, 2002). However, for PA metabolism to occur, transport of the phospholipid to the ER is required. Thus, an alternative explanation for the increased PA levels observed is reduced transport from the plasma membrane. One possible means of transport for PA, consistent with localisation of its production, could be through endocytosed caveolae/rafts and correlating with the theory that PA is the trigger for caveolae/raft endocytosis through activation of dynamin as proposed recently (Burger *et al.*, 2000) and that once internalised, caveolae/rafts can be transported to the ER (Nabi & Le, 2003;Henley *et al.*, 1998). Furthermore, as endocytosis of caveolae/rafts is reported to be cholesterol-dependent (Nabi & Le, 2003), this would be consistent with the effects of CD on PA levels observed – with PA remaining 'trapped' at the membrane. Further consistent with this, ET-1-stimulated PA levels were dramatically increased in caveolae/raft fractions over non-caveolae/raft fractions (**Fig. 7.6B**). As DGK activity is unaffected by cholesterol depletion, this suggests that PA produced is targeted to the caveolae/rafts and remains 'trapped' there in cholesterol-depleted RMSA. However, this theory is inconsistent with the effects of cholesterol-depletion in NA-stimulated RMSA as a more delocalised increase in PA levels across membrane fractions observed. Whilst this could reflect agonist-specific differences, a study in neutrophils reporting that PA is not transported to the ER by soluble lipid carriers or vesicular transport (Whatmore *et al.*, 1999) further suggests that other factors are involved in the increased [^{33}P]-PA levels observed in cholesterol-depleted RMSA.

4.4 Cholesterol depletion and VSM contraction

Previously unpublished work in RMSA found that cholesterol depletion enhanced the contractile response to NA (**Fig. 7.1**), contrary to previous studies in cultured aortic VSM cells reporting inhibition of α_1-adrenergic-stimulated contraction on depletion of cholesterol with CD (Je *et al.*, 2004) and in rat caudal artery reporting the vasoconstrictor response to α_1-adrenergic agonists was unaffected although the response to ET-1 and vasopressin was disrupted (Dreja *et al.*, 2002). This could be due to differences between tissue and cultured cells in the case of the former study or may reflect differences between the large and small arteries. Additionally, in the latter study, preparations were endothelium-denuded. Thus, any possible effects of cholesterol depletion on endothelium-dependent contractile regulation would be absent.

The enhanced contractile response to NA in cholesterol-depleted RMSA suggests that signalling pathways regulating VSM contraction have been affected, consistent with the effects of CD on NA -stimulated PI signalling discussed above. However, it cannot be said if this is also the case with ET-1, as the effect of cholesterol depletion on the ET-1 response in RMSA is not known. The enhanced metabolism of PIP_2 suggested above would, by extension, result in increased production of the second messengers IP_3 and DAG. As these second messengers are important in the development and regulation of agonist-induced contractile force through calcium mobilisation and PKC activation (Ganitkevich *et al.*, 2002), this could contribute towards the enhanced contractile response to NA observed. Furthermore, as DGK activity is unaffected by cholesterol depletion, this further suggests that PI-derived DAG would accumulate and lead to increased PKC activity and subsequent

activation of PKC-dependent pathways. Finally, PA has also been proposed to play a role in VSM contraction (Jones *et al.*, 1993;Ohanian *et al.*, 1990) and so increased cellular levels of PA observed in cholesterol-depleted RMSA could also contribute to the enhanced contractile response through hyperactivation of PA-regulated signalling cascades. One such possibility is hyperactivation of the Ras-Raf-Mek-ERK cascade as this has been implicated in VSM contraction through phosphorylating the actin-associated protein caldesmon (Morgan & Gangopadhyay, 2001;Khalil *et al.*, 1995). Consistent with this theory, a number of studies have reported hyperactivation of the ERK cascades in cholesterol-depleted Raf-1 cells (Furuchi & Anderson, 1998), fibroblasts and HeLa cells (Wang *et al.*, 2003) although in the latter case, this was attributed to disassembly of a high molecular weight phosphatase complex (Wang *et al.*, 2003) rather than hyperactivation of upstream activators. Furthermore, as it is unclear if PA is involved in the Ras-Raf-Mek-ERK cascade (**chapter 6**) in RMSA, the validity of this theory is unclear. Finally, as very few *in vivo* targets of PA have been identified, considerable further research is required to determine the PA-regulated signalling cascades before speculations into signalling pathways hyperactivated on cholesterol depletion can be made.

4.5 Other effects of cholesterol depletion

Since undertaking this study, considerable evidence has emerged that cholesterol depletion has more wide ranging effects than just disruption of caveolae/rafts. One such effect of particular relevance in VSM is the effect of cholesterol depletion on the actin cytoskeleton. Previous studies have reported that cholesterol sequestration caused release of the actin-binding and raft-associated annexin II and the cytoskeletal proteins α-actinin, ezrin and moeisin from the

membrane (Harder *et al.*, 1997), also reducing the actin-driven activity of regulatory cytoskeletal proteins at the cell surface (Caroni & Golub, 2002). Another study found that redistribution of PIP_2 at the plasma membrane induced by cholesterol depletion affected the actin cytoskeleton (Kwik *et al.*, 2002) although whether PIP_2 redistribution occurs in cholesterol-depleted RMSA is unclear as discussed above. However, as remodelling of the actin cytoskeleton has been implicated in sustained VSM contraction (Gerthoffer & Gunst, 2001;Woodrum & Brophy, 2001), the effects of cholesterol depletion on this process cannot be excluded from contributing to the altered NA response of RMSA. Finally, cholesterol depletion has other reported effects such as decreased caveolae endocytosis (Nabi & Le, 2003), decreased clathrin-coated pit endocytosis (Subtil *et al.*, 1999), altered lipid turnover (Leppimaki *et al.*, 1998) and reduced exocytosis (Lang *et al.*, 2001). Thus, these cannot be excluded from having an effect on contractile responses in NA-stimulated RMSA.

5.0 Summary and Conclusions

Cholesterol depletion had differential effects on NA and ET-1-stimulated PI signalling, consistent with a role for caveolae/rafts in agonist-stimulated RMSA contraction. However, as it is now clear that cholesterol depletion has other effects, the enhanced contractile response to NA in cholesterol-depleted RMSA (**Fig. 7.1**) may not be due solely to disruption of caveolae/raft domains. Furthermore, it is not clear if caveolae/rafts are fully disrupted in RMSA as suggested by the differential effects of CD treatment on caveolin-1, GM_1 and caveolin-3 distribution.

CHAPTER 8

Summary and Conclusions

The spatial regulation of signalling pathways is now understood to be a major component of agonist-induced signal transduction. Caveolae/rafts are specialised plasma membrane domains proposed to be involved in signal transduction and recently implicated in regulation of vascular tone. The data presented in this thesis supports the involvement of caveolae/rafts in signal transduction as sites of lipid signalling and supports a role for caveolae/rafts in VSM contraction as sites where agonist-stimulated PI turnover couples to intracellular signalling pathways. These results are summarised in a schematic model of lipid dynamics in VSM (**Fig. 8.1**).

In VSM, the agonist-induced turnover of PIP_2 is a major step for the development of agonist-induced contractile force. The localisation of this process solely to caveolae/rafts and the fact this occurs with both NA and ET-1 suggests that they play a role in the coupling of vasoconstrictor-stimulated PIP_2 turnover to intracellular signalling through localised production of second messengers as discussed in chapter 3. This theory would be strengthened if it were shown to occur with other vasoconstrictors. The localisation of NA and ET-1-stimulated DGK activity to caveolae/rafts further supports a role for these domains in PI turnover and lipid signalling. Consistent with this, both NA and ET-1-stimulated DGK- and PI-derived PA production was also localised to caveolae/rafts. This suggests that these domains are sites where lipid second messengers couple to intracellular signalling cascades although preliminary experiments with the *in vitro* PA targets Raf-1, PP1c and MYPT-1 indicated that they did not translocate to caveolae/rafts on agonist

stimulation. However, in order to understand how localised PA production may be involved in VSM contraction, considerable work identifying the *in vivo* targets of PA is required before the signalling pathways involved in VSM can be elucidated.

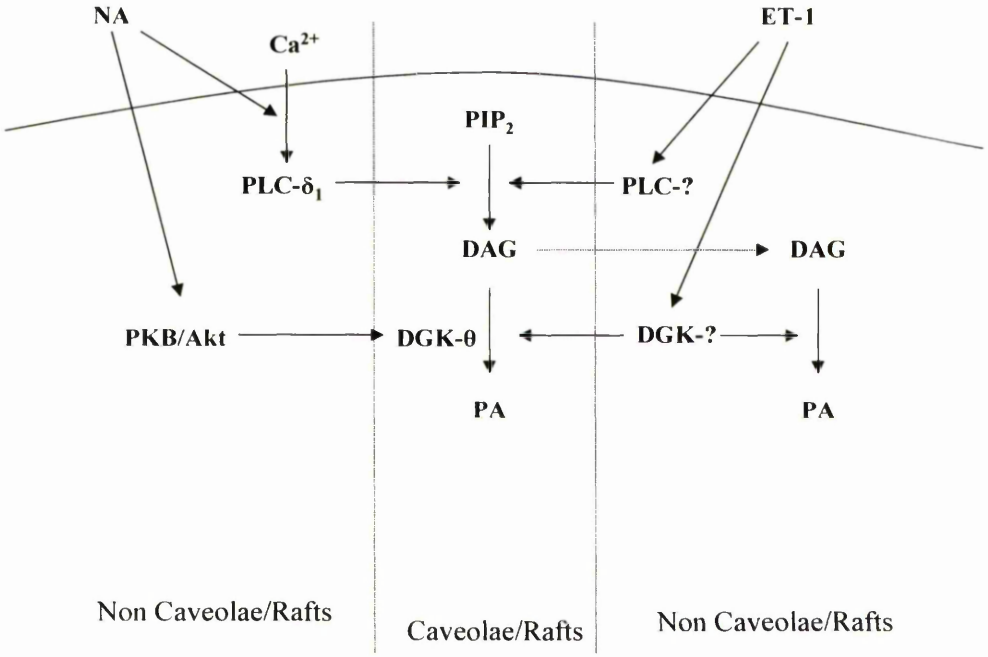


Fig. 8.1: Schematic model of lipid dynamics in RMSA. As this model shows, whilst caveolae/rafts are common sites of noradrenaline and endothelin-1-stimulated PIP_2 turnover, responses to these agonists diverge on subsequent metabolism of DAG to PA by DGK indicating that spatial dynamics of lipid production may be important for agonist-specific responses in vascular smooth muscle.

Although the results in this thesis all support a role for caveolae/rafts in vasoconstrictor-stimulated PI signalling, it is also suggested from this and other research that this role does not appear to be essential for agonist-stimulated VSM contraction. Experiments with cholesterol depletion in this study indicated an enhanced contractile response to NA, possibly as a consequence of altered PI signalling, rather than a blunted/inhibited contractile response. Furthermore, previous

experiments in cholesterol-depleted rat-tail artery reported only a moderate reduction of contraction in response to ET-1 with no effect on contraction stimulated by α_1-adrenergic agonists (Dreja *et al.*, 2002). Although these results must be interpreted with caution, as it is not clear if caveolae/rafts were disrupted in cholesterol-depleted RMSA and cholesterol depletion has effects other than caveolae/raft disruption, results in caveolin (-/-) knockout mice also suggest a non-essential role for caveolae/rafts in VSM contraction. Whilst previous research in caveolin-1 (-/-) knockout mice reported blunted contractile responses, this was attributed to enhanced NO-dependent vasodilation as a result of increased basal NO levels (Drab *et al.*, 2001; Razani *et al.*, 2001). These results all suggest that integrity of caveolae/rafts is not a prerequisite for agonist-induced development of contractile force but that these domains may play a more facilitatory role in VSM contraction. This could be through the regulation of signalling cascades important for sustained VSM contraction such as calcium sensitisation (Ganitkevich *et al.*, 2002). This is supported by previous research reporting that translocation of PKC-α and RhoA translocation, both involved in this process (Taggart *et al.*, 2000) is prevented by an inhibitory caveolin-1 peptide. Furthermore, PKC-dependent contractile regulation and carbachol-induced contraction of smooth muscle were both prevented by an inhibitory caveolin-1 peptide (Je *et al.*, 2004; Lee *et al.*, 2001).

Results in this thesis also show that the levels of the lipid second messenger PA are sustained for as long as 20 minutes – supporting a role for this lipid in the regulation of tonic contraction. However, these measurements were steady-state time averaged measurements whereas recent studies in pressurised RMSA have reported that tonic contraction in response to α_1-adrenergic agonists and endothelin-1 is accompanied

by dynamic second-by-second oscillations in intracellular calcium of individual smooth muscle cells. These oscillations are asynchronous (Shaw *et al.*, 2004) and are dependent on the IP_3 receptor (Lamont and Weir, 2004) suggesting that oscillating levels of IP_3 may be involved in the sustained phase of contraction. It would be important for future studies, should technical advances allow, to address whether similar asynchronous oscillations in lipid mediator levels occur as this would indicate a more dynamic regulation of agonist-stimulated lipid turnover at the membrane than anticipated and may also shed light as to which lipid second messengers and lipid species are important for regulation of VSM contraction. Additionally, this may provide a rationale for the localisation of contractile signalling to caveolae/lipid rafts i.e. these domains provide an environment where second messenger levels can be tightly regulated and efficiently coupled to intracellular signalling through co-localisation of effectors.

Results in this thesis also indicate agonist-specific differences in PI signalling in RMSA. The differential translocation of PLC-δ_1 to caveolae/rafts in response to NA and ET-1 indicated agonist-specific activation of PLC isoforms. Also, the differential dependence of NA and ET-1-stimulated DGK activity and PA production on PI3K indicates agonist-specific activation of DGK isoforms – in this case DGKθ - and results suggest that this is due to agonist-specific translocation of PKB/Akt to caveolae/rafts. These results suggest that agonist-specific recruitment of enzyme isoforms to caveolae/rafts is an important factor in specificity for vasoconstrictors utilising a common transmembrane signalling system, a theory that would be strengthened by further research with other vasoconstrictors.

Based on the results in this thesis, the schematic of lipid dynamics in VSM (**Fig. 8.1**) clearly suggests that caveolae/rafts are a focal point for initiation of agonist-stimulated PI turnover in VSM and that future studies must also consider the spatial localisation of lipid second messenger production when investigating lipid signalling in cellular function. The agonist-specific differences in DGK activity and PA production observed in this study further suggests that spatial regulation of lipid mediator production is an important mechanism for determining signalling specificity and that levels of lipid second messengers in both caveolae/rafts and non-caveolae/rafts must contribute to the cellular response. It is a necessity for future research to address how such spatial differences in lipid second messenger production are translated into differential regulation of contractile force in VSM.

There is increasing evidence that caveolae/rafts play a role in VSM contraction with results in this thesis indicating that these microdomains are also important for lipid signalling as well as protein signalling pathways in vascular tissue. Extending these studies to include other physiological vasoconstrictors would help to determine if caveolae/rafts play an agonist-specific role in VSM contraction as suggested by previous studies in rat tail artery (Dreja *et al.*, 2002). Also, the identification of further agonist-specific temporal, spatial and isoform differences may provide an opportunity for specific therapeutic targets. Additionally, an investigation of agonist-stimulated caveolae/raft signalling in vascular tissue in pathological states would determine if spatial disorganisation of signalling is a factor in the development of diseases such as hypertension, as has recently been suggested for atherosclerosis (Bergdahl & Sward, 2004) and may offer possible avenues of treatment. Finally, other lipid mediators have been implicated in regulation of vascular tone including

arachidonic acid, ceramide and sphingosine-1-phosphate (Dantas *et al.*, 2003; Castillo *et al.*, 2002; Zheng *et al.*, 2000). Given the localisation of PI signalling to caveolae/rafts reported in this thesis, future work directed at further examining the localisation and dynamics of production of these second messengers in VSM may shed further light on the role that caveolae/rafts play in regulation of vascular contractility.

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