REGULATION OF INOSITOL POLYPHOSPHATE METABOLISM IN AIRWAYS SMOOTH MUSCLE

Barbara Jane Lynch

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DECLARATION

The work presented in this thesis was carried out solely by the author, unless otherwise stated, under the supervision of Dr Edwin Chilvers. All experimental work was carried out in the Rayne Laboratories at the City Hospital, Edinburgh and the University of Edinburgh Medical School and was financed by the Chest, Heart and Stroke Association, U.K.

ABSTRACT

Agonist-induced contraction of airways smooth muscle is mediated by phosphoinositide hydrolysis and the production of the second messenger inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3). Muscarinic receptor-stimulation of bovine tracheal smooth muscle (BTSM) results in a transient increase in Ins(1,4,5)P_3 mass despite a sustained, non-desensitising hydrolysis of its precursor phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2). Hence the rapid metabolism of Ins(1,4,5)P_3 appears to be the major regulator of Ins(1,4,5)P_3 levels under agonist-stimulated conditions. A model system has been developed to facilitate detailed study of the pathways involved in this complex metabolism. BTSM slices were labelled to equilibrium with *myo*-[^{3}H]inositol in the presence of agonist and subsequent carbachol (CCh)- or histamine (Hist)-stimulations carried out in the presence of lithium ions to block InsP_1 breakdown. A delayed accumulation of [^{3}H]Ins1/3P and [^{3}H]Ins4P was observed under agonist-stimulated conditions. Moreover, there was no demonstrable phosphoinositide hydrolysis either following membrane-depolarisation, or secondary to a physiologically relevant increase in intracellular calcium in this tissue. The model therefore provides an appropriate system for the study of receptor-stimulated PtdIns(4,5)P_2-derived Ins(1,4,5)P_3 metabolism. Cell-free experiments confirmed that Ins(1,4,5)P_3 is metabolised primarily by two different pathways - a 3-kinase and a 5-phosphatase pathway - which yield mutually exclusive products. H.P.L.C. separation of the individual [^{3}H]inositol polyphosphate (InsPP) isomers accumulating in BTSM slices enabled the 3-kinase and 5-phosphatase metabolites to be quantified, and facilitated the determination of flux of the inositol headgroup through these two pathways. The pattern

of $Ins(1,4,5)P_3$ metabolism varies during the lifetime of the agonist-stimulated response: The 5-phosphatase enzyme is highly dominant especially at early time-points following agonist-stimulation, whilst the 3-kinase becomes increasingly important at later time-points. Several possible regulators of the InsPP metabolising enzymes were studied in order to try and elucidate the factors which may determine the fate of $Ins(1,4,5)P_3$ in BTSM. Kinetic analysis reveals that physiological concentrations of calcium ions have no effect on the activity of the 5-phosphatase and 3-kinase enzymes in crude BTSM extracts. It is generally thought that the 3-kinase may be activated by Ca^{2+}/calmodulin via an increase in its V_{max}; however this conclusion is based on studies pre-dating the discovery that 3-kinase may be a substrate for the calcium-activated neutral protease calpain. The inclusion of calpain inhibitors in my experiments may explain the conflicting results. The metabolism of $Ins(1,4,5)P_3$ in stimulated BTSM slices appears to be agonist-specific such that the proportion of $[^3H]Ins(1,4,5)P_3$ entering the 3-kinase pathway is greater in slices stimulated with Hist than with CCh. The protein kinase C-activating phorbol ester phorbol dibutyrate has a more potent inhibitory effect on the accumulation of $[^3H]$InsPPs stimulated by Hist (IC_{50} = 5 nM) than by CCh (IC_{50} = 230 nM). Similarly, the long-acting β_2-agonist salmeterol potently inhibits Hist-stimulated $[^3H]$InsPP accumulation (IC_{50} = 0.24 nM) but is without detectable effect on CCh-stimulated $[^3H]$InsPP formation. In addition to the well-characterised 3-kinase and 5-phosphatase pathway metabolites, an agonist-stimulated accumulation of a further $[^3H]InsP_2$, co-eluting with $[^3H]Ins(4,5)P_2$, was observed. Accumulation of this isomer exhibited a bell-shaped lithium concentration response curve and was maximal in the presence of 10 mM LiCl. These data indicate the possible existence of a novel 1-phosphatase enzyme for the removal of $Ins(1,4,5)P_3$ in this tissue.

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ABBREVIATIONS

ACh	Acetylcholine
AEBSF	2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid)
AF-DX-116	11[2-[(diethylamino)-methyl]1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido-[2,3-b][1,4]benzodiazepine-6-one
aPKC	Atypical protein kinase C
ASM	Airways smooth muscle
ATP	Adenosine 5'-diphosphate
ATPase	Adenosine 5'-triphosphate phosphatase
BCA	Bicinchoninic acid
BK_{Ca}	Calcium-activated potassium channel
$[Ca^{2+}]_{free}$	Free calcium ion concentration
$[Ca^{2+}]_i$	Intracellular free calcium ion concentration
cADP-ribose	cyclic adenosine diphosphate ribose
CaM kinase II	calcium/calmodulin-dependent kinase II
cAMP	adenosine 3',5'-cyclic monophosphate
CCh	carbachol
CICR	Calcium-induced calcium release
cGMP	guanosine 3',5'-cyclic monophosphate
CIF	Calcium influx factor
cPKC	Calcium-dependent protein kinase C
DAG	Diacylglycerol
4-DAMP	4-diphenylacetoxy-*N*-methylpiperidine methobromide
Dex	Dexamethasone
DPB	Deoxyisobutyrate

EC_{50}	Concentration of agonist producing 50% of maximal response
E_m	Membrane potential
eNANC	Excitatory non-adrenergic non-cholinergic
F-actin	Filamentous actin
G-actin	Globular actin
G_i	G-protein coupled to inhibition of adenylyl cyclase
G_s	G-protein coupled to stimulation of adenylyl cyclase
G_q	Novel class of G-protein which may couple to activation of PLC-β
GDP	Guanosine 5'-diphosphate
GroPIns	Glycerophosphoinositol
GTP	Guanosine 5'-triphosphate
GTPase	Guanosine 5'-triphosphate phosphatase
GTPγS	Guanosine 5'-*O*-(3-thiotriphosphate)
H_1	Type I histamine receptor
HHSiD	Hexahydrosiladifenidol
Hist	Histamine
H.P.L.C.	High performance liquid chromatography
IBMX	3-Isobutyl-1-methylxanthine
IC_{50}	Concentration of inhibitor causing 50 % maximal inhibition
IM	Ionomycin
iNANC	Inhibitory non-adrenergic non-cholinergic
$InsP_{1,2,3,4,5,6}$	D-*myo*-inositol mono-, bis-, tris-, tetrakis-, pentakis- and hexakisphosphates. Positional isomers, where specified, are given in parentheses.

$Ins(1:2c,4,5)P_3$	Inositol 1:2cyclic,4,5-trisphosphate
$InsP_5P$	Diphosphoinositol pentakisphosphate
$InsP_6P$	Bisdiphosphoinositol tetrakisphosphate
$InsP_3R$-1,2,3,4	$Ins(1,4,5)P_3$ receptor subtypes 1, 2, 3 or 4
KHB	Krebs-Henseleit buffer
K_i	Inhibition constant. The concentration of inhibitor which would occupy 50 % of the receptors if no ligand were present.
K_m	Michaelis constant. The concentration of substrate at which the reaction proceeds at half the maximal velocity.
LC_{20}	20 KDa myosin light chain
$M_{1,2,3,4,5}$	Muscarinic receptor subtypes 1, 2, 3, 4 or 5
m1,2,3,4,5	Genes encoding muscarinic receptor subtypes 1, 2, 3, 4 or 5
MKI	Myosin kinase inhibitor
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
nPKC	Novel protein kinase C
PDBu	Phorbol dibutyrate
PDGF	Platelet-derived growth factor
PIC	Phosphoinositidase C
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PMA	Phorbol myristate acetate
PLC	Phospholipase C
PtdIns	Phosphatidylinositol

PtdInsP(2)	Phosphatidylinositol (bis)phosphate. Positional isomers, where specified, are given in parentheses.
PMSF	Phenylmethylsulphonyl fluoride
PtdOH	Phosphatidate
QNB	Qunuclidinyl benzilate
[S]	Substrate concentration
Salb	Salbutamol
Salm	Salmeterol
SEM	Standard error of the mean
SR	Sarcoplasmic reticulum
TCA	Trichloroacetic acid
$t_{1/2}$	Half-life
V_{max}	Maximal reaction velocity
V_0	Initial reaction velocity

CHAPTER ONE
INTRODUCTION

Control of airways smooth muscle (ASM) tone is essential for regulating airways calibre and hence the resistance of the airways. There is also considerable evidence to suggest that airway wall remodelling, including hypertrophy and hyperplasia of ASM is an important contributer to airway responsiveness in asthmatic patients (Wiggs *et al.*, 1992). A variety of neurotransmitters (released from endogenous neurones), hormones and peptides (either blood-borne or produced locally by pro-inflammatory cells) can elicit contraction of ASM following their binding to, and stimulation of, surface receptors on the ASM cells. It is now well established that excitation-contraction coupling is achieved principally by a pharmacological process (i.e. pharmacomechanical coupling) rather than an electrical response (i.e. electromechanical coupling). Hence agonist stimulation of ASM is associated with the hydrolysis of a minor membrane lipid component namely phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), resulting in the formation of the second messengers inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG) (e.g. see Berridge, 1987; Rana and Hokin, 1990). DAG can activate protein kinase C (PKC) (e.g. Hug and Sarre, 1993; Wilkinson and Hallam, 1994) while Ins(1,4,5)P_3 can stimulate Ca^{2+} release from intracellular stores to provide the activator Ca^{2+} necessary for the initiation of contraction (see Berridge, 1993; Berridge and Irvine, 1989). The agonist-stimulated contraction appears to be unaltered in asthmatic compared to non-asthmatic subjects (Cerrina *et al.*, 1986; Coyle *et al.*, 1990; Goldie *et al.*, 1986; Whicker *et al.*, 1988). Nonetheless, the dominant role played by

$Ins(1,4,5)P_3$ in initiating agonist-mediated contraction in ASM necessitates an understanding of the factors regulating $Ins(1,4,5)P_3$ formation and metabolism in this tissue, and further research may facilitate the generation of clinical modulators of the $Ins(1,4,5)P_3$ response. Moreover, an understanding of the complex interplay between phosphoinositide hydrolysis and other second messenger systems may further our understanding of the mechanisms underlying both the maintainance of contraction of ASM and the relaxant action of currently used bronchodilators.

1.1 AIRWAYS SMOOTH MUSCLE PHYSIOLOGY

1.1.1 Structure of airways smooth muscle

In the foetus the trachealis consists of both outer (longitudinal) and inner (transverse) portions together with a mucosal layer (the muscularis mucosae) (Hakanson *et al.*, 1976); however during development the outer and mucosal layers are lost, and when fully developed only the transverse layer - the muscularis transversus tracheae - remains. The trachealis is classified as a multi-unit smooth muscle consisting of many individual cell units (myocytes) each with their own nerve supply, arranged into thick, non-branching, parallel-set bundles of smooth muscle which connect the ends of the C-shaped tracheal cartilage rings. The individual myocytes are typically 5-10 μm in diameter and 50-200 μm long (Stephens, 1987) and the bundles are physically separated from each other by spaces filled with connective tissue (Dixon and Small, 1983). While several cell-cell communications exist within each bundle in the form of intermediate junctions, the density of true gap junctions is very low in this tissue; furthermore there are almost no communications

linking the muscle cells bundles. The relative paucity of gap junctions confers poor electrical conductivity on the trachealis muscle (Dixon and Small, 1983) and the resting membrane potential (E_m) is very stable (-45 - -60 mV; Stephens, 1987) due to the strong rectifying properties of the plasmalemma (Kirkpatrick, 1975). Following neuronal or pharmacological stimulation ASM does not normally exhibit action potentials; in contrast, the pattern of innervation and the poor electrical conductivity of this tissue confer a slow, graded deplolarisation response in which the difference between the resting E_m and the mechanical threshold is typically only ~ 5 mV (Stephens, 1987).

1.1.2 Innervation of the airways

The innervation of ASM is complex, consisting in most species of two neuronal pathways mediating contraction and two linked to relaxation of the muscle. The trachealis muscle is richly innervated with afferent parasympathetic cholinergic neurones, stimulation of which results in contraction of the muscle. These neurones provide the dominant control of ASM tone, utilising acetylcholine (ACh) as a neurotransmitter. In addition, stimulation of an excitatory non-adrenergic non-cholinergic (eNANC) system can also facilitate contraction of ASM; the tachykinins substance P, neurokinin-A and neuropeptide-B have been localised to sensory afferent nerves and are believed to be the neurotransmitters for eNANC (Hua *et al.*, 1985; Lundberg *et al.*, 1984). Conversely inhibitory non-adrenergic non-cholinergic (iNANC) stimulation mediates relaxation of ASM. The precise identification of the neurotransmitter for this system remains uncertain, but strong candidates are vasointestinal peptide and nitric oxide (Belvisi, 1992; Li and Rand, 1991; Matsuzaki *et al.*, 1980). Direct sympathetic (adrenergic) innervation of ASM, employing

noradrenaline as a neurotransmitter appears to be species dependent; while it has been demonstrated in a number of animal species (although the innervation is often sparse), it appears to be entirely absent in man (Cabezas *et al.*, 1971; El-Bermani, 1987; O'Donnell and Saar, 1973; see Richardson, 1979). Fibres from cervical sympathetic ganglia do penetrate the airways however to supply vascular tissue, and have a regulatory influence at the parasympathetic ganglia level (see Richardson, 1979). In addition, ASM cells possess β-adrenoceptors, predominantly (80-90 %) β_2-adrenoceptors, and hence can elicit relaxation in response to circulating catecholamines (Barnes *et al.* 1983c; Popovich *et al.*, 1984).

1.1.3 Muscarinic acetylcholine receptors in airways smooth muscle

Receptor-binding and autoradiographic studies have identified a high density of muscarinic receptors in the smooth muscle layer of the trachea and large airways from a number of animal species (Barnes *et al.*, 1983a and b; Basbaum *et al.*, 1984; Cheng and Townley, 1982; Murlas *et al.*, 1982; Van Koppen *et al.*, 1985). There appears to be a muscarinic receptor gradient in the ASM of ferret lung as Barnes and colleagues (1983a and b) noted a decrease in binding of the muscarinic ligand [^{3}H]quinuclidinyl benzilate (QNB) from the large to the small airways.

Original studies in other tissues, measuring the binding affinity of the muscarinic antagonist pirenzepine, led to the division of muscarinic receptors into three functional subclasses M_{1-3} (Birdsall *et al.* 1980; Hammer, 1980). The arrival of more specific muscarinic agonists and antagonists however, has highlighted additional heterogeneity of the originally described M_2 subtype. A revised classification on the grounds of several structural and pharmacological criteria identifies at least four (M_{1-4}) receptor subtypes. The latter classification has now been

substantiated by the cloning, sequencing and expression of distinct but homologous genes (*m1-m4*) encoding these receptor subtypes (Bonner, 1989; Peralta *et al.*, 1987). A fifth muscarinic receptor gene (*m5*) has also been cloned from rat and human tissue but currently awaits its pharmacological equivalent (M_5) (Bonner *et al.*, 1988; Liao *et al.*, 1989). Further heterogeneity of muscarinic receptors is suggested by Bognar and colleagues (1992) who observed contraction of rabbit iris smooth muscle following stimulation of a muscarinic receptor with an antagonist-affinity profile distinct from M_{1-4} receptors or indeed that of the *m5* gene product.

It is now evident that many smooth muscles co-express multiple muscarinic receptor subtypes (e.g. Leiber *et al.*, 1990; Mahesh *et al.*, 1992). Displacement of [^{3}H]QNB binding to ASM preparations by M_2-selective (AF-DX-116) and M_3-selective (HHSiD and 4-DAMP) receptor antagonists has identified both M_2 and M_3 receptor subtypes in this tissue, with the former representing up to 89 % of the total muscarinic receptor pool (Fernandes *et al.*, 1992; Mahesh *et al.*, 1992).

Expression of cloned muscarinic receptors has facilitated the identification of the signal transduction mechanisms employed by the receptor subtypes. Generally M_3 receptors couple to stimulation of phosphoinositide hydrolysis (e.g. Pinkas-Kramarski *et al.*, 1988; Wess *et al.*, 1989) wheras activation of M_2 receptors couple via a pertussis toxin-sensitive G-protein (G_i) to inhibtion of adenylyl cyclase (e.g. Ashkenazi *et al.*, 1987, 1989). In accordance with these data, activation of M_3 receptors in ASM results in the generation of contraction, secondary to phosphoinositide hydrolysis (Roffel *et al.*, 1990a), however there is considerable controversy concerning the role of the predominant M_2 receptor pool. It has been suggsted that since M_2 receptors are coupled to

the inhibition of adenylyl cyclase their activation may explain (at least in part) the relative resistance of muscarinic cholinoceptor-mediated contraction to the relaxant effects of β-adrenoceptor agonists (see Eglen *et al.*, 1994b and c). It has also been suggested however, that the extent of β-adrenoceptor mediated relaxation is controlled instead by the level of M_3 receptor-mediated contractile tone (see Roffel *et al.*, 1994).

Pre-junctional muscarinic autoreceptors may modulate ACh release, thereby exerting a regulatory role on muscarinic receptor-mediated contraction of ASM. Such inhibitory autoreceptors have been identified in the pre-junctional neurones serving many smooth muscles including human and guinea-pig airways (D'Agnostino *et al.*, 1990; Minette and Barnes, 1988). While there is some uncertainty over the identity of this autoreceptor (either M_2 or M_4) current evidence favours an M_2 or 'M_2-like' subtype (see Watson, 1994).

1.1.4 Non-muscarinic contractile receptors in airways smooth muscle

In addition to muscarinic cholinergic receptors, H_1 histaminergic receptors have been localised to the smooth muscle of both the small and large airways where occupancy of the H_1 receptor with histamine (Hist) is closely related to the contractile response (Grandordy and Barnes, 1987). A wide range of regulatory peptides which elicit a contractile response, including substance P, tachykinins and gallanin, have also been identified in ASM (see Polak and Bloom, 1986) although their signal transducing mechanisms are less well characterised.

1.2 MECHANISM OF AIRWAYS SMOOTH MUSCLE CONTRACTION

The force induced by ASM following agonist activation can be divided into two components - a rapid initial increase in force (phasic contraction), followed by a slower, maintained contractile phase (tonic contraction). The mechanisms governing the generation and regulation of these two contractile states may differ, and although great advances in our understanding of force generation in smooth muscle have been made in recent years, the mechanisms underlying force maintenance in this tissue remain poorly understood.

1.2.1 Contractile proteins in airways smooth muscle

As in striated muscle, actin represents the major contractile protein present in ASM. This protein is highly conserved in eukaryotic cells and is readily demonstrable in smooth muscle (e.g. Fatigati and Murphy, 1984). Actin monomers (globular actin or G-actin) polymerise to form F (filamentous)-actin with two linear chains of actin coiled into a right-handed double helix. Each G-actin monomer contains a binding site for a second contractile protein, myosin. This latter protein is present in smooth muscle in much lower proportions than in striated muscle, being some 10-20 fold less abundant than actin. Myosin is a hexameric protein, comprising a single pair of heavy peptide chains folded into a globular 'head' and a 'tail', and two pairs of light chains (17 KDa and 20 KDa) located at the head region. Myosin molecules combine to form thick, bipolar filaments, each globular head containing actin-binding sites and two Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase activities (myosin ATPase). Further actin-binding proteins which may play a role in regulating contraction have been identified in smooth muscle including

tropomyosin (Cummins and Perry, 1973; Fatigati and Murphy, 1984), caldesmon (see Walsh, 1990) and calponin (Takahashi *et al.*, 1988).

1.2.2 Force generation in airways smooth muscle

Evidence to support the increase in the free intracellular calcium concentration ($[Ca^{2+}]_i$) as being the primary initiator of the contractile response was first presented by Filo *et al.* (1965) in permeabilised vascular smooth muscle preparations. This finding was later substantiated in smooth muscle by Taylor (1989) and it is now recognised that a 4-10 fold increase in free intracellular Ca^{2+} ($[Ca^{2+}]_i$) from a basal level of 70-180 nM is necessary for the initiation of contraction (Felbel *et al.*, 1988; Fujiwara *et al.*, 1988; Kotlikoff *et al.*, 1987; Takuwa *et al.*, 1988; Taylor and Stull, 1988; Taylor *et al.*, 1988).

In resting smooth muscle myosin forms weak contacts with actin molecules, however following stimulation, the myosin heads form transient, but tighter attachments to actin (cross-bridges) before detachment and re-attachment to a G-actin monomer further along the actin filament (cross-bridge cycling). The resulting sliding of the thick and thin filaments over each other results in contraction, with the magnitude of force related to the degree of interdigitation of the filaments. The energy required for the cross-bridge cycling is provided by ATP hydrolysis catalysed by the myosin ATPase, and hence force-generation is dependent on activation of myosin ATPase. In contrast to striated muscle in which myosin ATPase is constitutively active unless regulated by the actin-binding proteins troponin and tropomyosin, a mixture of partially purified smooth muscle actin and myosin exhibits little myosin ATPase activity (see Giembycz, 1994). These data suggest the presence in smooth muscle of a Ca^{2+}-regulated factor responsible for the

activation of myosin ATPase which is removed during the purification procedure.

It is now known that activation of the calmodulin-dependent enzyme myosin light chain kinase (MLCK) is both necessary and sufficient to facilitate smooth muscle contraction (Itoh *et al.*, 1989). MLCK is inactive in the absence of Ca^{2+} ions, however it has a high affinity (K_d ~ 1 nM) for Ca^{2+}/calmodulin, and in the presence of raised $[Ca^{2+}]_i$ forms a $Ca^{2+}{}_4$:calmodulin:MLCK complex which exhibits phosphotransferase activity (see Adelstein and Eisenberg, 1980). MLCK shows strict substrate specificity for the 20 KDa light chain of myosin (LC_{20}) which, when phosphorylated, facilitates actin-mediated activation of myosin ATPase (Kamm and Stull, 1985). Taylor *et al.* (1989) reported a direct correlation between $[Ca^{2+}]_i$ and LC_{20} phosphorylation irrespective of the source of $[Ca^{2+}]_i$; furthermore the peak in LC_{20} phosphorylation precedes maximal force generation (de Lanerolle *et al.*, 1982; de Lanerolle and Stull, 1980; Gerthoffer and Murphy, 1983; Kamm and Stull, 1985; Silver and Stull, 1984). These data indicate that LC_{20} phosphorylation, catalysed by Ca^{2+}/calmodulin-activated MLCK, is the primary biochemical determinant for force generation in ASM.

1.2.3 Force maintenance in airways smooth muscle

As described above contraction of ASM can be divided into a phasic and a tonic component. These contractile states reflect a phasic and tonic $[Ca^{2+}]_i$ response whereby agonist stimulation results in a rapid and transient $[Ca^{2+}]_i$ peak followed by a plateau of $[Ca^{2+}]_i$ slightly above basal levels. During the tonic phase of smooth muscle contraction the level of LC_{20} phosphorylation decreases from that associated with phasic contraction, and plateaus at a concentration slightly above basal. Hence maintained

tension can be achieved in ASM despite the $[Ca^{2+}]_i$ and phosphorylated LC_{20} concentrations being relatively low.

Force is maintained during tonic contraction, whereas shortening velocity decreases rapidly from that obtained in phasic contraction to reach a relatively low steady-state value. Since force is governed by the number of attached cross-bridges, and shortening velocity is determined by the rate of cross-bridge cycling, it would appear that in the maintained contractile state cross-bridge cycling is dramatically slowed. Hence tonic contractions are energetically economical, utilising relatively little ATP.

Much interest has focussed on the regulation of myosin phosphorylation during tonic contraction in order to gain some insight into the factors governing maintained force. The degree of myosin phosphorylation is determined by the relative activities of MLCK and myosin light chain phosphatase (MLCP). There is evidence to suggest that during tonic contraction of smooth muscle MLCK may be phosphorylated by Ca^{2+}-calmodulin kinase (CaM kinase II) (Stull *et al.*, 1990), cyclic AMP-dependent protein kinase (PKA) (Conti and Adelstein, 1981; Nishikawa *et al.*, 1984, 1985; Stull *et al.*, 1990) and protein kinase C (Nishikawa *et al.*, 1985). Phosphorylation by the former two enzymes results in a decrease in the affinity of MLCK for Ca^{2+}/calmodulin and hence a decrease in its activity. If this process occurs *in vivo* it may serve to limit cross-bridge formation and hence decrease shortening velocity.

Several different MLCPs have been purified and characterised from smooth muscle (see Cai *et al.*, 1994) some of which are tightly associated with myosin filaments. It has been proposed that G-proteins may decrease MLCP activity since G-protein receptor-linked contractile agonists and GTPγS increase myosin phosphorylation and inhibit MLCP in skinned vascular smooth muscle fibres (Kitazawa *et al.*, 1991a and b).

In addition to the absolute level of myosin phosphorylation, a change in the flux of phosphorylated LC_{20} may serve to regulate ASM tone, since it has been observed that myosin kinase inhibitor, or MKI (an inhibitor of both MLCK and MLCP), inhibits shortening velocity in skinned taenia coli (Strauss *et al.*, 1992). This data suggests that shortening velocity may vary despite levels of LC_{20} phosphorylation remaining fairly constant.
While a direct relationship between LC_{20} phosphorylation and force generation has been clearly demonstrated in ASM, correlative studies have failed to demonstrate a tight temporal association between force, shortening velocity and LC_{20} phosphorylation during maintained contractions (Gunst *et al.*, 1992; Merkel *et al.*, 1990; Ozaki *et al.*, 1990; Shieh *et al.*, 1991; Silver and Stull, 1984). Hence it has been proposed that additional Ca^{2+}-regulated mechanisms may be responsible for controlling tonic contraction, and much research has focussed on the potential role of the thin filament-associated proteins caldesmon and calponin in such a process. Caldesmon can bind to calmodulin, myosin and tropomyosin. When bound to the latter caldesmon is an effective inhibitor of myosin ATPase (see Walsh, 1990). Phosphorylation of caldesmon *in vitro* removes its inhibitory action on myosin ATPase (Adam *et al.*, 1990). Like caldesmon, calponin can bind to actin and tropomyosin and can inhibit myosin ATPase activity (Abe *et al.*, 1990; Winder and Walsh, 1990). Phosphorylation of calponin by CaM kinase II or PKC reverses this inhibiton (Winder and Walsh 1990) which can be restored by the action of calponin phosphatase (Winder *et al.*, 1992). A preliminary report suggests that caldesmon may be phosphorylated during contraction of intact tracheal smooth muscle (Pohl *et al.*, 1991). Thus the phosphorylation state of these thin filament-binding proteins may play a critical role in

regulating smooth muscle contraction, although details of whether such regulation is applicable *in vivo* remain to be determined.

PKC has been implicated as playing a major role in the maintenance of smooth muscle contraction. Phorbol 12-myristate 13-acetate (PMA, 500 nM) can both activate PKC and induce tonic contraction, an effect which is greatly enhanced by the presence of Ca^{2+}-channel activators or Ca^{2+} ionophores (Park and Rasmussen, 1985). In agreement, application of low concentrations ($\leq 1\ \mu M$) of the phorbol esters PMA and 12-deoxyphorbol 13-isobutyrate (DPB) induce contraction of rabbit trachea which can be abolished by Ca^{2+} channel antagonists (Schramm and Grunstein, 1989). The contraction evoked by low concentrations of phorbol ester can be abolished by Ca^{2+} channel antagonists (Schramm and Grunstein, 1989) indicating that PKC-induced contraction is mediated, at least in part, by the activation of voltage-operated Ca^{2+} channels. The tonic contraction induced by PKC activation is associated with phosphorylation of the same proteins (including caldesmon) as those observed during the tonic contraction induced by carbachol (CCh) (Park and Rasmussen, 1986). It should be noted however that in the study of Schramm and Grunstein (1989) the application of higher concentrations of phorbol esters mediated ASM relaxation rather than contraction, pointing to either a more complex role of PKC in regulating ASM contraction or non-PKC-mediated effects at high phorbol ester concentrations.

1.3 SOURCE OF ACTIVATOR CALCIUM

ASM exhibits poor electrical conductivity and while a slow graded membrane depolarisation is observed following agonist stimulation, this effect is unlikely to account for the very rapid events leading to the initiation of tone in this tissue. The use of fluorescent calcium probes such as Fura-2 have facilitated temporal studies of the agonist-stimulated $[Ca^{2+}]_i$ response. The response is biphasic, consisting of a rapid, sharp rise in $[Ca^{2+}]_i$ which, in the continued presence of agonist falls to a plateau level slightly above basal (Kotlikoff *et al.*, 1987; Murray and Kotlikoff, 1991; Pannettieri *et al.*, 1989; Senn *et al.*, 1990; Shieh *et al.*, 1991). It is now established that the increase in $[Ca^{2+}]_i$ responsible for force generation (the initial transient increase in cytosolic Ca^{2+}) in ASM arises from a non-mitochondrial intracellular store (Twort and van Breemen, 1989), whereas the influx of extracellular Ca^{2+} may be more important for the maintenance of tone. Thus in ASM agonist-induced phasic contractions are generally very resistant to the removal of extracellular Ca^{2+} and are insensitive to dihydropyridine Ca^{2+} channel blockers; by contrast depolarisation-induced contractions (or the tonic phase of agonist-induced contractions) are more critically dependent on extracellular Ca^{2+} (Kirkpatrick, 1975; Ritchie *et al.*, 1984; Weichman *et al.*, 1982, 1983).

Electron probe X-ray microanalysis has identified the terminal cisternae of the sarcoplasmic reticulm (SR) as the major source of activator Ca^{2+} in smooth muscle (Bond *et al.*, 1984; see Somlyo, 1981), and Bond *et al.* (1984) have shown that this store contains sufficient Ca^{2+} to elicit maximal contraction of guinea-pig portal vein. Recent studies in ASM have highlighted a heterogeneity of the Ca^{2+} store, such that the available Ca^{2+} may be divided between multiple discrete pools released by $Ins(1,4,5)P_3$,

ryanodine or GTPγS, although passage of Ca^{2+} between the pools may occur under some circumstances (Chopra *et al.*, 1991).

1.3.1 The inositol 1,4,5-trisphosphate-sensitive calcium store

One of the most important mediators of intracellular Ca^{2+} release known is Ins(1,4,5)P_3, a water-soluble compound formed by phosphoinositidase C-mediated hydrolysis of PtdIns(4,5)P_2 (see Berridge, 1993). Ins(1,4,5)P_3 has been shown to release Ca^{2+} from a non-mitochondrial intracellular store in permeabilised canine (Hashimoto *et al.*, 1985), rabbit (Chopra *et al.*, 1991) and human (Twort and van Breemen, 1989) ASM preparations. These studies show that application of exogenous Ins(1,4,5)P_3 to permeabilised tissue results in a rapid (90% release by 12 sec, Twort and van Breemen, 1989) and concentration-dependent (EC_{50} = 0.8-2.3 μM; maximal effect at 2-30 μM) release of intracellular Ca^{2+}. A maximally effective concentration of Ins(1,4,5)P_3 released a high proportion (84 %) of the ATP-dependent intracellular Ca^{2+} store in cultured human ASM cells, a quantity which relates to that released in intact cells in response to 10 μM Hist (Twort and van Breemen, 1989).

A protein with highly specific binding properties for Ins(1,4,5)P_3 has been identified in many cell types including ASM (Chilvers *et al.*, 1990b; Schramm *et al.*, 1992 and see Ferris and Snyder, 1992). Reconstitution studies, in which the Ins(1,4,5)P_3 binding proteins purified from rat cerebellar membranes (Ferris *et al.*, 1989) or aortic smooth muscle (Mayrleitner *et al.*, 1991) were incorporated into artificial membranes, demonstrated that the Ins(1,4,5)P_3 receptor protein has an intrinsic Ca^{2+} channel activity; moreover the binding affinity of a series of inositol polyphosphates for this protein correlated with their ability to induce $^{45}Ca^{2+}$ flux in these vesicles (Ferris *et al.*, 1989). It has now been shown

that the vicinal (4,5)-bisphosphate group is crucial for Ca^{2+}-release (Nahorski and Potter, 1989). In accordance with a role in Ins(1,4,5)P_3-mediated Ca^{2+} release from intracellular stores the receptor has been localised predominantly to the endoplasmic reticulum (Mignery *et al.*, 1989; Ross *et al.* 1989), however recent studies have also identified an Ins(1,4,5)P_3 receptor in the nucleus (Malviya *et al.*, 1990) and in some cells an Ins(1,4,5)P_3 receptor has been shown to be associated with the plasma membrane (Khan *et al.*, 1992; Kuno and Gardner, 1987). This latter observation has prompted speculation for a direct role for Ins(1,4,5)P_3 in agonist-dependent Ca^{2+} influx. The plasma membrane-associated Ins(1,4,5)P_3 receptors exhibit less specificity for Ins(1,4,5)P_3 over receptors localised to the endoplasmic reticulum (Kalinoski *et al.*, 1992) and recently attention has therefore been drawn to the potential role of Ins(1,3,4,5)P_4 in Ca^{2+} influx (Irvine 1991).

The high density of Ins(1,4,5)P_3 receptors found in cerebellar Purkinje cells facilitated their purification and cloning (Danoff *et al.*, 1991; Furuichi *et al.*, 1989; Nakagawa *et al.*, 1991; Supattapone *et al.*, 1988). More recent cloning studies however, have identified four subtypes of receptor (InsP_3R-1-4) encoded by distinct but related genes, with the originally described cerebellar receptor designated as InsP_3R-1. Complete cDNA sequences are also now available for both InsP_3R-2 (Sudhof *et al.*, 1991) and InsP_3R-3 (Blondel *et al.*, 1993; Maranto, 1994), and a partial sequence has been determined for InsP_3R-4 (Danoff *et al.*, 1991). The mRNA molecules for these Ins(1,4,5)P_3 receptor isoforms are differentially expressed in various tissues (Blondel *et al.*, 1993; Mignery *et al.*, 1990; Ross *et al.*, 1992) and a study of the Ins(1,4,5)P_3-binding properties of the receptors shows the neuronal sites generally display a lower affinity for Ins(1,4,5)P_3 (K_d = 20-70 nM) than peripheral receptors (K_d = 2-8 nM) (see

Willcocks *et al.*, 1990). Two major splice sequences, SI and SII, have been identified within the $InsP_3R$-1 subtype leading to further heterogeneity of $Ins(1,4,5)P_3$ receptors with splice variants expressed in both a developmentally- and tissue-specific manner (Danoff *et al.*, 1991; Nakagawa *et al.*, 1991).

The $Ins(1,4,5)P_3$ receptors exhibit extensive sequence and structural similarity with the ryanodine receptor of skeletal and cardiac muscle clustered around the transmembrane domains which form the Ca^{2+}-channel (Mignery *et al.*, 1989; Takeshima *et al.*, 1989). The native protein exists as a tetramer (Maeda *et al.*, 1991; Mignery *et al.*, 1990) and hence several forms of the receptor may exist in a single cell-type arising from different combininations of $Ins(1,4,5)P_3$ receptor monomers.

In many cell types $Ins(1,4,5)P_3$-induced Ca^{2+} flux exhibits a biphasic dependence on the cytoplasmic Ca^{2+} concentration with nanomolar and micromolar $[Ca^{2+}]_i$ potentiating and attenuating Ca^{2+} release respectively (Bezprozvanny *et al.*, 1991; Loomis-Husselbee and Dawson, 1993; Missiaen *et al.*, 1992, 1994). Initial studies by Worley *et al.* (1987) identified an inhibitory effect of Ca^{2+} ions on $Ins(1,4,5)P_3$ binding to the cerebellar receptor, suggesting that the inhibitory effect of Ca^{2+} at high concentrations might be due to regulation of ligand-binding to the receptor. Danoff *et al.* (1988) however, observed that Ca^{2+} did not regulate $Ins(1,4,5)P_3$ binding to its receptor directly but required a secondary protein termed calmedin. Moreover, Mignery *et al.* (1992), unable to demonstrate such a Ca^{2+}-mediated control of $Ins(1,4,5)P_3$ binding in cerebellum, proposed that calmedin may be a Ca^{2+}-sensitive isoform of PIC. The results observed by Worley *et al.* (1987) would therefore simply reflect a Ca^{2+}-stimulated production of endogenous $Ins(1,4,5)P_3$ which displaced radiolabelled $[^3H]Ins(1,4,5)P_3$ from the receptor. In agreement

with this proposal Chilvers *et al.* (1990b) showed Ins(1,4,5)P_3 binding to the BTSM receptor was unaffected by the Ca^{2+} ion concentration.

Ins(1,4,5)P_3-induced Ca^{2+} release seems to occur in discrete quanta (Bootman *et al.*, 1992; Ferris *et al.*, 1992), a phenomenon which may reflect, in part, an interaction between luminal Ca^{2+} and Ins(1,4,5)P_3 (Missiaen *et al.*, 1992, 1994; Oldershaw and Taylor, 1993; Parys *et al.*, 1993). Depletion of Ca^{2+} stores reduces the affinity of the receptor for Ins(1,4,5)P_3 (Oldershaw and Taylor, 1993) and increases the EC_{50} for Ca^{2+} release (Nunn and Taylor, 1990). There is also some evidence to suggest that luminal Ca^{2+} may modulate the regulatory effects of cytoplasmic Ca^{2+} on the Ins(1,4,5)P_3 receptor (Missiaen *et al.*, 1992, 1994).

1.3.2 The ryanodine-sensitive calcium store

Ryanodine is a naturally occurring plant alkaloid which has been shown to release Ca^{2+} from the SR of cardiac (Wier *et al.*, 1985) and skeletal (Sutko *et al.*, 1985) muscle. A similar action of this compound has been observed in airways smooth muscle (Chopra *et al.*, 1991) although the Ca^{2+} release effect is quantitatively much smaller. Since ryanodine binding to its receptor is absolutely dependent on Ca^{2+} (e.g. Michelak *et al.*, 1988; Pessah *et al.*, 1985) ryanodine receptors have been implicated in Ca^{2+}-induced Ca^{2+} release (see section 1.3.3). In cultured ASM cells pre-treatment with a maximally effective concentration of ryanodine abolishes Ca^{2+} release in response to GTPγS and caffeine suggesting that the latter pools are a subset of the ryanodine-releasable Ca^{2+} pool (Chopra *et al.*, 1991). In addition ryanodine pre-treatment of these cells results in an inhibition of any subsequent Ins(1,4,5)P_3 response indicating that the ryanodine releasable Ca^{2+} pool may be a subset of the Ins(1,4,5)P_3 releasable pool.

A further agent which which is now recognised to release Ca^{2+} from ryanodine-sensitive stores is cyclic adenosine diphosphate-ribose (cADP-ribose). The ability of this pyridine metabolite to mobilise Ca^{2+} was first observed in sea urchin eggs (Clapper *et al.*, 1987) and has more recently been demonstrated in mammalian cells (Koshiyama *et al.*, 1991; Takasawa *et al.*, 1993). Ca^{2+} mobilisation in response to cADP-ribose occurs independently of Ins(1,4,5)P_3 receptor-activation (see Galione, 1993) and since pretreatment with modulators of the ryanodine receptor can regulate cADP-ribose-induced Ca^{2+} release (see Galione, 1992), this agent has been proposed to mediate Ca^{2+} efflux from ryanodine-sensitive sites. It is not known if cADP-ribose is formed following agonist stimulation of ASM and hence the physiological relevance of this Ca^{2+}-release mechanism in this tissue is not clear.

1.3.3 Calcium-induced calcium release

Ca^{2+}-induced Ca^{2+} release (CICR) has been demonstrated in permeabilised skeletal, cardiac and vascular smooth muscle where small increases in the [Ca^{2+}] of the bathing medium elicit substantial Ca^{2+} release (Endo, 1975, 1977; Endo *et al.*, 1970; Fabiato, 1983; Ford and Podolski, 1970; Saida, 1981, 1982). A localised increase in $[Ca^{2+}]_i$ has been proposed to activate large conductance cation channels on the SR close to the plasma membrane to mediate CICR which has been implicated as the mechanism underlying the propagation of intracellular Ca^{2+} waves (Berridge and Irvine, 1989). In other muscle types, caffeine appears to release Ca^{2+} from intracellular stores by sensitising the CICR mechanism (see Endo, 1977; Iino, 1987; Leijten and van Breemen, 1984; see Martinosi, 1984). Although there is no direct evidence for CICR in ASM, the presence of such a mechanism is suggested by the ability of caffeine to

induce a slow release of Ca^{2+} in permeabilised cultured ASM cells (Chopra *et al.*, 1991).

1.3.4 The guanine-nucleotide-sensitive calcium store

The ability of guanine-nucleotides to release intracellular calicum was originally observed in hepatic and neuronal cells (Chueh and Gill, 1986; Dawson, 1989; Gill *et al.*, 1986). A similar action of GTPγS, independent of G-protein activation of phosphoinositidase C and hence $Ins(1,4,5)P_3$ formation, has more recently been observed in cultured ASM cells (Chopra *et al.*, 1991). Studies in the DDT_1 MF-2 smooth muscle cell line suggest that GTP acts to translocate Ca^{2+} from an $Ins(1,4,5)P_3$-insensitive to an $Ins(1,4,5)P_3$-sensitive compartment (Ghosh *et al.*, 1989; Mullaney *et al.*, 1988). While the presence of such a mechanism in ASM is questionable since GTPγS-induced Ca^{2+} release (unlike $Ins(1,4,5)P_3$-mediated Ca^{2+} release) is unaffected by heparin (Chopra *et al.*, 1989), the GTPγS-sensitive pool does appear to communicate with the $Ins(1,4,5)P_3$-sensitive pool and may be able to regulate its size.

In addition to storing Ca^{2+} which may be released into the cytosol following agonist stimulation, in non-excitable cells the SR may also serve to regulate Ca^{2+} influx into the cell. A model has been proposed (the capacitative model) in which depletion of intracellular Ca^{2+} stores can, through an unknown mechanism, increase the permeability of the plasma membrane to Ca^{2+} ions (Putney, 1986, 1991). In support of such a mechanism, Randriamampita and Tsien (1993) have isolated a putative messenger from Jurkat cells which is released into the cytosol from intracellular organelles following depletion of Ca^{2+} stores. The messenger can induce Ca^{2+} influx when applied exogenously to

macrophages, astrocytoma cells and fibroblasts and hence has been termed calcium influx factor, or CIF. This small molecular weight (< 500 Da) molecule is negatively charged and appears to contain both a phosphate group (essential for stimulation of Ca^{2+} influx) and two hydroxyl groups on adjacent carbon atoms. In addition, independent data from electrophysiological experiments in *Xenopus* oocytes identify a plasma membrane Ca^{2+} current which is activated by a diffusible factor following depletion of intracelluar Ca^{2+} stores, and is inhibited by a phosphatase (Parekh *et al.*, 1993). The capacitative model of Ca^{2+} entry provides a possible mechanism for agonist-stimulated Ca^{2+} influx (secondary to intracellular Ca^{2+} release), and may be important in the maintenance of contraction in ASM.

1.4 INVOLVEMENT OF PHOSPHOINOSITIDE HYDROLYSIS IN PHARMACOMECHANICAL COUPLING IN AIRWAYS SMOOTH MUSCLE

Michell (1975) was the first to propose a link between agonist-stimulated phosphoinositide metabolism and Ca^{2+}-signalling, based on the following observations from a variety of tissues:-

1. Certain receptors controlled the activation of inositol lipid metabolism in many tissues regardless of the cellular response.
2. These receptors did not generally activate adenylyl cyclase and the post-receptor signalling mechanism for these receptors was unknown.
3. Stimulation of these receptors always appeared to cause an increase in $[Ca^{2+}]_i$.

4. Studies in adrenal medulla, platelets and parotid gland suggested that the inositol lipid metabolism was not secondary to the rise in $[Ca^{2+}]_i$.
5. Receptor stimulation caused tissues to become partially depleted of PtdIns.
6. Inositol lipid breakdown occured with a rapidity to that required of a signalling reaction.
7. Inositol lipid breakdown showed a concentration-response curve that corresponded to receptor occupation by the applied agonist rather than the functional effect.

Dawson (1971) demonstrated that a phosphoinositide-specific phospholipase C (PIC) was responsible for this phosphoinositide metabolism leading to the formation of DAG and water-soluble inositol phosphates (InsPs). Durrel *et al.* (1969) suggested that in guinea-pig brain the polyphosphoinositides may also be metabolised in addition to PtdIns - a finding that was later substantiated in iris smooth muscle by Abdel-Latif and collegues, (see Abdel-Latif, 1986). All of these proposals are now well accepted as a means of agonist-stimulated second messenger formation (see Berridge, 1987; Rana and Hokin, 1990). PtdIns(4,5)P_2, formed by the sequential actions of a 4- and 5-kinase on PtdIns, is recognised to be the primary substrate for PIC resulting in the formation of DAG and Ins(1,4,5)P_3. DAG remains within the plane of the membrane and can activate certain PKC isoforms and hence facilitate phosphorylation of a range of effector molecules (see Wilkinson and Hallam, 1994), whereas Ins(1,4,5)P_3 is released into the cytosol and can facilitate intracellular Ca^{2+} release by binding to its specific receptor on the ER (see Berridge, 1993).

The identification of Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 isomers in tissue extracts was thought initially to suggest the presence of additional 3-

phosphorylated phosphoinositides from which these compounds might be derived (Batty *et al.*, 1985; Downes *et al.*, 1986; Nahorski and Batty, 1986; Nahorski *et al.*, 1986). Although a series of 3-phosphorylated phosphoinositides (PtdIns3P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3) have now been identified in membranes from a variety of cell types (see Stephens *et al.*, 1993), they appear to represent minor phosphoinositide components that are resistant to hydrolysis by PIC (Lips *et al.*, 1989; Serunian *et al.*, 1989). Recently, much research has been focussed on the role of these 3-phosphorylated phosphoinositides leading to the proposal that they may represent components of a separate phosphoinositide signalling pathway, whereby PtdIns(4,5)P_2 is phosphorylated by a 3-kinase to yield PtdIns(3,4,5)P_2 (Stephens *et al.*, 1991b). PtdIns(3,4,5)P_3 has now been implicated in a variety of cellular processes including cell growth (Fantl *et al.*, 1993; Valius and Kaslauskas, 1993), chemotaxis and cytoskeletal rearrangement (Eberle *et al.*, 1990), glucose transport (Okada *et al.*, 1994a) and neutrophil respiratory burst activity (e.g. Eberle *et al.*, 1990; Okada *et al.*, 1994b). The precise second messenger function of PtdIns(3,4,5)P_3 however remains uncertain.

Chilvers *et al.* (1991a) could not detect any isomeric heterogeneity of the PtdInsP_2 pool in BTSM which appeared to consist solely of PtdIns(4,5)P_2. Conversely analysis of the PtdInsP_1 pool by these authors showed the presence of both the dominant PtdIns4P isomer and a minor (17 %) PtdInsP_1 constituent identified as PtdIns3P. As yet however, no evidence exists to suggest the presence of higher 3-phosphorylated phosphoinositides in ASM.

1.4.1 Contractile agonist-stimulated phosphoinositide metabolism in airways smooth muscle

The involvement of phosphoinositide hydrolysis in the coupling of contractile receptor stimulation to Ca^{2+} release and ASM contraction was first suggested in a study by Baron *et al.* (1984). These authors noted enhanced $[^{32}P]P_i$ incorporation into PtdIns following CCh stimulation of canine trachealis, accompanied by an overall decrease in PtdIns mass and a parallel increase in the accumulation of DAG and its metabolite phosphatidate (PtdOH). A similar agonist-stimulated incorporation of $[^3H]$inositol into PtdIns, PtdInsP and $PtdInsP_2$ has also been observed in tracheal smooth muscle (Baron *et al.*, 1989; Chilvers *et al.*, 1989a). Studies in $[^3H]$inositol-labelled BTSM have demonstrated a rapid ($t_{1/2}$ = 14 sec) and sustained, CCh-stimulated decrease in the mass of $PtdInsP_2$ (Takuwa *et al.*, 1986) and more specifically $PtdIns(4,5)P_2$ (Chilvers *et al.*, 1991a). The former study (Takuwa *et al.*, 1986) demonstrated that in tracheal smooth muscle, as in most other tissues, $PtdInsP_2$ appears to be the major membrane phosphoinositide hydrolysed following agonist stimulation. Accumulation of $[^3H]$InsPs in the presence of 5-10 mM LiCl (a potent, non-competitive inhibitor of the inositol monophosphatase - see section 1.5.1) has also been used as an index of phosphoinositide hydrolysis in $[^3H]$inositol pre-labelled ASM. Using this approach, agonist-stimulated $[^3H]$InsP accumulation has been observed in response to a variety of contractile stimuli including full and partial muscarinic agonists (Chilvers *et al.*, 1989a, 1990a; Duncan *et al.*, 1987; Hall and Hill, 1988; Meurs *et al.*, 1988; Offer *et al.*, 1991; Roffel *et al.*, 1990a), histamine (Hall and Hill, 1988), bradykinin (Chilvers *et al.*, 1989a), 5-hydroxytryptamine (Lemoine *et al.*, 1988), endothelin (Hay, 1990), leukotrienes C_4, D_4 and E_4 (Grandordy *et al.*, 1987; Mong *et al.*, 1988), substance P and neurokinins A

and B (Grandordy *et al.*, 1988). A similar finding was also observed following both electrical field stimulation of [^{3}H]inositol-labelled BTSM strips (Miller-Hance *et al.*, 1988), and co-incubation of [^{3}H]inositol-labelled canine ASM membranes with histamine and GTP (Murray *et al.*, 1989).

1.4.2 Agonist-stimulated inositol 1,4,5-trisphosphate accumulation in airways smooth muscle

[^{3}H]Ins(1,4,5)P_3 has been shown to mediate Ca^{2+} release from intracellular stores in permeabilised canine tracheal smooth muscle cells (Hashimoto *et al.*, 1985). To substantiate a role for this second messenger in stimulating the release of activator Ca^{2+} for the contractile response, Duncan *et al.* (1987) demonstrated a rapid (1 sec) ACh-stimulated accumulation of [^{3}H]InsP_3 in canine ASM which preceded the detectable onset of contraction. Similarly, increases in [^{3}H]InsP_3 in bovine ASM have been detected as early as 500 msec following electrical field stimulation (Miller-Hance *et al.*, 1988).

One of the major defects of these early studies however was the failure to identify the precise isomeric composition of [^{3}H]InsP_3. Using both H.P.L.C. and enzymic methods the [^{3}H]InsP_3 fraction accumulating in bovine tissue has been shown to consist of both [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4)P_3 with the latter isomer predominating even at early time-points after agonist stimulation (Chilvers *et al.*, 1990a; Kennedy *et al.*, 1989). Nonetheless, Chilvers *et al.* (1990a) demonstrated a rapid (albeit transient) increase in [^{3}H]Ins(1,4,5)P_3 in BTSM strips following stimulation with CCh. In agreement, subsequent studies utilising a specific radioreceptor binding assay for the determination of Ins(1,4,5)P_3 mass (Palmer *et al.*, 1989) identified a substantial CCh-stimulated increase

in $Ins(1,4,5)P_3$ mass, apparent by 2 sec, peaking after 5 sec and returning to basal and sub-basal levels by 30 sec in BTSM (Chilvers *et al.*, 1989b).

Rosenberg and co-workers (1991) demonstrated a relationship between both the EC_{50} and magnitude of the CCh-stimulated $Ins(1,4,5)P_3$ response and differences in the contractile response observed between immature and adult rabbit ASM. Furthermore, a direct relationship has been observed between [^{3}H]InsP formation and contraction induced by a range of muscarinic agonists in bovine trachealis (Meurs *et al.*, 1988). Hence stimulation of $Ins(1,4,5)P_3$ production in ASM appears to occur with a time- and concentration dependence compatible with the contractile response. While $Ins(1,4,5)P_3$ has not been shown directly to mimic contraction in ASM, such experiments have been carried out in vascular and gastric smooth muscle where liberation of caged $Ins(1,4,5)P_3$ can elicit contraction in permeabilised tissue (Bitar *et al.*, 1986; Somlyo *et al.*, 1985, 1992; Walker *et al.*, 1987).

Therefore much evidence has accumulated to implicate the receptor-mediated generation of $Ins(1,4,5)P_3$ in pharmacomechanical coupling in ASM. This compound is generated rapidly enough and with a concentration-dependence consistent with the contractile reponse, and by binding to its specific receptor on the SR can elicit sufficient Ca^{2+} release to initiate contraction. Also in support of a second messenger role for $Ins(1,4,5)P_3$, it should be noted that inhibition of $Ins(1,4,5)P_3$ generation by phorbol esters (Baba *et al.*, 1989) and cAMP (see section 1.6.3) is associated with an attenuation of agonist-induced contraction.

In BTSM, CCh-stimulated hydrolysis of $PtdIns(4,5)P_2$ appears to persist (> 20 min) in the continued presence of agonist (Chilvers *et al.*, 1991a), hence the transient $Ins(1,4,5)P_3$ accumulation response observed in this tissue must reflect extensive agonist-stimulated metabolism of

$Ins(1,4,5)P_3$. Such rapid metabolism is to be expected of a putative second messenger. An alternative pattern of agonist-stimulated $[^3H]Ins(1,4,5)P_3$ formation however, has been observed in rabbit tracheal smooth muscle where endothelin-1 stimulated a sustained, rather than transient, increase in $Ins(1,4,5)P_3$ accumulation (Grunstein *et al.*, 1991). The mechanisms underlying such agonist- and/or species-specific differences in the $Ins(1,4,5)P_3$ response in this tissue are unclear. They may reflect intrinsic differences in the enzymes present in the tissues (e.g. the PIC isozymes recruited by the receptors) or in the regulation of these enzymes (e.g. the use of different G-proteins to couple the receptors to PIC) (see sections 1.4.3 and 1.4.4). Alternatively the contrasting receptor-mediated $Ins(1,4,5)P_3$ responses may arise as a result of differential feedback or crosstalk mechanisms from other signal transducing pathways, which could affect both the production and metabolism of $Ins(1,4,5)P_3$.

1.4.3 Phosphoinositidase C studies in airways smooth muscle

Studies in various tissues have identified four, and possibly five, biochemically, structurally and immunologically distinct subclasses of PIC enzymes (α, β, γ, δ and ε) (see Crooke and Bennett, 1989; Meldrum *et al.*, 1991; Rhee *et al.*, 1989, 1991). Homma *et al.* (1989) identified mRNA encoding at least four discrete PIC isozymes in rat lung homogenates. Two groups have subsequently reported partial resolution of at least three peaks of PIC activity in soluble fractions of bovine, porcine and canine trachealis (Chilvers *et al.*, 1992; Murray *et al.*, 1991b). The enzyme activity in all three peaks in bovine trachealis was found to be Ca^{2+}-dependent. Furthermore, the ability of platelet-derived growth factors PDGF-AB and PDGF-BB to stimulate $[^3H]InsP$ generation and antisense PIC-β_1 cDNA to inhibit agonist-stimulated phosphoinositide hydrolysis in ASM suggests

the presence of at least PIC-γ and PIC-β_1 isozymes in this tissue (see Chilvers, 1994).

1.4.4 Involvement of G-proteins in agonist-stimulated phosphoinositide hydrolysis in airways smooth muscle

G-proteins, or guanine-nucleotide-binding proteins, are a large family of hetrotrimeric proteins (consisting of α, β and γ subunits) which serve to couple a wide range of receptors to their effector enzymes (for review see Helper and Gilman, 1992). Under resting conditions the α subunit is bound to GDP which can slowly dissociate and be exchanged for GTP. Interaction of the G-protein with agonist-bound receptor dramatically enhances the rate of GDP/GTP transfer and following GTP binding the G-protein dissociates into α-GTP and $\beta\gamma$ subunits. Until recently, the released α-GTP has been thought to be the principal modulator of effector enzymes, however evidence has accumulated indicating the importance of the $\beta\gamma$ subunit in regulation of certain responses, most notably stimulation of PLC-β_2 (Boyer *et al.*, 1992; Camps *et al.*, 1992) and binding of receptor kinases, leading ultimately to receptor desensitisation (Kameyama *et al.*, 1993; Koch *et al.*, 1993). Termination of α-GTP mediated responses follows GTP hydrolysis by the intrinsic GTPase activity of the α subunit.

The first suggestion that G-proteins might be involved in receptor-mediated phosphoinositide signalling was provided by the observation that guanine nucleotides could alter the binding affinity of agonists linked to Ca^{2+}-mobilising receptors in human astrocytoma cells (Evans *et al.*, 1985). This observation has been substantiated in ASM where muscarinic agonist binding affinity is regulated by a range of stable GTP analogues (Grandordy *et al.*, 1986; Lucchesi *et al.*, 1990). GTPγS has since

been observed to directly stimulate phosphoinositide hydrolysis in ASM membranes and permeabilised ASM cells (Grunstein *et al.*, 1991; Murray *et al.*, 1989). The ability of fluoroaluminate (a non-selective G-protein activator which mimics the γ-phosphate of GTP) to elicit concentration-dependent contractions and [^{3}H]InsP formation in BTSM provided further evidence for the involvement of G-proteins in coupling receptors to PIC activation (Hall *et al.*, 1990a). While the precise identity of the G-protein(s) involved in phosphoinositide signalling in ASM remains to be determined, the inability of pertussis toxin to modulate high affinity oxotremorine binding (Lucchesi *et al.*, 1990) or to affect CCh-, Hist- or bradykinin-stimulated [^{3}H]InsP accumulation in ASM (Chilvers, 1991; Pyne and Pyne, 1992) implies the involvement of a member of the recently identified pertussis toxin-insensitive G_q subclass ($G_{q/11/14/15/16}$) of G-proteins (Strathmann and Simon, 1990). Furthermore Pyne and Pyne (1992) have detected the α subunit of G_q/G_{11} in guinea-pig tracheal smooth muscle using specific peptide-directed antibodies. By contrast, leukotriene-D_4-stimulated [^{3}H]InsP accumulation in guinea-pig tracheal smooth muscle cells is pertussis-toxin-sensitive, although it should be noted that this effect was observed at a very high concentration (10 μg/ml) of pertussis toxin (Howard *et al.*, 1992). This data may suggest either the presence of more than one PIC isozyme coupled differentially to discrete G-proteins, or the recruitment alternative G-protein isoforms in response to activation of different receptors in guinea-pig ASM.

1.5 INOSITOL 1,4,5-TRISPHOSPHATE METABOLISM

As detailed above, CCh stimulation of BTSM results in a transient accumulation of Ins(1,4,5)P_3 despite ongoing hydrolysis of PtdIns(4,5)P_2, indicating a rapid agonist-stimulated metabolism of Ins(1,4,5)P_3 in this tissue. Studies in other tissues show that the immediate and downstream metabolism of Ins(1,4,5)P_3 is highly complex, resulting in the formation of a large array of InsPP isomers (for reviews see Majerus *et al.*, 1988; Shears 1989, 1991, 1992). In some cases subsecond oscillations of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 have been observed (e.g. Breer *et al.*, 1990; Raha *et al.*, 1993). In mammalian cells the metabolism of Ins(1,4,5)P_3 proceeds predominantly via two main pathways involving the Ins(1,4,5)P_3 3-kinase and Ins(1,4,5)P_3 5-phosphatase enzymes. These '3-kinase' and '5-phosphatase' pathways are shown in figure 1.5 and outlined below. The possible regulatory mechanisms governing the activities of these enzymes are presented in chapter three.

1.5.1 The 5-phosphatase pathway

A Mg^{2+}-dependent inositol polyphosphate 5-phosphatase enzyme has been identified in many cell types which can specifically remove a phosphate group from the 5-position of the inositol ring of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, Ins(1:2cyc,4,5)P_3 and Ins(4,5)P_2 (e.g. Downes *et al.*, 1982; Mitchell *et al.*, 1989; Takimoto *et al.*, 1989). In the majority of tissues studied the enzyme appears to be predominantly membrane bound, forming tight (probably hydrophobic) interactions with the membrane - in particular the plasma membrane, and can be liberated following treatment with detergent (Downes *et al.*, 1982; Hansbro *et al.*, 1994; Laxminarayan *et al.*, 1993; Shears *et al.*, 1988).

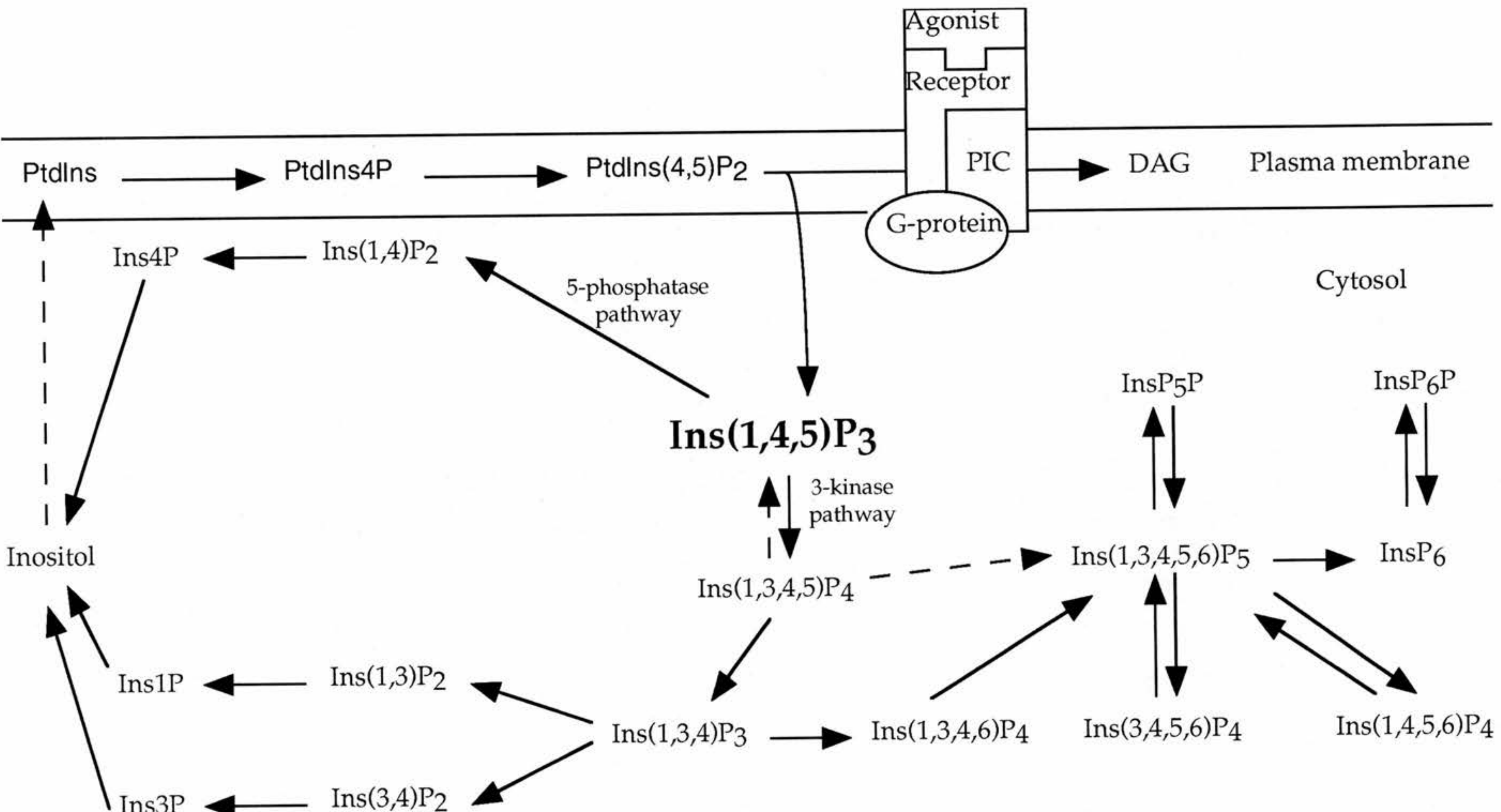

Figure 1.5 Pathways of inositol 1,4,5-trisphosphate formation and metabolism

Two distinct soluble 5-phosphatase activities have been identified in a variety of tissues including brain (Erneux *et al.*, 1989; Hansen *et al.*, 1987), skeletal muscle (Hansbro *et al.*, 1994), and neutrophils (Kennedy *et al.*, 1990). These isoforms were originally designated, according to their substrate specificity, as Type I (hydrolyses both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4) and Type II (very low affinity for Ins(1,3,4,5)P_4 and therefore *in vivo* probably metabolises only Ins(1,4,5)P_3) (Hansen *et al.*, 1987). The Type I 5-phosphatases generally have a molecular mass of 40-45 KDa and are smaller than the Type II enzymes (70-160 KDa). However the possibility of a further 5-phosphatase isoform has been suggested by the presence in platelets (Mitchell *et al.*, 1989; Ross *et al.*, 1991) and erythrocytes (Hodgkin *et al.*, 1994) of a 5-phosphatase activity which can hydrolyse both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 but which has a higher molecular mass (70-75 KDa) than the previously identified Type I enzymes. Hodgkin and co-workers (1994) noted that these enzymes had lower affinity for Ins(1,4,5)P_3 than their smaller 40-45 KDa counterparts, leading to the conclusion that they may represent a third isoform designated as Type Ib (the 40-45 KDa isoform was redesignated as Type Ia). Further evidence to suggest that these isoforms may be distinct enzymes was provided by Ross *et al.* (1991) who demonstrated that human platelet Type Ia and Type Ib enzymes are immunologically distinct. To date, Type Ib 5-phosphatase activity has only been identified in platelets and erythrocytes, suggesting that they may be restricted to cells of haematopoietic lineage.

In general, where the membrane-associated 5-phosphatase has been studied, it has been found to be very similar, both physically and biochemically to the Type Ia enzyme (e.g. Erneux *et al.*, 1989; Hodgkin *et al.*, 1994; Laxminarayan *et al.*, 1993, 1994; Takimoto *et al.*, 1989), and

Verjans *et al.* (1990) have shown that these two activities are immunologically indistinguishable.

Following dephosphorylation of Ins(1,4,5)P_3 to Ins(1,4)P_2, the latter compound is further dephosphorylated by a 1-phosphatase to Ins(4)P (Balla *et al.*, 1986). This enzyme has been purified from bovine brain and found to have a molecular mass of 44 KDa (Inhorn and Majerus, 1987, 1988). It specifically removes the 1-phosphate from both Ins(1,4)P_2 and Ins(1,3,4)P_3, is Mg^{2+}-dependent and is inhibited by lithium ions. The product of this reaction - Ins4P - is metabolised to free inositol by a monophosphatase enzyme (Gee *et al.*, 1988) which can additionally utilise Ins3P, Ins4P, Ins5P and Ins6P as substrates. Lithium is a potent (K_i < 1mM), uncompetitive inhibitor of the monophosphatase enzyme (Gee *et al.*, 1988; Hallcher and Sherman, 1980) and therefore prevents the generation of free inositol and traps inositol monophosphates within the cytosolic fraction of the cell.

1.5.2 The 3-kinase pathway

An Ins(1,4,5)P_3 3-kinase enzyme which phosphorylates Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 has been purified and cloned from both brain (Choi *et al.*, 1990; Johanson *et al.*, 1988; Lee *et al.*, 1990; Sim *et al.*, 1990; Takazawa *et al.*, 1989, 1990b, 1991a and b) and platelets (Lin *et al.*, 1990) and shown to have a molecular mass of approximately 50 KDa. Takazawa *et al.* (1991a and b) identified two isozymes in a human hippocampus cDNA library with calculated molecular masses of 50,988 and 53,451 Da. Other Ins(1,4,5)P_3 3-kinase activities with a lower molecular weight have also been identified (Lin *et al.*, 1990; Takazawa *et al.*, 1989, Lee *et al.*, 1990); however these proteins may represent proteolysis products of the higher molecular weight enzyme (Lee *et al.*, 1990). Indeed Takazawa *et al.* (1991a)

demonstrated that a truncated 3-kinase cDNA transcript from human hippocampus encoded an active protein which when expressed in *E. coli* had a molecular mass of 36-38 KDa. These smaller, active 3-kinase proteins can be formed *in vivo* by calpain digestion (Lee *et al.*, 1990). The presence of a further 3-kinase isozyme is suggested from studies in porcine aortic smooth muscle, in which a higher molecular weight (93,000 Da, calculated by SDS-PAGE) 3-kinase activity has been purified (Yamaguchi *et al.*, 1988). The molecular mass of this protein estimated by gel-filtration agrees with that above, indicating the protein is a monomer and not simply a dimer of two 50,000 Da subunits.

The most generally accepted and well-characterised route of Ins(1,3,4,5)P_4 metabolism is by a Type I 5-phosphatase, as described in section 1.5.1, to form Ins(1,3,4)P_3. Phosphorylation of Ins(1,3,4,5)P_4 by a novel 6-kinase identified in turkey erythrocytes and *Chlamydomonas eugametos* (Irvine *et al.*, 1992) or dephosphorylation by a 3-phosphatase (e.g. Hughes and Shears, 1990; Nogimori *et al.*, 1991) may represent additional albeit more minor routes of Ins(1,3,4,5)P_4 metabolism. However it is unknown how widespread the former enzyme is in the animal kingdom, and the latter enzyme appears to be localised to the endoplasmic reticulum and not to be active within the cytosolic compartment under *in vivo* conditions (Ali *et al.*, 1993).

The metabolism of Ins(1,3,4)P_3 can also proceed through multiple pathways. Two dephosphorylation pathways have been identified in which Ins(1,3,4)P_3 may be metabolised to either Ins(1,3)P_2 by a 4-phosphatase (Bansal *et al.*, 1987, 1990; Guse *et al.*, 1989), or to Ins(3,4)P_2 by the same 1-phosphatase that degrades Ins(1,4)P_2. As noted above, this enzyme is inhibited by lithium ions; the potency of inhibiton is greater when Ins(1,3,4)P_3 is used as a substrate compared to Ins(1,4)P_2 (K_i < 1 mM

and ~ 6 mM respectively). The metabolism of $Ins(1,3)P_2$ proceeds by the action of a 3-phosphatase (Bansal *et al.*, 1987; Caldwell *et al.*, 1991) to produce Ins1P, while $Ins(3,4)P_2$ is metabolised to Ins3P by the same 4-phosphatase that dephosphorylates $Ins(1,3,4)P_3$ (Bansal *et al.*, 1990). As detailed above (section 1.5.1), both Ins1P and Ins3P are finally dephosphorylated to free inositol by the inositol monophosphatase enzyme.

An additional route of $Ins(1,3,4)P_3$ metabolism, demonstrated in a variety of cell types, is phosphorylation by a 6-kinase (e.g. Balla *et al.*, 1987; Hansen *et al.*, 1988; Hughes *et al.*, 1989; Shears *et al.*, 1987a).

The $Ins(1,4,5)P_3$ 3-kinase and 5-phosphatase enzymes therefore provide mechanisms for both the removal of $Ins(1,4,5)P_3$ and the recycling of inositol back into the phosphoinositide pool. There are several reasons however, why the differential routing of $Ins(1,4,5)P_3$ metabolism through the 3-kinase and 5-phosphatase pathways may be important. The 5-phosphatase pathway is generally thought to be simply an inactivating pathway, since $Ins(1,4)P_2$ does not mobilise Ca^{2+} from intracellular stores (Berridge and Irvine, 1989). By contrast, the 3-kinase pathway may provide additional second messengers.

A topic of much debate over recent years has been the involvement of $Ins(1,3,4,5)P_4$ in Ca^{2+} entry. Such an action has been the subject of a great deal of controversy (see Irvine, 1991), nonetheless, the proposed second messenger role for $Ins(1,3,4,5)P_4$ acting, either alone or in concert with $Ins(1,4,5)P_3$, to facilitate Ca^{2+} entry into the cell (e.g. Changya *et al.*, 1989; De Lisle *et al.*, 1992; Irvine and Moor, 1986; Luckhoff and Clapham, 1992) is substantiated by the presence of specific intracellular binding sites for $Ins(1,3,4,5)P_4$ (Bradford and Irvine, 1987; Enyedi and Williams, 1988;

Theibert *et al.*, 1990). Ins(1,3,4,5)P_4 has also been implicated in intracellular Ca^{2+} release (Joseph *et al.*, 1989; Ely *et al.*, 1990; Gawler *et al.*, 1990).

The 6-kinase route of Ins(1,3,4)P_3 metabolism may provide even more functional inositol polyphosphate molecules. For example Ins(1,3,4,6)P_4 is a substrate for the synthesis of Ins(1,3,4,5,6)P_5 (Balla *et al.*, 1989) and $InsP_6$ (Ji *et al.*, 1989; Stephens *et al.*, 1991a). Due to the apparently slow metabolism of these latter compounds (i.e. their labelling to isotopic equilibrium with [^{3}H]inositol takes several days) and their almost unchanging mass, Ins(1,3,4,5,6)P_5 and $InsP_6$ were originally thought to be metabolically inert. Passive roles have therefore been suggested for these highly polar inositol phosphates, for example as Ca^{2+} chelators (Luttrell, 1992) or antioxidants (Graf and Eaton, 1990). More recently however, a rapid, PLC-stimulated accumulation of the Ins(1,3,4,5,6)P_5 metabolite Ins(3,4,5,6)P_4 has been demonstrated (Balla *et al.*, 1989; Barker *et al.*, 1992; Menniti *et al.*, 1990; Stephens *et al.*, 1988) and in [^{3}H]inositol labelling studies the specific activity of this compound following [^{3}H]inositol-labelling always co-varies with [^{3}H]Ins(1,3,4,5,6)P_5. This observation led to the suggestion that a separate Ins(1,3,4,5,6)P_5/$InsP_6$ signalling pathway may exist, although a role for the putative messenger - Ins(3,4,5,6)P_4 has not been identified.

Ins(1,3,4,5)P_4 and its enantiomer Ins(1,4,5,6)P_4 will bind with high affinity to a protein purified from rat brain (Kanematsu *et al.*, 1992). It should be noted that Ins(1,4,5,6)P_4 is also a cellular constituent which may be formed *in vitro* by the action of a 3-phosphatase on Ins(1,3,4,5,6)P_5 (Nogimori *et al.*, 1991), and that accumulation of this isomer may increase following either cell transformation with the *src* oncogene

(Mattingly *et al.*, 1991) or treatment with PIC-linked agonists (Barker *et al.*, 1992).

A high affinity $InsP_6$ binding protein can be found in the nervous system which contains identical amino acid sequences to, and is recognised by antibodies raised against the α subunit of the the clathrin assembly protein AP-2 (Timerman *et al.*, 1992; Volgmaier *et al.*, 1992). In addition Beck and Keen (1991) have shown that $InsP_6$ inhibits clathrin assembly. These data suggest that $InsP_6$ may play a regulatory role in endocytosis/exocytosis cycles involving clathrin coated vesicles.

Further roles for Ins(1,3,4,5,6)P_5 and $InsP_6$ may be in the formation of inositol pyrophosphates. $InsP_5P$ and $InsP_6P$ (i.e. compounds containing a diphosphate on the inositol ring) are formed by ATP-dependent phosphorylation of Ins(1,3,4,5,6)P_5 and $InsP_6$ and are rapidly turned over in AR4-2J cells (Menniti *et al.*, 1993). The standard free energy of hydrolysis of the inositol pyrophosphates isolated from *Dictyostelium discoideum* are 6.5-6.6 kcal mol^{-1} (Stephens *et al.*, 1992), values similar to the pyrophopshate bonds of ATP. It is likely therefore that these compounds represent novel cellular stores of high energy phosphate bonds.

Hence, in addition to governing the pattern of Ins(1,4,5)P_3 formation, regulation of the Ins(1,4,5)P_3 5-phosphatase and 3-kinase enzymes may serve to regulate the formation of a host of other functional molecules. Understanding of the factors which could determine the routing of Ins(1,4,5)P_3 through these two pathways in ASM could give valuable insight into Ca^{2+} signalling in this tissue and its resultant contractile effect.

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1.6. RELAXATION OF AIRWAYS SMOOTH MUSCLE

As with the maintenance of ASM contraction, the mechanisms governing ASM relaxation are not clear. From our current understanding of the role of cross-bridges in the regulation of ASM tone, it would appear that when the rate of force generation is less than the rate of cross-bridge detachment the muscle will relax. Myosin phosphorylation is the rate-limiting factor for force generation (see section 1.2.2) but the mechanisms controlling the rate and extent cross-bridge detachment are not clear. If smooth muscle is relaxed by the removal of a spasmogenic stimulus a close correlation between myosin dephosphorylation and relaxation is observed (de Lanerolle and Stull, 1980; Gerthoffer and Murphy, 1983), however little myosin dephosphorylation accompanies relaxation induced by agents which decrease $[Ca^{2+}]_i$ (Gerthoffer, 1986; Tansey *et al.*, 1990).

It has been appreciated for some time that β-adrenoceptor agonists (e.g. isoprenaline) can induce ASM relaxation and these agents represent the major class of clinical drugs used in the treatment of bronchospasm. β-adrenoceptors couple via G_s to activation of adenylyl cyclase with the resultant formation of the second messenger cAMP which can in turn activate protein kinse A (PKA). The effects of β-adrenoceptor agonists on ASM tone have generally been attributed to their ability to stimulate PKA. PKA in turn has been thought to effect relaxation by phosphorylating a number of functionally relevant substrates resulting in increases in Ca^{2+}-ATPase and Na^+/K^+-ATPase activity, activation of BK_{Ca} channels, inhibition of PIC and modulation of the contractile machinery (see sections 1.6.1-4 below). In accordance with this theory it has been observed that cAMP analogues can relax ASM (Francis *et al.*, 1988; Heaslip *et al.*, 1987; Torphy *et al.*, 1985) and that the bronchorelaxant action of β-

adrenoceptor agonists can be mimicked and potentiated by cyclic nucleotide phosphodiesterase inhibitors (Bryson and Rodger, 1987; Harris *et al.*, 1989; Polson *et al.*, 1982; Shahid *et al.*, 1991; Small *et al.*, 1989; Torphy *et al.*, 1988).

Recent evidence however has challenged the dogma that cAMP-mediated activation of PKA is the sole mechanism underlying the relaxant effects of β-adrenoceptor agonists (see Torphy, 1994). For example, cGMP-dependent protein kinase (PKG) from canine tracheal smooth muscle shows little selectivity for cGMP over cAMP *in vitro* (Torphy *et al.*, 1982) and the increase in cAMP levels following isoprenaline treatment of bovine coronary arteries results in simultaneous activation of both PKG and PKA (Jiang *et al.*, 1992). Similarly, preliminary evidence indicates that treatment of canine trachealis with forskolin (a direct activator of adenylyl cyclase) also produces activation of PKC and PKG (see Torphy and Hall, 1994). These data suggest that cAMP may exert some of its second messenger action through PKG. Moreover, Francis *et al.* (1988) showed that the ability of a range of cyclic nucleotide analogues to relax guinea-pig trachealis correlated with their ability to activate PKG and not PKA. In support for a role of PKG in relaxation of ASM it has been observed that increases in the concentration of cGMP, or the application of non-hydrolysable analogues of cGMP, induce relaxation of pre-contracted tracheal smooth muscle preparations (e.g. Heaslip *et al.*, 1987; Szaduykis-Szadurski and Berti, 1972; Szaduykis-Szadurski *et al.*, 1972).

The demonstration that a greater increase in cAMP content is required to produce a similar degree of relaxation in canine trachealis treated with forskolin as compared with isoprenaline also suggests that additional β-adrenoceptor-mediated, cAMP-independent mechanisms may contribute to the relaxant response. Some of the possible mechanisms by which β-

adrenoceptor agonists may exert their bronchorelaxant effect are discussed below.

1.6.1 Regulation of membrane potential

β-adrenoceptor stimulation of trachealis is accompanied by hyperpolarisation of the plasma membrane (Allen *et al.*, 1985; Fujiwara *et al.*, 1988; Honda *et al.*, 1986), an effect which is antagonised by charybdotoxin, a selective inhibitor of the Ca^{2+}-activated potassium channel (BK_{Ca}) (Jones *et al.*, 1990; Murray *et al.*, 1991a) which is densely distributed in ASM membranes (McCann and Welsh, 1986). Charybdotoxin treatment also attenuates the relaxant effect of isoprenaline on ASM (Huang *et al.*, 1993; Jones *et al.*, 1990; Miura *et al.*, 1992). The observation that the open-state probability of BK_{Ca} is increased in rabbit tracheal myocytes following application of either isoprenaline or exogenous PKA (Kume *et al.*, 1989) led to the suggestion that this effect might underlie β-adrenoceptor-mediated relaxation of ASM. In addition it would appear that PKG is also able to regulate BK_{Ca} channel activity: Hamaguchi *et al.* (1992) reported that charybdotoxin inhibits the relaxation of bovine trachealis in response to agents which activate guanylyl cyclase and increase the tissue PKG content. Furthermore, BK_{Ca} is phosphorylated by PKG in canine coronary smooth muscle cells (Taniguchi *et al.*, 1993) and rabbit basilar arteries (Robertson *et al.*, 1993). Hence activation of the BK_{Ca} channel activity may contribute to the relaxant effect of β-adrenoceptors on ASM, however part of the relaxant response is charybdotoxin-insensitive indicating that other relaxant mechanisms must also exist in this tissue (Huang *et al.*, 1993; Jones *et al.*, 1990; Miura *et al.*, 1992). In addition to the BK_{Ca} channel, β-adrenoceptor agonists can stimulate the plasma membrane Na^{+}/K^{+}-

ATPase (Gunst and Stropp, 1988) which can also result in membrane hyperpolarisation and may lower $[Ca^{2+}]_i$ by a consequent increase in Na^+/Ca^{2+} exchange.

1.6.2 Regulation of intracellular calcium concentration

cAMP and cGMP elevating agents, when added immediately prior to or after the addition of agonist, can attenuate the spasmogen-induced Ca^{2+} response in ASM (e.g. Felbel *et al.*, 1988; Takuwa *et al.*, 1987; Taylor *et al.*, 1989). This effect may reflect the ability of cAMP to promote both Ca^{2+} extrusion and uptake into intracellular stores in smooth muscle preparations (Itoh *et al.*, 1982; Mueller and Van Breemen, 1979; Schied and Fay, 1984). Furthermore, the hyperpolarisation induced by β-adrenoceptor agonists could reduce Ca^{2+} entry through the classical dihydropyridine-sensitive voltage operated Ca^{2+} channels present in ASM (Worley and Kotlikoff, 1991). While a continued decrease in Ca^{2+} entry may eventually deplete intracellular Ca^{2+} stores (and hence the amount of Ca^{2+} available for the initial spasmogen-induced Ca^{2+} transient), such a decrease in Ca^{2+} entry would be first expected to result in inhibition of the tonic $[Ca^{2+}]_i$ response. The plateau phase of the Hist-induced Ca^{2+} response, at least in cultured human airway myocytes however, is insensitive to organic Ca^{2+} channel blockers (Murray and Kotlikoff, 1991). Ca^{2+} entry in these cultured cells appears to proceed via a receptor-operated Ca^{2+} channel rather than a voltage-operated mechanism which explains the latter result (Murray *et al.*, 1992). It is not clear whether there are major differences between the Ca^{2+} entry pathways of cultured cells and intact human ASM tissue; nonetheless organic voltage-operated Ca^{2+} channel blockers have minimal efficacy as clinical bronchodilators.

1.6.3 Regulation of phosphoinositide hydrolysis

In ASM, cAMP elevating agents have been shown to inhibit agonist-stimulated [^{3}H]InsP formation (Hall and Hill, 1988; Hall *et al.*, 1989; Madison and Brown, 1988; Offer *et al.*, 1991). The InsP response stimulated by the non-specific G-protein activator sodium fluoride is also inhibited by increases in cAMP suggesting a post-receptor mechanism is involved in this effect (Hall *et al.*, 1990a). However β-adrenoceptor stimulation does not seem to affect the initial Ins(1,4,5)P_3 response since the transient increase in the concentration of this second messenger is unaffected by isoprenaline pre-treatment in BTSM strips (Challiss and Boyle, 1994). A differential ability of β-adrenoceptor agonists to cause ASM relaxation has been observed, depending on the nature and concentration of the contractile agonist (Jenne *et al.*, 1987; Russel, 1984; van Amsterdam *et al.*, 1989). The differential relaxant responses are mirrored by a differential ability of β-adrenoceptor agonists to inhibit spasmogen-induced phosphoinositide hydrolysis. Muscarinic cholinoceptor-stimulated contraction seems particularly resistant to β-adrenoceptor-mediated relaxation and InsP responses elicited by maximally effective concentrations of full muscarinic agonists are unaffected by β-adrenoceptor pre-stimulation (Hall and Hill, 1988; Madison and Brown, 1988; Offer *et al.*, 1991). However, when tissue cAMP levels are increased using cyclic nucleotide phosphodiesterase inhibitors, an attenuation of the [^{3}H]InsP response to low concentrations of the muscarinic agonist CCh can be observed (Hall *et al.*, 1990b; Offer *et al.*, 1991).

One possible explanation for the relative resistance of muscarinic receptor-stimulated phosphoinositide hydrolysis to inhibition by β-adrenoceptors is the large M_3 muscarinic receptor reserve present in

tracheal smooth muscle (Gunst *et al.*, 1989; van Amsterdam *et al.*, 1989). This hypothesis however, has been challenged by the observation that some, but not all, partial muscarinic agonist-stimulated [^{3}H]InsP responses are susceptible to β-adrenoceptor-mediated inhibition (Offer *et al.*, 1991). It is possible that a differential stimulation of M_2 receptors (rather than M_3 receptors) by these partial agonists may determine their susceptibility to β-adrenoceptor inhibition. As detailed earlier, ASM expresses a large proportion of M_2 muscarinic receptors which are coupled via G_i to the inhibition of adenylyl cyclase. Indeed both basal and relaxant agonist-stimulated PKA activity in tracheal smooth muscle preparations are decreased by muscarinic agonists (Langlands and Rodger, 1992; Torphy *et al.*, 1985), and pre-treatment of canine tracheal smooth muscle with pertussis toxin renders muscarinic agonist-contracted tissue more sensitive to β-adrenoceptor-mediated relaxation (Mitchell *et al.*, 1993). In addition, it has been suggested recently that muscarinic cholinoceptor stimulation may inhibit adenylyl cyclase activity not only by stimulation of G_i, but also via a functional inactivation of G_s (Pyne *et al.*, 1992).

1.6.4 Regulation of contractile machinery

As detailed above, PKA can phosphorylate MLCK resulting in a reduction in its affinity for Ca^{2+}/calmodulin and hence a decreased catalytic activity. PKA-mediated phosphorylation of MLCK would therefore be expected to result in a decrease in force generation. In support of such a mechanism being involved in β-adrenoceptor-mediated relaxation of ASM, de Lanerolle *et al.* (1984) reported that forskolin treatment of canine tracheal smooth muscle increased both cAMP accumulation and phosphorylated MLCK. It is now clear that isoprenaline-induced relaxation of BTSM

occurs without a change in the affinity of Ca^{2+}/calmodulin for MLCK (Miller *et al.*, 1983; Tang *et al.*, 1992) and hence the effects of β-adrenoceptor stimulation on the contractile machinery may be secondary to Ca^{2+}-regulated events.

1.7 AIMS OF THE THESIS

The past decade has witnessed clear advances in our understanding of the involvement of phosphoinositide metabolism in ASM contraction. However no detailed studies have examined inositol polyphosphate metabolism in ASM, and there has been no investigation into the potential mechanisms involved in regulating Ins(1,4,5)P_3 accumulation, especially at early time-points. The aim of this study was to set up a model system (bovine tracheal smooth muscle) in which the inositol polyphosphate isomers formed following receptor stimulation could be identified and quantified. The protocol facilitates an assessment of the relative contributions of the 3-kinase and 5-phosphatase pathways to Ins(1,4,5)P_3 metabolism and so permits a study of the differential routing of Ins(1,4,5)P_3 under various conditions - looking at different time-points following agonist stimulation, the response to different contractile agonists, and the response observed when phosphoinositide hydrolysis is modulated by the pre-treatment of BTSM with activators of both PKC and β_2-adrenoceptors. An important finding obtained during the course of these studies was the agonist-stimulated accumulation of a novel InsP_2 isomer, namely Ins(4,5)P_2. Experiments were therefore undertaken to characterise the kinetics of its accumulation and the possible routes for its formation and metabolism. In addition kinetic studies were undertaken to address the relative affinities of the 3-kinase and 5-phosphatase

enzymes for $Ins(1,4,5)P_3$ and to assess the effect of Ca^{2+} ions on these enzymes.

CHAPTER 2
MATERIALS AND METHODS

2.1 MATERIALS

Fresh bovine tissue samples (tracheas or adrenal glands) were obtained from the local abattoir. All radiochemicals were purchased from NEN (Du Pont) (Stevenage, Herts., U.K.). Dowex anion exchange resins were from Bio-Rad (Watford, Herts., U.K.) and H.P.L.C. anion exchange columns and pre-columns from Whatman (Maidstone, Kent, U.K.). Scintillation cocktails were supplied by Packard (Pangbourne, Berks., U.K.), D-inositol 1,4,5-trisphosphate by Reasearch Biochemicals International (St Albans, Herts., U.K.), calpain inhibitors by Calbiochem (Nottingham, U.K.) and the BCA protein assay kit by Pierce (Chester, Cheshire, U.K.). Salmeterol was a kind gift from Glaxo (Ware, Herts., U.K.). All other reagents were purchased from either Sigma Chemical Company Limited (Poole, Dorset, U.K.) or BDH (Lutterworth, Leics., U.K.).

2.2 PREPARATION OF BOVINE TRACHEAL SMOOTH MUSCLE SLICES

BTSM slices were prepared according to the method described by Chilvers *et al.* (1989a). Fresh bovine tracheas were obtained from the local abbattoir and the trachealis muscle isolated and transported at 4 °C in pre-oxygenated (95% O_2/5% CO_2) modified Krebs-Henseleit buffer (KHB) (118 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 11.7 mM glucose). Prostaglandins, formed by the action of cyclo-oxygenase on arachidonic acid (a metabolite of DAG) have been proposed

to act in an autocrine/paracrine fashion in a variety of cell types including uterine smooth muscle (Goureau *et al.*, 1990; Okawa *et al.*, 1993) to affect, either directly or indirectly via adenylyl cyclase, the formation of inositol polyphosphates. In order to ensure that no such modulation of receptor-mediated InsPP production occurred in my system the potent cyclo-oxygenase inhibitor flurbiprofen (1 μM) was included in the buffer (KHB) for all BTSM preparations. The muscle was dissected free of any overlying epithelium or connective tissue and chopped into 300 x 300 μm slices on a wooden block with a McIlwain tissue chopper. The tissue slices were then washed three times in 200 ml KHB and pre-incubated, in bulk, in 500 ml oxygenated KHB for 60 min at 37 °C in a shaking water bath. Buffer was replaced and re-equilibrated every 15 min over this pre-incubation period.

2.3 LABELLING OF BOVINE TRACHEAL SMOOTH MUSCLE SLICES WITH *MYO*-[^{3}H]INOSITOL

Myo-[^{3}H]inositol (specific activity 17-25 Ci/mmole) was used to label BTSM slices since incorporation of this molecule facilitates labelling of both the membrane-bound phosphoinositides and the water soluble inositol phosphates subsequently formed by the hydrolysis of these lipids. In addition, inositol is not readily metabolised by any other pathways than those involved in the inositol polyphosphate metabolism under study. Two different protocols were utilised to achieve steady-state radiolabelling of the phosphoinositide pool: agonist-stimulated labelling requires only a short (one hour) labelling period (Chilvers *et al.*, 1989a), whereas overnight labelling has the advantages of using much less radioactivity, thereby being more economical, and of producing far more

labelled slices from each BTSM preparation, so enabling a larger scale experiment to be carried out.

2.3.1 Agonist-stimulated labelling

Following pre-incubation (2.2) BTSM slices were allowed to settle and 3 ml of gravity packed slices were transferred to a 25 ml Erlenmeyer flask in a total volume of 12 ml oxygenated KHB containing 1 μM CCh and 30 μCi (or 150 μCi for experiments involving H.P.L.C.) cleaned *myo*-[^{3}H]inositol[1] (i.e. 0.5 or 2.5 μCi [^{3}H]inositol/50 μl BTSM slices respectively). The slices were then incubated at 37 °C for 60 min in a shaking water bath, re-oxygenating the labelling medium (95% O_2/5% CO_2) every 15 min. This labelling period was followed by extensive washing of the slices with fresh KHB over 30 min to remove any agonist and unincorporated label. Subsequent stimulations were carried out in 6 ml flat bottomed vials. Aliquots (50 μl) of gravity packed slices were incubated in the presence of an identical concentration of *myo*-[^{3}H]inositol to that described above, in a final volume of 300 μl oxygenated KHB at 37 °C in a shaking water bath. Unless otherwise stated LiCl was added 10 min prior to agonists, each being added in a 10 μl volume. In most experiments CCh and histamine were used at their maximally effective concentrations (100 μM and 1 mM respectively, as shown in figure 2.3.1.1). Figure 2.3.1.2 shows the radioactivity associated with [^{3}H]InsPs and [^{3}H]inositol phospholipids over a 30 min time-course following stimulation with 100 μM CCh. In agreement with Chilvers (1991), with the above labelling protocol there is little change in the labelling of the lipids and an approximately linear accumulation of the [^{3}H]InsPs, suggesting that steady-state labelling of the agonist-sensitive phosphoinositide pool has indeed been achieved.

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[1]The *Myo*-[^{3}H]inositol label was previously cleaned of polar contaminants by passing through a 250 μl Dowex AG 1-X8 column (100-200 mesh, formate form).

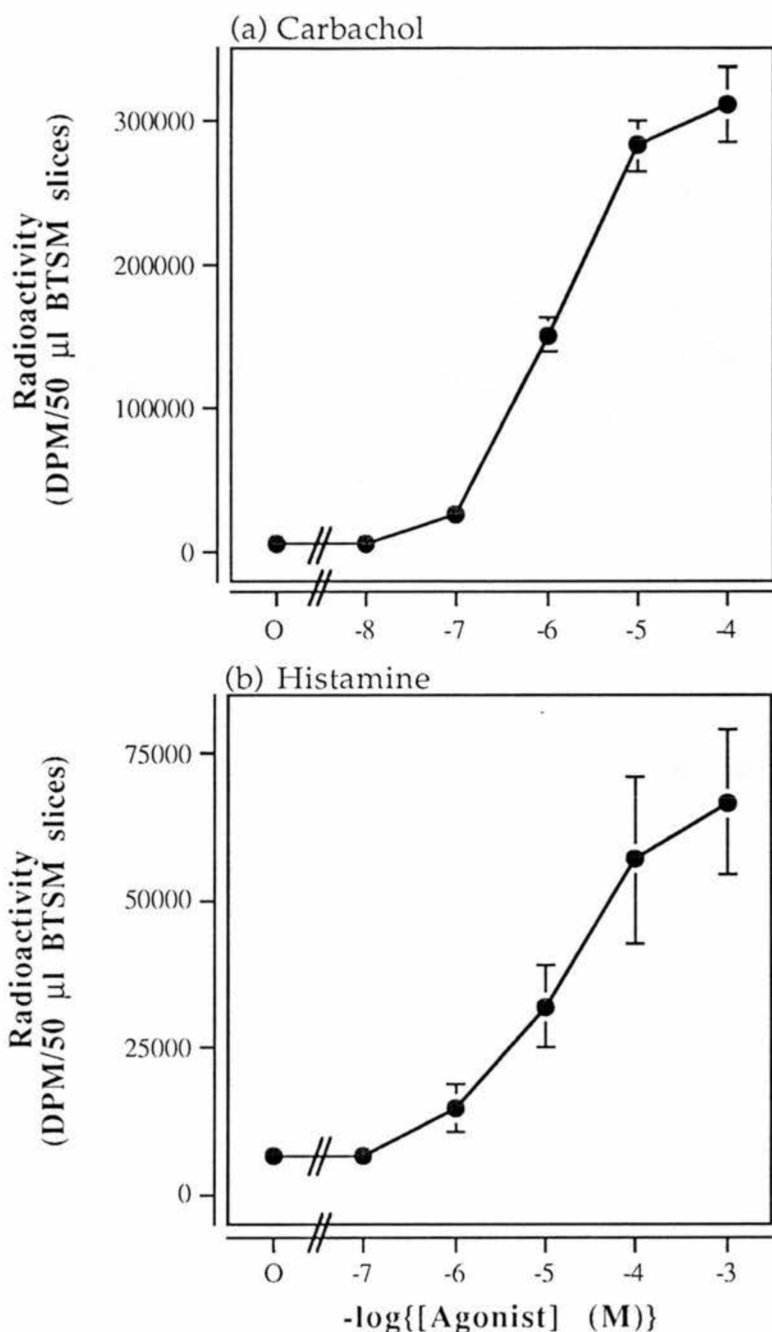

Figure 2.3.1.1a & b Concentration-response relationship for carbachol- and histamine -stimulated total [^{3}H]inositol phosphate accumulation

BTSM slices were pre-labelled for 24 hours with 0.5 µCi [^{3}H]inositol/50 µl slices (2.3.2) prior to stimulation with the indicated concentration of agonist for 30 min in the presence of 10 mM LiCl. [^{3}H]InsPs in neutralised TCA extracts (2.4.2) were were quantified using Dowex anion exchange chromatography (2.5.1). Data represent mean ± SEM of two separate experiments.

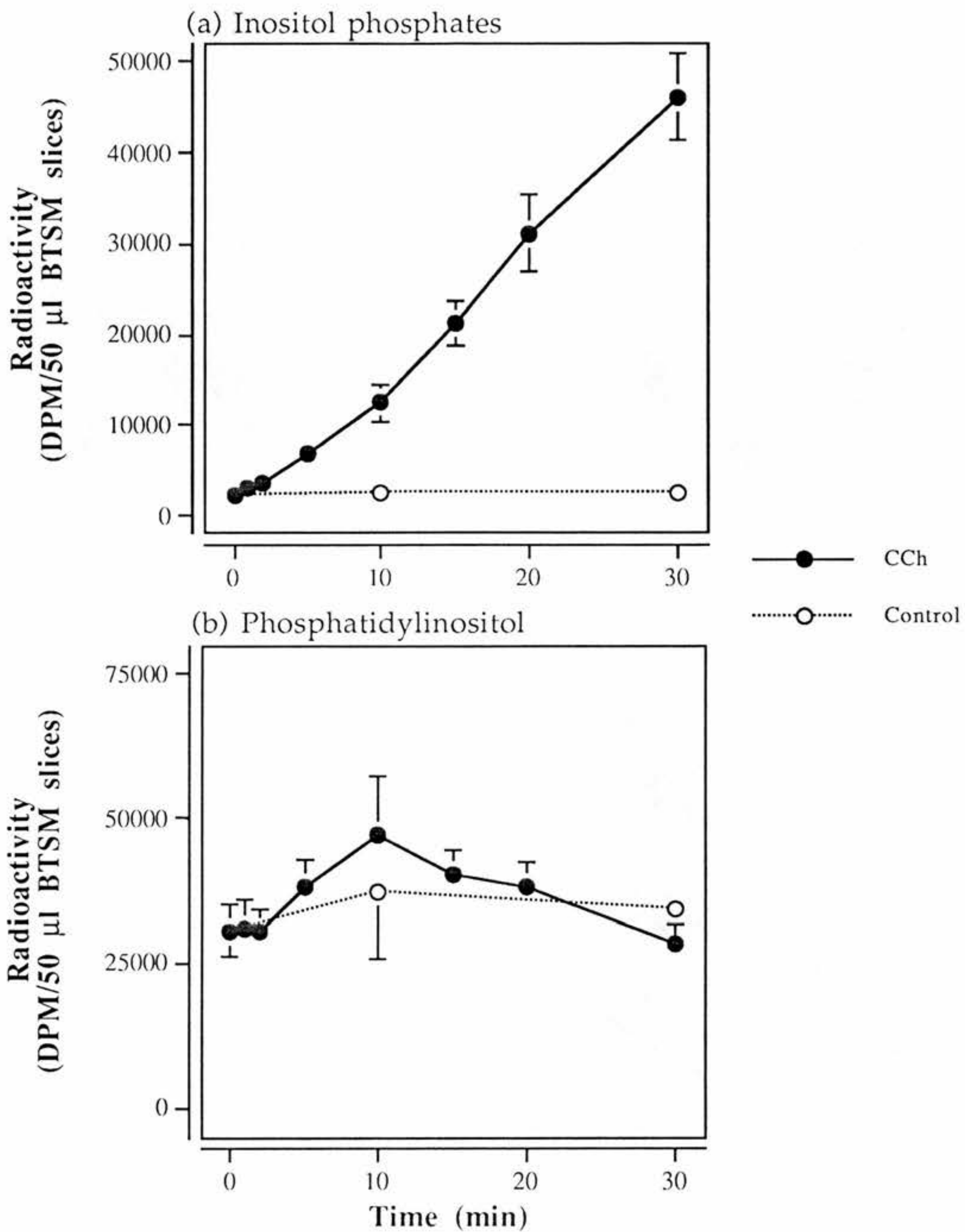

Figure 2.3.1.2a & b Time course of carbachol-stimulated [^{3}H]inositol incorporation into inositol phosphates and inositol phospholipids
Aliquots (50 µl) of BTSM slices were pre-labelled (short-term) with [^{3}H]inositol in the presence of 1 µM CCh (2.3.1). After extensive washing, slices were stimulated in the presence of 5 mM LiCl with 100 µM CCh or vehicle for the times indicated, in a final volume of 300 µl. Reactions were terminated with 0.94 ml chloroform/methanol (1:2 v/v) and total [^{3}H]InsPs and [^{3}H]PtdIns(P)s separated and quantified as detailed (2.4.1, 2.5). Data represent mean ± SEM of four separate experiments.

However, when total $[^3H]$InsPPs were assessed over a more detailed, shorter time-course following CCh stimulation (figure 2.3.1.3), an attenuation of $[^3H]$InsPP accumulation was seen after 30 seconds. This may represent a slight decrease in the specific radioactivity of the phosphoinositides or a small degree of receptor desensitisation, but is more likely due to an inevitable retardation of the extremely rapid initial hydrolysis of $[^3H]PtdIns(4,5)P_2$. Such rapid metabolism of this lipid cannot be maintained since its resynthesis by the sequential phosphorylation of $[^3H]$PtdIns and $[^3H]$PtdIns4P proceeds at a slower rate (Chilvers *et. al.* 1991a).

2.3.2 Overnight labelling

BTSM slices (2.2) were washed twice in 20 ml M199 medium containing 50 units/ml of each of penicillin and streptomycin. Aliquots (50 µl) of gravity packed slices were then transferred to 24 well tissue culture plates containing M199 medium and 0.92-3.00 µCi/ml *myo*-$[^3H]$inositol and incubated at 37 °C for 24 hours in a humidified atmosphere of 5% CO_2/95% air. Subsequent stimulations were carried out with the plate standing on a dry block at 37 °C (returning slices to the incubator when possible) in a final volume of 1 ml. Unless otherwise stated LiCl was added to the wells 10 min prior to agonists, each being added in a 10 µl volume.

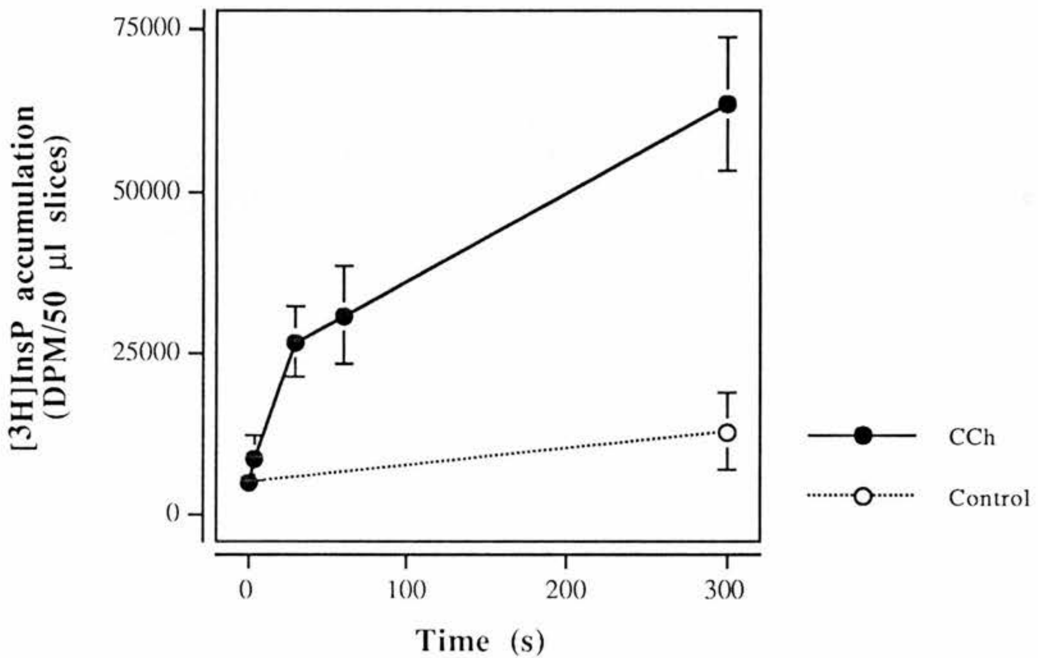

Figure 2.3.1.3 Detailed early time-course of carbachol-stimulated [^{3}H]inositol phosphate accumulation

Aliquots (50 µl) of BTSM slices were pre-labelled with [^{3}H]inositol in the presence of 1 µM CCh (2.3.1). After extensive washing slices, were stimulated in the presence of 0.5 mM LiCl with 100 µM CCh or vehicle for the times indicated. [^{3}H]InsPs were separated from neutralised neutralised TCA extracts (2.4.2) using H.P.L.C. (2.6.3), and the radioactivity in each fraction quantified by liquid scintillation counting. Results are presented as radioactivity associated with total [^{3}H]InsPPs against time. Data represent mean ± SEM of three separate experiments.

2.4 EXTRACTION OF [^{3}H]INOSITOL PHOSPHATES

2.4.1 Neutral chloroform/methanol extraction

For experiments where both [^{3}H]inositol phospholipids and [^{3}H]InsPs were to be measured a chloroform/methanol extraction was employed. Neutral extracts were prepared as described by Bligh and Dyer (1959) with incubations (300 μl) terminated by the addition of 0.94 ml chloroform/methanol (1:2, v/v). Following the addition of a further 0.31 ml of each of chloroform and dH_2O, samples were left to extract at room temperature for 10 min before partitioning of the resulting aqueous and organic phases by centrifugation (3,000 g, 20 min, 25 °C). Aliquots (0.75 ml) of the upper aqueous phase were diluted with 2.25 ml dH_2O and stored at 4 °C for subsequent separation and quantification of [^{3}H]InsPs (see section 2.6).

2.4.2 Trichloroacetic acid extraction

For experiments where [^{3}H]InsPs only, or [^{3}H]InsPs and [^{3}H]polyphosphoinositides were to be measured, a trichloroacetic acid extraction (as developed by Sharps and McCarl, 1982) was employed. This acid extraction protocol has the advantage of faithfully recovering the higher InsPPs (especially $InsP_3$ and above) which are not so readily isolated with a neutral chloroform/methanol extraction, and of facilitating a further acidic chloroform/methanol extraction on the tissue pellet as is necessary for recovery of the polyphosphoinositides (see section 2.5). Reactions were terminated with an equal volume of ice-cold 1 M trichloroacetic acid (TCA) (except for those experiments performed in 24 well tissue culture plates, in which 1 ml incubations were terminated with 300 μl ice-cold 3 M TCA). Samples were left to extract on ice for 10-20 min and, if necessary, transferred to 6 ml flat bottomed vials. After

vortex mixing and centrifugation (3000 g, 20 min, 25 °C) the TCA was removed from the extracts using 1,1,2-trichloro-1,2,2-trifluoroethane (freon)/tri-n-octylamine according to the method described by Downes and Wusteman (1983). Portions of the supernatants were transferred to 1.5 ml Eppendorf tubes containing an equal volume of freon/octylamine (1:1, v/v, made fresh) and a quarter volume of 10 mM EDTA (pH 7); samples were vortexed vigorously, microfuged (10,000 g, 5 min, 25 °C) and aliquots of the upper aqueous layer neutralised with a 1/5 volume 60 mM $NaHCO_3$. Neutralised TCA extracts were stored at 4 °C for subsequent separation and quantification of $[^3H]$InsPs (2.6). Since neutralisation is achieved by the removal of acid rather than the addition of alkali there is no increase in the amount of salt in the final samples. The extracts are also free of any insoluble protein or solvents and therefore this protocol produces samples ideally suited for subsequent H.P.L.C. analysis.

2.5 EXTRACTION AND QUANTIFICATION OF $[^3H]$PHOSPHO-INOSITIDES

$[^3H]$PtdIns may be isolated by neutral chloroform/methanol extraction as detailed in section 2.4.1: following centrifugation, aliquots of the lower organic phase are mixed with Emulsifier-Safe scintillation fluid and radioactivity in the samples determined by liquid scintillation counting. The polyphosphoinositides however are highly negatively charged and tend to associate with protein, possibly via interactions with Ca^{2+} or Mg^{2+} ions, and therefore do not readily enter the organic phase following neutral chloroform/methanol extraction. Accurate quantification of these polyphosphoinositides requires acidic conditions to supress their

negative charge and prevent the formation of salt-bridges, thereby facilitating their partitioning into the organic phase with the other phospholipids (see Hawthorne and White, 1975).

2.5.1 Acidic chloroform/methanol extraction

TCA precipitated tissue pellets (2.4.2) were washed according to the method of Creba *et al.* (1983), with 1 ml 5% (w/v) TCA in 1 mM EDTA, followed by 1 ml dH_2O. [^{3}H]Polyphosphoinositides were extracted from this pellet as detailed by Downes and Wusteman (1983): 0.94 ml chloroform/methanol (2:1, v/v)/100 mM HCl was added to the pellet and samples left on ice to extract for 10-15 minutes. A further 0.31 ml chloroform and 0.56 ml 0.1 M HCl were added to the samples prior to vortexing and centrifugation (3,000 g, 20 min, 4 °C). The upper aqueous layer was gently aspirated and discarded. Aliquots of the lower organic phase were evaporated to dryness under nitrogen. The radioactivity in these samples was determined by redissolving in Emulsifier-Safe scintillation fluid and liquid scintillation counting, or alternatively the dried lipids could be stored under nitrogen at -20 °C overnight for subsequent deglyceration (see section 2.5.2).

2.5.2 Alkaline hydrolysis of [^{3}H]phosphoinositides

Base hydrolysis (deglyceration) of [^{3}H]polyphosphoinositides, according to a method modified from Grado and Ballou (1961), was used to produce a series of [^{3}H]InsPP standards for characterisation of the H.P.L.C. separation system. Pooled triplicates of acidic chloroform/methanol extracts (2.5.1) (previously dried under nitrogen) were redissolved in 0.25 ml 1 M KOH, and placed in a boiling water bath for 30 min. Samples were then cooled on ice for five minutes prior to centrifugation (3,000 g, 20 min, 4 °C). Excess alkali was removed by passing the supernatant through a 0.5 ml

acidified formate Dowex AG 1-X8, 200-400 mesh column. After washing the column with 2.25 ml dH_2O the combined eluates were extracted, as described by Clark and Dawson (1981), with 3 ml butan-1-ol/petroleum ether (5:1 v/v) followed by a further 1.875 ml butan-1-ol/petroleum ether to remove any contaminating fatty acids and other lipids. Aliquots (2 ml) of the lower phase were sampled with a long-reach pipette, dried down under nitrogen and stored at -20 °C for subsequent H.P.L.C. analysis (2.6.3).

2.6 SEPARATION AND QUANTIFICATION OF [^{3}H]INOSITOL PHOSPHATES

2.6.1 Separation of total [^{3}H]inositol phosphates

The total [^{3}H]InsP fraction extracted from BTSM slices was separated using formate Dowex AG 1-X8 (200-400 mesh) anion exchange chromatography according to a method modified from Rooney and Nahorski (1986). Open glass columns (5 mm internal diameter, ASA Derby U.K.) were plugged with glass wool and loaded under water with a 1 ml volume of dH_2O pre-washed anion exchange resin (50% w/v slurry). Neutralised TCA extracts (2.4.2) or the aqueous phase from neutral chloroform/methanol extracts (2.4.1) were applied to the column and unbound [^{3}H]inositol removed by washing with 20 ml dH_2O. [^{3}H]GroPIns was eluted from the column using 4 ml 0.025 M NH_4COOH, and the remaining [^{3}H]InsPs collected by elution with 10 ml 1 M NH_4COOH/0.1 M HCOOH. This latter buffer is of sufficiently high concentration to remove [^{3}H]$InsP_{1-4}$. More extensive washing with 0.025 M ammonium formate resulted in early elution of [^{3}H]InsPs present in tissue extracts, and hence figure 2.6.1 shows that with such prolonged

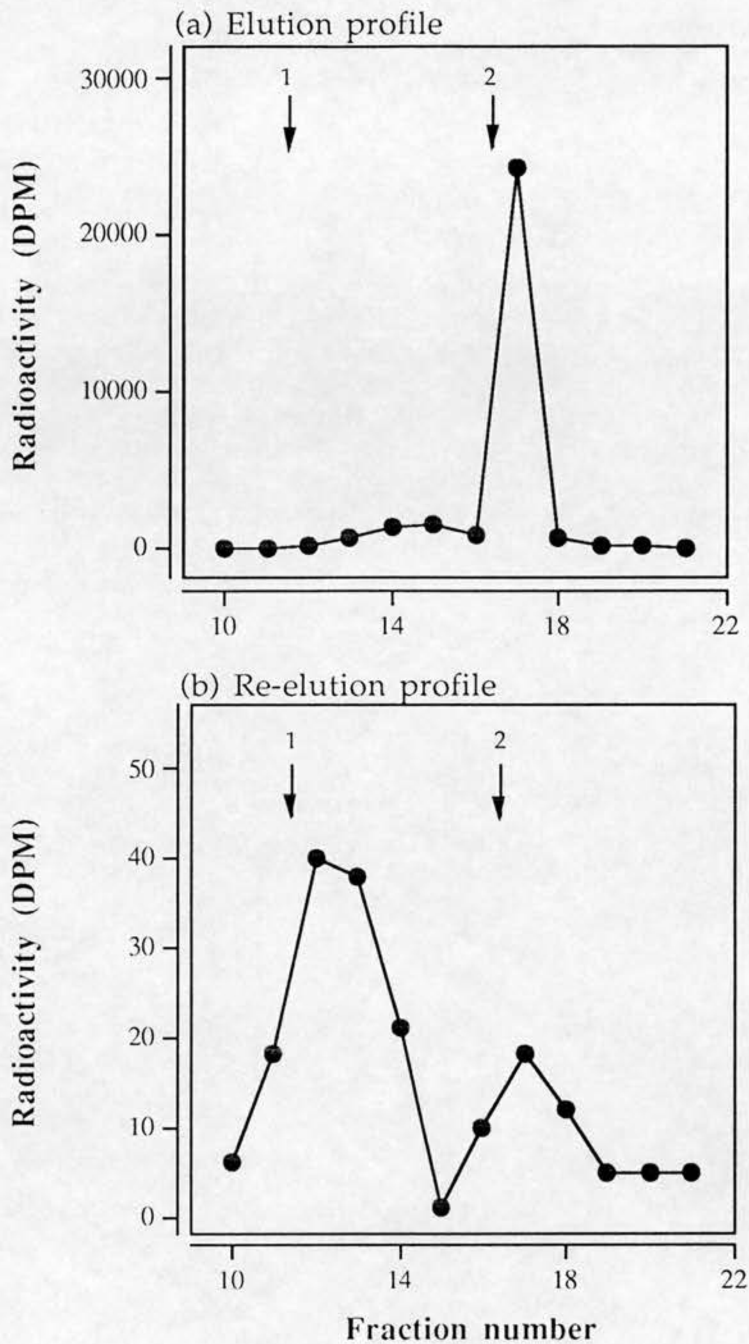

Figure 2.6.1 Purity of the [^{3}H]GroPIns fraction from Dowex anion exchange chromatograpy

(a) Neutralised TCA extracts (2.4.2) from [^{3}H]inositol labelled BTSM slices (2.3.1) stimulated with CCh (30 min) were applied to formate Dowex AG 1-X8 columns and eluted with 10 x 2 ml dH_2O, 5 x 2 ml 0.025 M NH_4COOH (1) and 5 x 2 ml 1 M NH_4COOH/0.1 M HCOOH (2).

(b) Samples 12-16 were combined, diluted ten-fold and re-eluted exactly as described above. Radioactivity in the eluate was determined by liquid scintillation counting.

washing there is a significant contamination of the [^{3}H]GroPIns fraction with [^{3}H]InsPs. Higher [^{3}H]InsPs (i.e. [^{3}H]InsP$_5$ and [^{3}H]InsP$_6$) can be removed from the column with 10 ml 2 M NH_4COOH/0.1 M HCOOH, however no such compounds could be detected in neutralised tissue extracts obtained from tissue labelled under short-term (60 min) agonist-stimulated conditions (2.3.1) (results not shown). Radioactivity associated with the separated [^{3}H]InsPs was quantified by liquid scintillation counting of 2 ml samples of eluant mixed with 18 ml Emulsifier-Safe.

2.6.2 Separation of [^{3}H]inositol mono-, bis-, tris- and tetrakisphosphates

[^{3}H]Inositol polyphosphate fractions were separated using Dowex anion exchange chromatography and ammonium formate/formic acid buffers as detailed by Batty *et al.* (1985). Neutralised TCA extracts (2.4.2) were applied to 1 ml formate Dowex AG 1-X8 columns as described above. Typically [^{3}H]Ins, [^{3}H]GroPIns, [^{3}H]InsP$_1$, [^{3}H]InsP$_2$, [^{3}H]InsP$_3$ and [^{3}H]InsP$_4$ were eluted in a sequential manner with 20 ml dH_2O, 4 ml 0.025 M NH_4COOH, 14 ml 0.2 M NH_4COOH, 14 ml 0.5 M NH_4COOH/0.1 M HCOOH, 14 ml 0.75 M NH_4COOH/0.1 M HCOOH and 10 ml 1 M NH_4COOH/0.1 M HCOOH respectively. Due to significant batch-batch variation in the Dowex anion exchange resin purchased, the exact concentration and quantity required of each buffer was reassessed for each new batch resin acquired and varied accordingly. Figure 2.6.2 shows the separation of [^{3}H]Ins1P, [^{3}H]Ins(1,4)P$_2$ and [^{3}H]Ins(1,4,5)P$_3$ standards achieved by this technique. For the 3-kinase and 5-phosphatase experiments in which, by design, there was little or no degradation of [^{3}H]InsPs to free inositol the dH_2O and 0.025 M NH_4COOH washes were omitted.

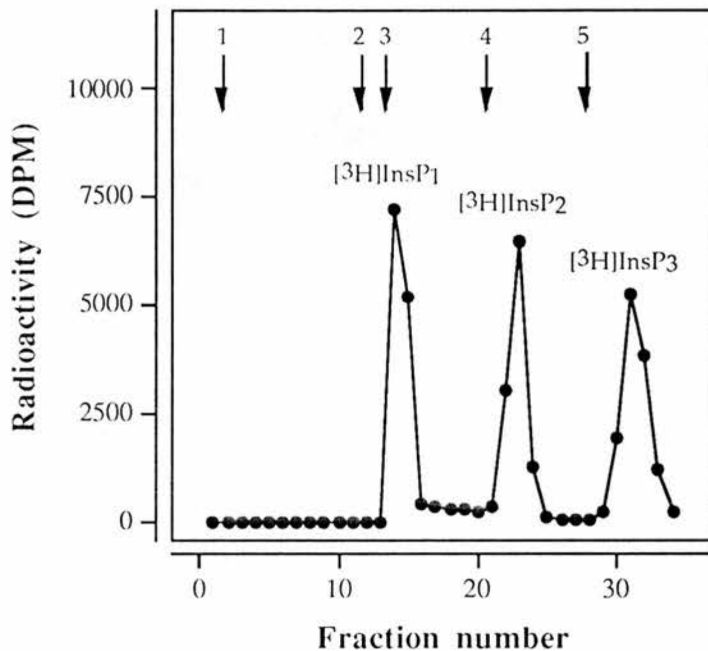

Figure 2.6.2 Separation of [^{3}H]inositol phosphate standards by Dowex anion exchange chromatography

10,000 DPM each of [^{3}H]Ins1P, [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins(1,4,5)P_3 were added to BTSM tissue extract (2.4.2) and applied to a formate Dowex AG 1-X8 column. The column was then washed with with the following buffers: 10 x 2 ml dH_2O (1), 2 x 2ml 0.025 M NH_4COOH (2), 7 x 2 ml 0.2 M NH_4COOH (3), 7 x 2 ml 0.5 M NH_4COOH/0.1 M HCOOH (4) and 7 x 2 ml 0.75 M NH_4COOH/0.1 M HCOOH (5). The radioactivity associated with each 2 ml sample of eluant was quantified by liquid scintillation counting.

2.6.3. Separation of individual [^{3}H]inositol polyphosphate isomers

Individual [^{3}H]inositol polyphosphate isomers were separated using H.P.L.C. according to a method modified from Batty *et al.* (1989). Neutralised TCA extracts (2.4.2) were spiked with 50 μM each of adenosine-, and guanosine- mono, di, tri, and tetraphosphates and adjusted to a final volume of 2.5 ml with dH_2O. Aliquots (2 ml) were then injected onto a Partisphere 5 SAX anion exchange column equipped with a cartridge pre-column containing anion exchange resin. Nucleotides were added to provide internal standards for each H.P.L.C. run. Following sample injection the column was washed for 20 min with dH_2O, and the [^{3}H]InsPPs and nucleotides eluted with five consecutive gradients (i-v) of dH_2O (A) and 1.4 M $NH_4H_2PO_4$ adjusted to pH 3.7 with H_3PO_4 (B) buffers, at a flow rate of 1 ml/min: (i) linear gradient 0-5% B over 30 min, (ii) isocratic elution at 14% B for 15 min, (iii) linear gradient 14-21% B over 15 min, (iv) isocratic elution at 35% B for 35 min and (v) isocratic elution at 100% B for 5 min (see figure 2.6.3.1). Nucleotides were detected by continuous U.V. monitoring of the column eluate at 254 nm - a typical trace from the on line uvicord (Pharmacia) is shown in figure 2.6.3.1. The eluate was collected in 0.5 ml fractions and each mixed with 4.5 ml Flo-Scint IV for quantification of radioactivity by liquid scintillation counting. Identification of [^{3}H]InsPPs was based on co-elution with standards, both commercial and those generated by the mild alkaline hydrolysis of [^{3}H]Ins lipids (2.5.2) (see figure 2.6.3.2), or by comparison with previous studies (e.g. Batty *et al.*, 1989; Wong *et al.*, 1992).

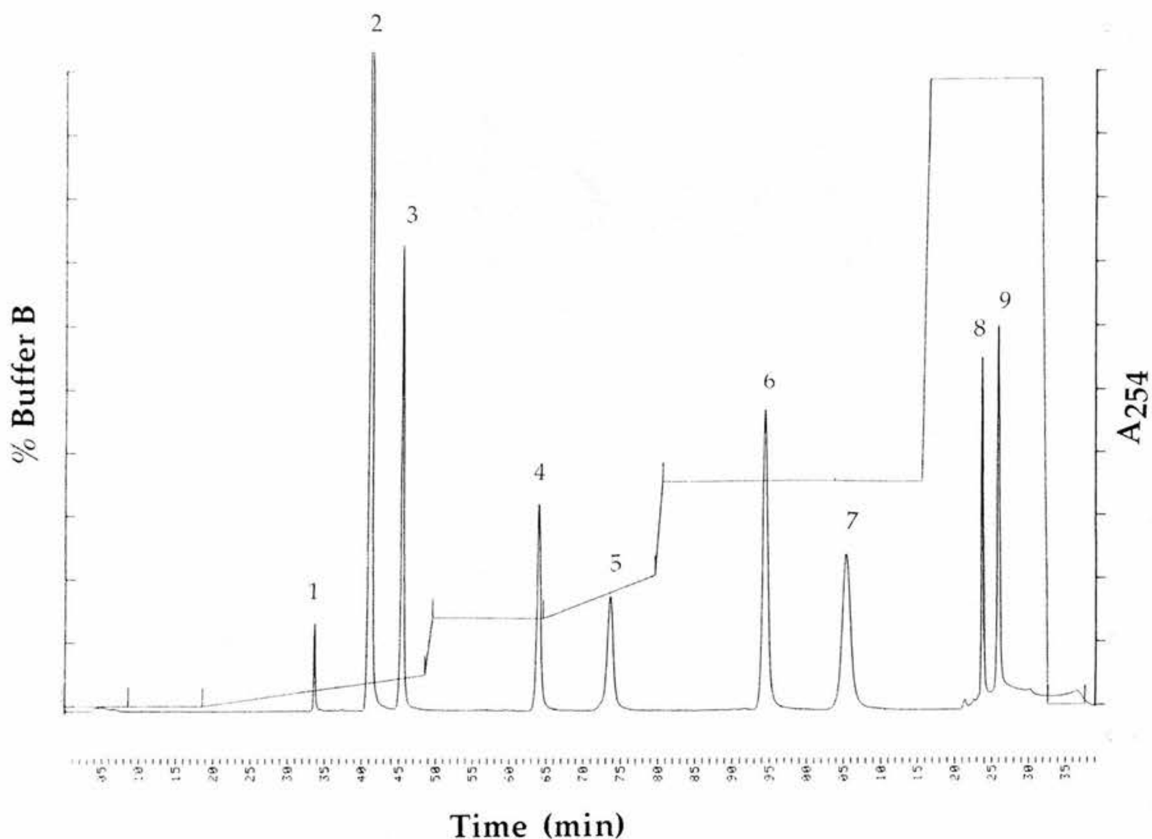

Figure 2.6.3.1 H.P.L.C. separation of adenosine and guanosine nucleotides A distilled water sample (2 ml) was spiked with 20 µM of each nucleotide (1-9) and loaded onto a Partisphere 5 SAX H.P.L.C. column equipped with a Whatman cartridge anion exchange pre-column. The column was washed for 20 min with dH_2O before eluting the nucleotides with five consecutive gradients of dH_2O (A) and 1.4 M $NH_4H_2PO_4$ adjusted to pH 3.7 with H_3PO_4 (B) buffers, as detailed in section 2.6.3 and shown on the % buffer B trace. Nucleotides were monitored using an on line U.V. spectrophotometer and are identified as cAMP (1), AMP (2), GMP (3), ADP (4), GDP (5), ATP (6), GTP (7), A-4-P (8) and G-4-P (9).

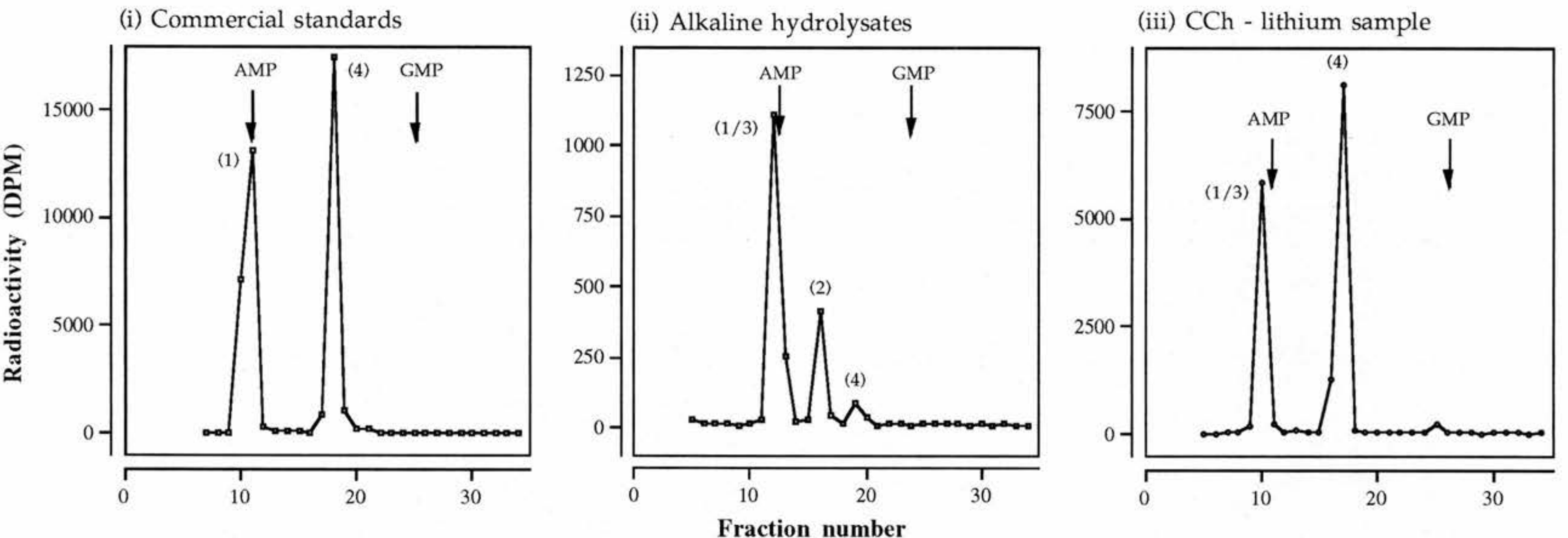

Figure 2.6.3.2a Typical H.P.L.C. elution profiles : MONOPHOSPHATE FRACTION

Samples (2 ml) of (i), (ii) and (iii) were spiked with nucleotides, loaded onto a Whatman Partisphere 5 SAX column and eluted with $NH_4H_2PO_4/dH_2O$ buffers as detailed previously (2.6.3). Eluent was collected over 30s time intervals and the radioactivity in each fraction determined by liquid scintillation counting. The positions of phosphate groups on the inositol ring are given in parentheses for each isomer.

(i) 20,000 DPM of each of [^{3}H]Ins1P, [^{3}H]Ins4P, [^{3}H]Ins(1,4)P_2, [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 diluted in tissue extract (2.4.2).

(ii) [^{3}H]InsPPs formed by mild alkaline hydrolysis (2.5.2) of pooled triplicate inositol lipid extracts (2.5.1) isolated from BTSM slices previously labelled with 3 μCi [^{3}H]inositol/50 μl slices (2.3.2).

(iii) Pooled triplicate TCA extracts (2.4.2) from [^{3}H]inositol labelled BTSM slices (2.3.1) stimulated with 100 μM CCh for 30 min.

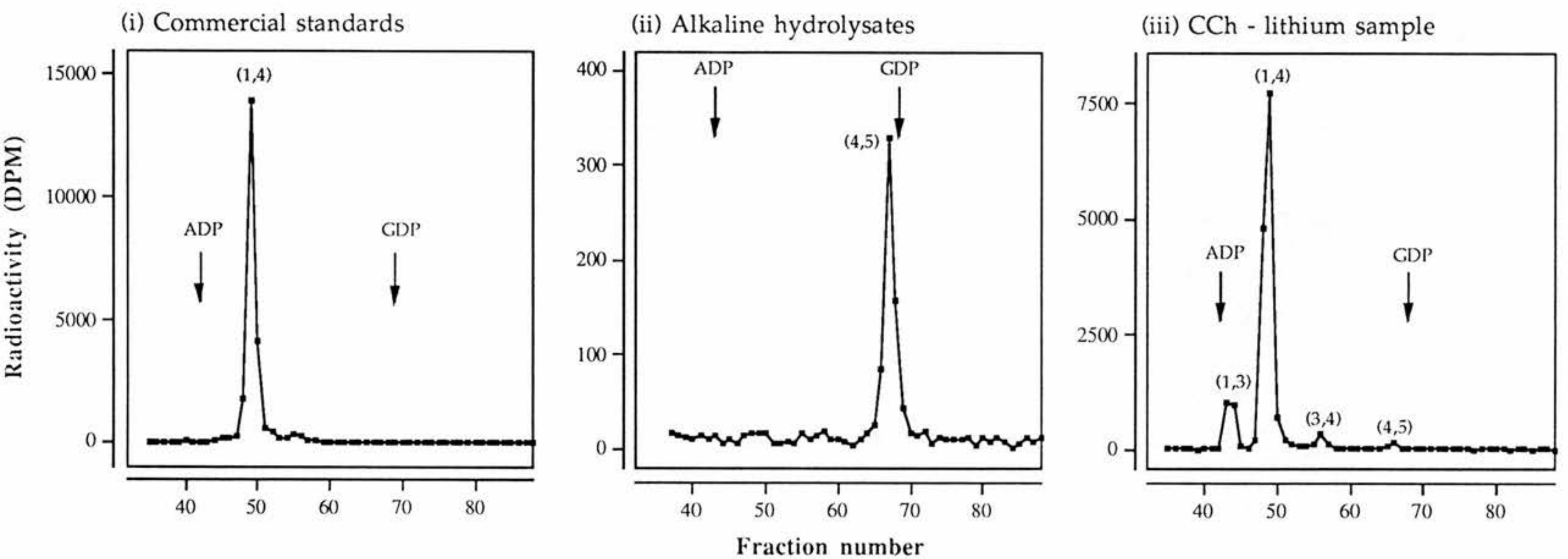

Figure 2.6.3.2b Typical H.P.L.C elution profiles: BISPHOSPHATE FRACTION
See legend to figure 2.6.3.2a.

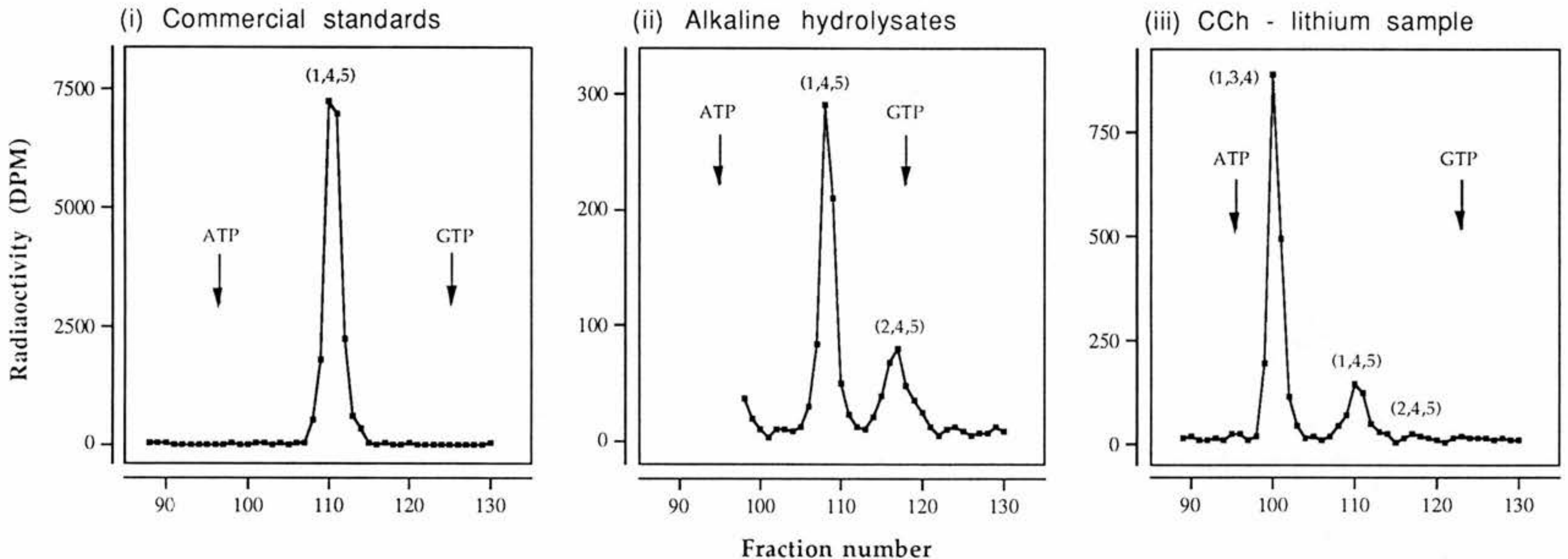

Figure 2.6.3.2c Typical H.P.L.C. elution profiles: TRISPHOSPHATE FRACTION
See legend to figure 2.6.3.2a.

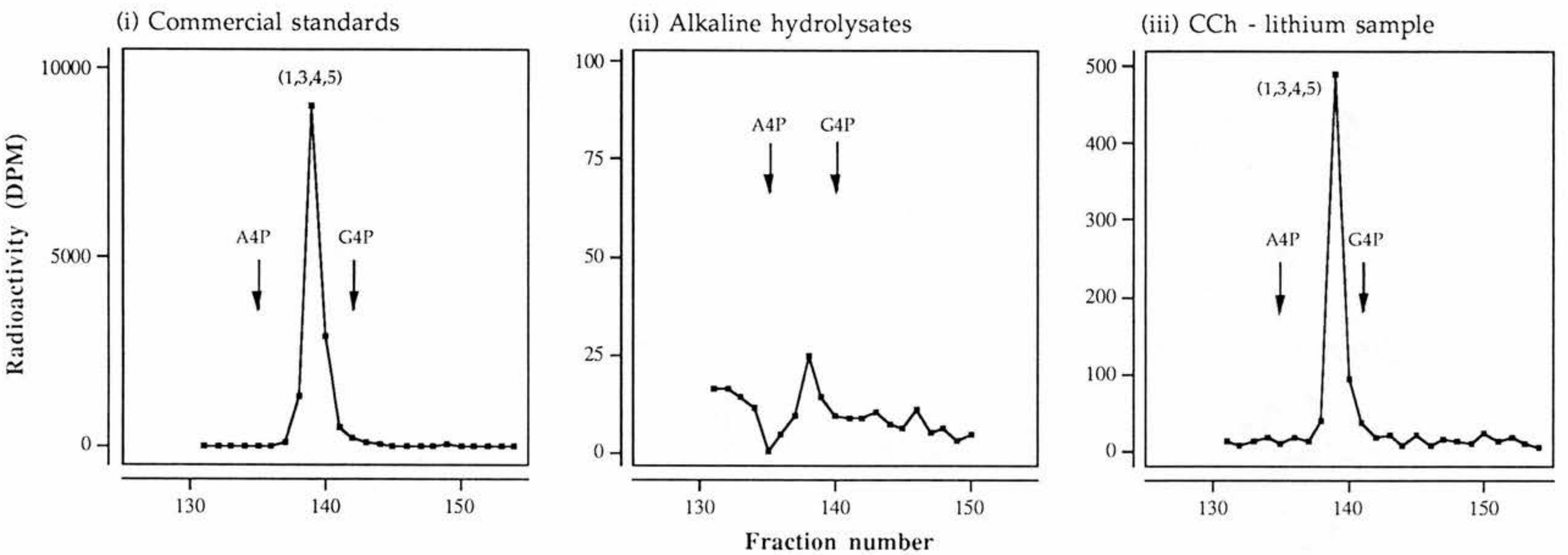

Figure 2.6.3.2d Typical H.P.L.C. elution profiles: TETRAKISPHOSPHATE FRACTION
See legend to figure 2.6.3.2a.

2.7 DETERMINATION OF INOSITOL 1,4,5-TRISPHOSPHATE MASS

D-Ins(1,4,5)P_3 mass was quantified using a specific radioreceptor assay according to the method of Challiss *et al.* (1988, 1990). The assay uses a bovine adrenal cortex preparation which contains a well characterised, single high affinity [^{3}H]Ins(1,4,5)P_3 binding site and is capable of distinguishing fmolar concentrations of [^{3}H]Ins(1,4,5)P_3.

2.7.1 Preparation of samples for inositol 1,4,5-trisphosphate mass determination

BTSM slices (300 x 300 μm) were prepared and pre-incubated in oxygenated KHB for 60 min as described in section 2.2. Aliquots (50 μl) of gravity packed slices were transferred to 6 ml flat bottomed vials containing 230 or 240 μl of either (a) oxygenated KHB for same-day stimulations or (b) RPMI medium containing 50 units/ml each of penicillin and streptotomycin for next-day stimulations.

(a) Samples were capped and incubated at 37 °C in a shaking water bath for 10 min prior to addition of agonists in a 10 μl volume. Reactions were terminated at the appropriate times with 300 μl ice-cold 1 M TCA and neutralised extracts (2.4.2) stored at 4 °C.

(b) Samples were incubated for 24 hours at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Subsequent stimulations were carried out exactly as described above (a).

The amount of protein in the TCA extracted tissue pellets was determined following solubilisation overnight in 2 M NaOH using the BCA protein assay.

2.7.2 Preparation of inositol 1,4,5-trisphosphate binding protein

Fresh bovine adrenal glands were demedullated and decapsulated to obtain 60-80 g of adrenal cortex. Tissue was homogenised (Polytron) in 8 volumes ice-cold 20 mM $NaHCO_3$, 1 mM dithiothreitol (DTT), pH 8.0 and centrifuged at low speed for 15 min (5,000 g, 4 °C). The supernatant was removed and the pellet rehomogenised in a further 4 volumes of buffer for re-centrifugation as above. The pooled supernatant fraction was spun at 38,000 g (20 min, 4 °C) and the resulting pellet washed in 20 mM $NaHCO_3$, 1 mM DTT, pH 8.0. The final pellet was resuspended in the above buffer at a protein concentration of 15-20 mg/ml, as determined using the BCA protein assay and stored in aliquots at -20 °C.

2.7.3 Inositol 1,4,5-trisphosphate mass assay

Assays were performed in LP3 tubes at 4 °C in a final volume of 120 µl. Aliquots (30 µl) of neutralised TCA extract (2.4.2) or dH_2O containing standard amounts of D-Ins(1,4,5)P_3 (0.036-36000 nmoles, i.e. 0.3-300 nM final concentration), or 1.2 nmoles (10 µM final concentration) D-Ins(1,4,5)P_3 (to define non-specific binding), were added to 30 µl 100 mM Tris-HCl, 4 mM EDTA buffer (pH 8.0) and 30 µl water containing 8-10,000 DPM [^{3}H]Ins(1,4,5)P_3. Finally 30 µl of adrenal cortex binding protein was added to the tubes which were vortexed and left to incubate on ice for 30-60 min. Bound and free [^{3}H]Ins(1,4,5)P_3 were separated by rapid filtration through Whatman GF/B glass fibre filters with 2 x 3 ml washes of ice-cold 25 mM Tris-HCl, 5 mM $NaHCO_3$, 1 mM EDTA (pH 8.0). Emulsifier-Safe (4 ml) was added to the filter discs and the radioactivity associated with bound [^{3}H]Ins(1,4,5)P_3 determined, after a 12 hour extraction period, by liquid scintillation counting. Unknown Ins(1,4,5)P_3 concentrations were calculated from the internal standard curve (figure 2.7.3) using a Packard

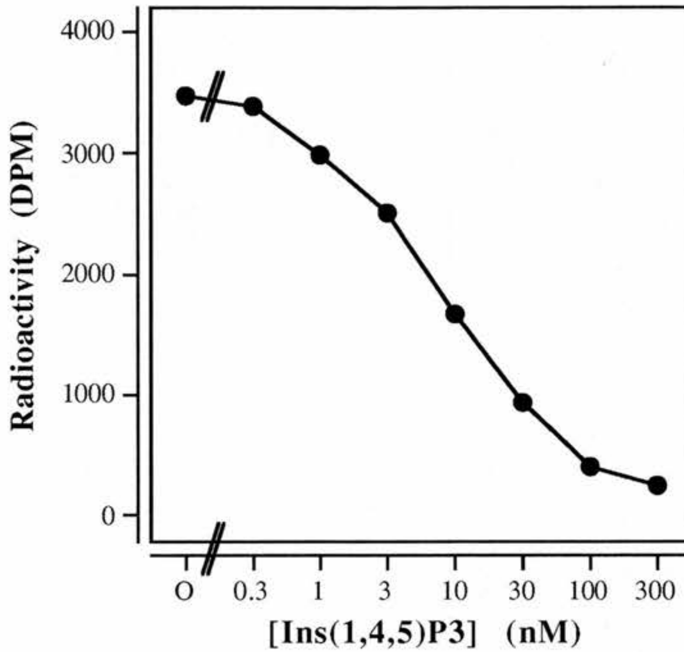

Figure 2.7.3 Inositol 1,4,5-trisphosphate mass assay: standard curve
Ins(1,4,5)P_3 was incubated, at the concentrations indicated, with Ins(1,4,5)P_3 binding protein (2.6.2) and 8-10,000 DPM [^{3}H]Ins(1,4,5)P3 as described in section 2.6.3. Bound and free [^{3}H]Ins(1,4,5)P3 were separated by filtration and the bound [^{3}H]Ins(1,4,5)P_3 quantified by liquid scintillation counting. Values represent means of duplicate samples. Standard errors ($\leq$ 5.4% of the mean) lie within the data points. Non-specific binding = 1.4%, total [^{3}H]Ins(1,4,5)P_3 bound in absence of added unlabelled Ins(1,4,5)P_3 = 40.7%.

radioimmunoassay programme and corrected for protein content for each 50 µl aliquot of BTSM slices (2.7.1). This method gave consistent standard curves for the binding of D-Ins(1,4,5)P_3 to the bovine adrenal cortex binding protein: EC_{50} = 14.21 ± 0.83 nM, non-specific binding = 1.33 ± 0.07%, D-[^{3}H]Ins(1,4,5)P_3 bound in absence of competing unlabelled D-Ins(1,4,5)P_3 = 40.27 ± 1.84% (Data represent mean ± SEM of 12 separate experiments).

2.8 METABOLISM OF [^{3}H]INOSITOL POLYHOSPHATES BY BOVINE TRACHEAL SMOOTH MUSCLE CELL FREE EXTRACTS

2.8.1 Preparation of bovine tracheal smooth muscle cytosol extract

BTSM strips were dissected free of overlying epithelium and surrounding connective tissue. Portions (9 g) of tissue were minced with scissors, and both washed in (5 x 15 ml), and homogenised in (36 ml), ice-cold isotonic buffer (10 mM Tris-maleate, 150 mM sucrose buffer, adjusted to pH 7.5 with NaOH). After centrifugation at low speed for 10 min (5,000 g, 4 °C), the upper fatty layer was aspirated and discarded and the supernatants respun at high speed (48,400 g, 90 min, 4 °C). Aliquots of the final supernatant were stored at -80 °C.

2.8.2 Preparation of bovine tracheal smooth muscle homogenates

BTSM was homogenised and spun at low speed as described above (2.8.1). Following centrifugation, the upper fatty layer was discarded and the remaining supernatant and pellet rehomogenised. Aliquots of homogenate were stored at -80 °C.

2.8.3 Assay conditions for cell free incubations

Reactions were performed in 1.5 ml Eppendorf tubes in an intracellular-like 100 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$[1] and 25 mM HEPES buffer, adjusted to pH 7.4 using KOH. Standard [^{3}H]Ins(x)PP label (10-60,000 DPM) was diluted in the above buffer and incubated with 20 µl cell free extract in a final volume 100 µl at 37 °C in a shaking water bath. Reactions were terminated by the addition of 100 µl ice-cold 1 M TCA and neutralised extracts prepared as detailed in section 2.4.2 stored at 4 °C for subsequent separation of [^{3}H]InsPPs using AG 1-X8 Dowex open-column chromatography (2.6.2) or H.P.L.C. (2.6.3).

2.9 INOSITOL 1,4,5-TRISPHOSPHATE 3-KINASE INCUBATIONS

An Ins(1,4,5)P_3 3-kinase enzyme purified from rat brain has been shown to be susceptible to degradation by the calcium dependent neutral cysteine protease calpain (Lee *et al.*, 1990), and a cDNA clone of the 3-kinase from this tissue reveals the presence of six 'PEST' regions, i.e sequences common to proteins susceptible to calpain (Choi *et al.*, 1990). In order to prevent such a degradation of the BTSM Ins(1,4,5)P_3 3-kinase in my studies calpain inhibitors were included in the buffers at all stages of both preparation and assay of the enzyme. These compounds potently inhibit the calpains (K_i = 0.12-0.23 nM) but were used at high (µM) concentrations since the inhibtion exhibited by these agents is reversible.

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[1] $MgCl_2$ was included in the incubation buffer since the 5-phosphatase has an absolute requirement for Mg^{2+}. A concentration of 2 mM $MgCl_2$ was chosen as this value lies within the range of Mg^{2+} concentrations (1-5 mM) generally required for optimal activity of Ins(1,4,5)P_3 5-phosphatase (e.g. Connolly *et al.*, 1985; Kennedy *et al.*, 1990; Sasaguri *et al.*, 1985; Seyfred *et al.*, 1984).

2.9.1 Preparation of bovine tracheal smooth muscle cell free extract for the assay of inositol 1,4,5-trisphosphate 3-kinase enzyme activity

BTSM was dissected free of overlying epithelium and surrounding connective tissue. Portions (9 g) of tissue were minced with scissors, and both washed in (5 x 15 ml), and homogenised in (36 ml), ice-cold (10 mM Tris-maleate, 150 mM sucrose buffer (pH 7.5, NaOH) containing the following agents to prevent proteolytic digestion: 100 nM 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) (AEBSF), 0.1% (v/v) 2-mercaptoethanol, 17 μg/ml (i.e. 46 μM) calpain inhibitor I and 7 μg/ml (i.e. 18 μM) calpain inhibitor II. After centrifugation at low speed for 10 min (5,000 g, 4 °C), the upper fatty layer was discarded and the supernatants respun at high speed (48,400 g, 90 min, 4 °C). Aliquots of the final supernatant (8.23 ± 0.23 mg protein/ml[1]) were stored at -80 °C.

2.9.2 Assay conditions for 3-kinase incubations

In order to optimise the conditions for study of the BTSM Ins(1,4,5)P_3 3-kinase, a buffer was chosen which would facilitate this enzyme activity, but inhibit dephosphorylation of both its substrate and product. Assays of 3-kinase activity have been performed in a variety of other tissues including rat brain (Irvine *et al.*, 1986) in which it has been identified that the 3-kinase is both ATP- and Mg^{2+}-dependent, and therefore these agents were included in the incubation buffer. In addition, ATP (Hansen *et al.*, 1987; Shears *et al.*,1987b) and Mg^{2+} ions, when present in high enough concentrations (Connolly *et al.*, 1985; Hansen *et al.*, 1987; Kennedy *et al.*, 1990; Seyfred *et al.*, 1984), have an inhibitory action on the 5-phosphatase. This enzyme is also potently inhibted by 2,3-diphosphoglycerate (2,3-DPG)

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[1] Protein concentration determined using the bicinchoninic acid (BCA) protein assay (Smith *et al.*, *Anal. Biochem.* **150**: 76-85, 1985) and bovine serum albumin as a standard. Results represent mean ± SEM of three separate BTSM 3-kinase preparations.

(Downes *et al.*, 1982; Hansen *et al.*, 1987; Kennedy *et al.*, 1990; Shears *et al.*, 1987b).

Ins(1,4,5)P_3 3-kinase activity was assayed according to a method modified from Batty (1987). Reactions were performed in 1.5 ml Eppendorf tubes in 50 mM Tris-maleate, 20 mM $MgCl_2$, 10 mM ATP, 5 mM 2,3-diphosphoglyceric acid, 5 mM EGTA and appropriate concentrations of $CaCl_2$[1], pH 7.5 containing 17 μg/ml calpain inhibitor I, and 7 μg/ml calpain inhibitor II. [^{3}H]Ins(1,4,5)P_3 label was diluted in the above buffer and incubated with 40 μl of the cytosol extract prepared for assay of 3-kinase activity (2.9.1) (diluted appropriately in the reaction buffer) in a final volume of 200 μl at 37 °C in a shaking water bath. Reactions were terminated by the addition of 200 μl ice-cold 1 M TCA, and 20 μl 5% (w/v) bovine serum albumin added to the samples to aid precipitation of the protein pellet. Neutralised TCA extracts (2.4.2) were stored at -80 °C for subsequent separation of [^{3}H]InsPPs.

2.10 INOSITOL 1,4,5-TRISPHOSPHATE 5-PHOSPHATASE INCUBATIONS

2.10.1 Preparation of bovine tracheal smooth muscle cell free extracts for assay of inositol 1,4,5-trisphosphate 5-phosphatase enzyme activity

BTSM was dissected free of overlying epithelium and surrounding connective tissue. Portions (9 g) of tissue were minced with scissors, and both washed in (5 x 15 ml), and homogenised in (36 ml), ice-cold 10 mM Tris-maleate, 150 mM sucrose buffer, adjusted to pH 7.5 with NaOH, containing 1 mM phenylmethylsulphonyl fluoride (PMSF)[2] and 0.1%

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[1] $[Ca^{2+}]_{free}$ was calculated with the use of software based on the work of Perrin and Sayce (1967).

[2] A high concentration of PMSF was used since it has a short half-life in aqueous solutions.

(v/v) 2-mercaptoethanol. After centrifugation at low speed for 10 min (5,000 g, 4 °C), the upper fatty layer was discarded and the supernatants respun at high speed (48,400 g, 90 min, 4 °C). Aliquots of the final supernatant (containing the soluble 5-phosphatase enzyme activity, 8.37 ± 0.87 mg protein/ml[1]) were stored at -80 °C. The pellet was solubilised in 5 ml of the above buffer containing 1% Nonidet (NP40) (v/v) at 4 °C for two hours and re-centrifuged to remove any remaining insoluble material (48,000 g, 90 min, 4 °C). Aliquots of the final supernatant (containing solubilised particulate 5-phosphatase enzyme activity, 5.84 ± 0.28 mg protein/ml[2]) were stored at -80 °C.

2.10.2 Assay conditions for 5-phosphatase incubations

Reactions were performed in 1.5 ml Eppendorf tubes in 100 mM KCL, 20 mM NaCl, 2 mM $MgCl_2$ and 25 mM HEPES buffer, adjusted to pH 7.4. using KOH. $[^3H]Ins(1,4,5)P_3$ (10-12,000 DPM) was diluted in the above buffer and incubated with 40 µl of the cytosol extract prepared for assay of 5-phosphatase activity (2.10.2) (diluted appropriately in the reaction buffer) in a final volume of 200 µl at 37 °C in a shaking water bath. Reactions were terminated by the addition of 200 µl ice-cold 1 M TCA, and 20 µl 5% (w/v) bovine serum albumin added to the samples to aid precipitation of the protein pellet. Neutralised TCA extracts (2.4.2) were stored at -80 °C for subsequent separation of $[^3H]$InsPPs. For experiments undertaken at determined $[Ca^{2+}]_{free}$, 1mM EGTA and appropriate concentrations of $CaCl_2$ were added to the reaction buffer[3].

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[1] Protein concentration determined using BCA protein assay and bovine serum albumin as a standard. Results represent mean ± SEM of three separate BTSM soluble 5-phosphatase preparations.

[2] Protein concentration determined using BCA protein assay and bovine serum albumin as a standard. Results represent mean ± SEM of three separate BTSM solubilised particulate 5-phosphatase preparations.

[3] $[Ca^{2+}]_{free}$ was calculated with the use of software based on the work of Perrin and Sayce (1967).

CHAPTER 3

MUSCARINIC CHOLINOCEPTOR-STIMULATED ACCUMULATION OF [^{3}H]INOSITOL POLYPHOSPHATES IN BOVINE TRACHEAL SMOOTH MUSCLE

3.1 INTRODUCTION

In BTSM, stimulation with the muscarinic cholinoceptor agonist carbachol (CCh) results in a rapid increase in Ins(1,4,5)P_3 (Chilvers *et al.*, 1990a) prior to Ca^{2+} release (Takuwa *et al.*, 1987). This in turn precedes myosin light chain phosphorylation and the development of tone (Kamm and Stull, 1985). Although CCh stimulation results in only a transient increase in Ins(1,4,5)P_3 accumulation in BTSM, PtdIns(4,5)P_2 hydrolysis is persistent in the continued presence of agonist (Chilvers *et. al.*, 1991a), suggesting that Ins(1,4,5)P_3 is rapidly removed from the system by its metabolising enzymes and is under stringent regulatory control.

Studies in other tissues (for reviews see Shears, 1991, 1992) have shown that two major pathways exist for the metabolism of Ins(1,4,5)P_3 - namely the 3-kinase and 5-phosphatase pathways. The latter is initiated by the removal of a phosphate group from the 5-position of the inositol ring to produce Ins(1,4)P_2, whereas the 3-kinase pathway proceeds by phosphorylating the inositol ring on the 3-position to form Ins(1,3,4,5)P_4. Both metabolites are subject to further sequential dephosphorylation to free inositol which may be recycled into the phosphatidylinositol pool. The production and degradation of these Ins(1,4,5)P_3 metabolites has been studied extensively in other tissues, however characterisation of the individual inositol polyphosphate isomers formed following receptor

stimulation, and the relative importance of the 3-kinase and 5-phosphatase pathways to Ins(1,4,5)P_3 metabolism is lacking in BTSM. Chilvers *et al.* (1990a) have demonstrated a rapid and transient CCh-stimulated increase in [^{3}H]Ins(1,4,5)P_3 accumulation in BTSM which is followed by a more delayed increase in [^{3}H]InsP_2 and [^{3}H]InsP_1. In addition, H.P.L.C. and enzymic analyses of the [^{3}H]InsP_3 isomers showed a progressive and sustained accumulation of [^{3}H]Ins(1,3,4)P_3 in BTSM slices stimulated with 100 μM CCh. This compound represented the predominant (> 80 %) [^{3}H]InsP_3 isomer present at all time-points greater than 1 min following CCh stimulation, suggesting that the 3-kinase pathway operates in BTSM and may contribute substantially to Ins(1,4,5)P_3 metabolism.

I have sought to separate, identify and quantify the individual inositol polyphosphate isomers produced in BTSM in order to determine the routes of Ins(1,4,5)P_3 metabolism employed in this tissue and the flux of the inositol headgroup through these pathways. Four major pre-requisites for such experiments are:-

(1) that there are no agonist-stimulated changes in [^{3}H]phosphoinositide (and hence [^{3}H]InsP) specific radioactivity;

(2) that the individual inositol polyphosphates formed can be readily separated and quantified;

(3) that the enzyme pathways responsible for the metabolism of Ins(1,4,5)P_3 yield mutually exclusive products; and

(4) that there is no major contribution to inositol polyphosphate accumulation from either PLC-mediated breakdown of PtdIns or PtdIns4P, or from PLD action on the inositol-containing phospholipids.

The first two considerations are in part resolved by steady-state labelling of the phosphoinositides with [^{3}H]inositol as detailed in section 2.3.1 and

including lithium ions during the agonist-incubation period. Lithium is an uncompetitive inhibitor of the inositol monophosphatase enzyme responsible for the degradation of inositol monophosphates to free inositol (Gee *et al.*, 1988; Hallcher and Sherman, 1980). Inclusion of lithium ions in the experimental system therefore prevents the total degradation of [^{3}H]inositol phosphates, so trapping them within the cytosolic fraction of the cell. The efficient trapping of [^{3}H]InsP$_1$s by Li^+ using this protocol is partially validated by the linear accumulation of total [^{3}H]InsPs following CCh stimulation (0-30 min) of BTSM slices (see figure 2.3.1a). [^{3}H]InsPPs may be extracted with TCA and separated using H.P.L.C.. Very clear separations (at least five fractions between each [^{3}H]InsPP isomer) were achieved using the complex H.P.L.C. gradient system detailed in section 2.6.3, and the radioactivity in each fraction was quantified by liquid scintillation counting.

The experiments detailed in this chapter were designed to assess whether or not the requirements (3) and (4) above are satisfied in BTSM, to determine the flux of [^{3}H]Ins(1,4,5)P$_3$ through it's metabolic pathways and to evaluate the relative importance of these pathways during a 0-30 min agonist-stimulated response.

3.2 DETERMINATION OF THE ROUTES OF [^{3}H]INOSITOL 1,4,5-TRISPHOSPHATE METABOLISM IN BOVINE TRACHEAL SMOOTH MUSCLE

As detailed previously, two major pathways exist for the metabolism of [^{3}H]Ins(1,4,5)P$_3$. Chilvers *et al.* (1990a) have identified [^{3}H]Ins(1,3,4,5)P$_4$ and [^{3}H]Ins(1,3,4)P$_3$ accumulation in [^{3}H]inositol labelled BTSM slices stimulated with 100 μM CCh, indicating the presence of a 3-kinase

pathway in this tissue. In all other cell types studied the 3-kinase pathway has led to the production of $Ins(1,3,4,5)P_4$, $Ins(1,3,4)P_3$, $Ins(1,3)P_2$, $Ins(3,4)P_2$, Ins1P and Ins3P isomers, whereas in most tissues the sole products of the 5-phosphatase pathway are $Ins(1,4)P_2$ and Ins4P. Hence the two pathways give rise to mutually exclusive metabolic products. In order to determine whether the same pathways of InsPP metabolism also occur in BTSM, exogenous [^{3}H]InsPPs were incubated with BTSM cell free extracts (both cytosol extracts and homogenates), under conditions which prevent further phosphorylation, and the resulting [^{3}H]InsPP degradation products separated and quantified.

3.2.1 Degradation of [^{3}H]inositol 1,4,5-trisphosphate by bovine tracheal smooth muscle cytosol extract

To determine optimal conditions for subsequent H.P.L.C. separations the degradation of [^{3}H]$Ins(1,4,5)P_3$ by BTSM cytosol extract was assessed over a 30 min time-course and the resulting [^{3}H]InsPs separated and quantified using Dowex AG 1-X8 anion exchange chromatography. Reactions were performed in an 'intracellular-like' buffer containing 2 mM $MgCl_2$ (to support 5-phosphatase activity, see section 2.8.3), in the absence of added ATP to prevent further phosphorylation of the [^{3}H]$Ins(1,4,5)P_3$ substrate. Figure 3.2.1.1 shows that under these conditions there is a rapid degradation of [^{3}H]$InsP_3$ ($t_{1/2}$ = 2.4 min) followed by a transient accumulation of [^{3}H]$InsP_2$ (maximal after 2 min incubation with cytosol extract), and secondary increases in [^{3}H]$InsP_1$ and [^{3}H]inositol. In control reactions in which the cytosol extract had been previously heat-inactivated (100 °C, 30 min) no such degradation of [^{3}H]$InsP_3$ was observed. These results suggest that in BTSM, [^{3}H]$Ins(1,4,5)P_3$ may be metabolised by a sequential dephosphorylation reaction via [^{3}H]$InsP_2$ and

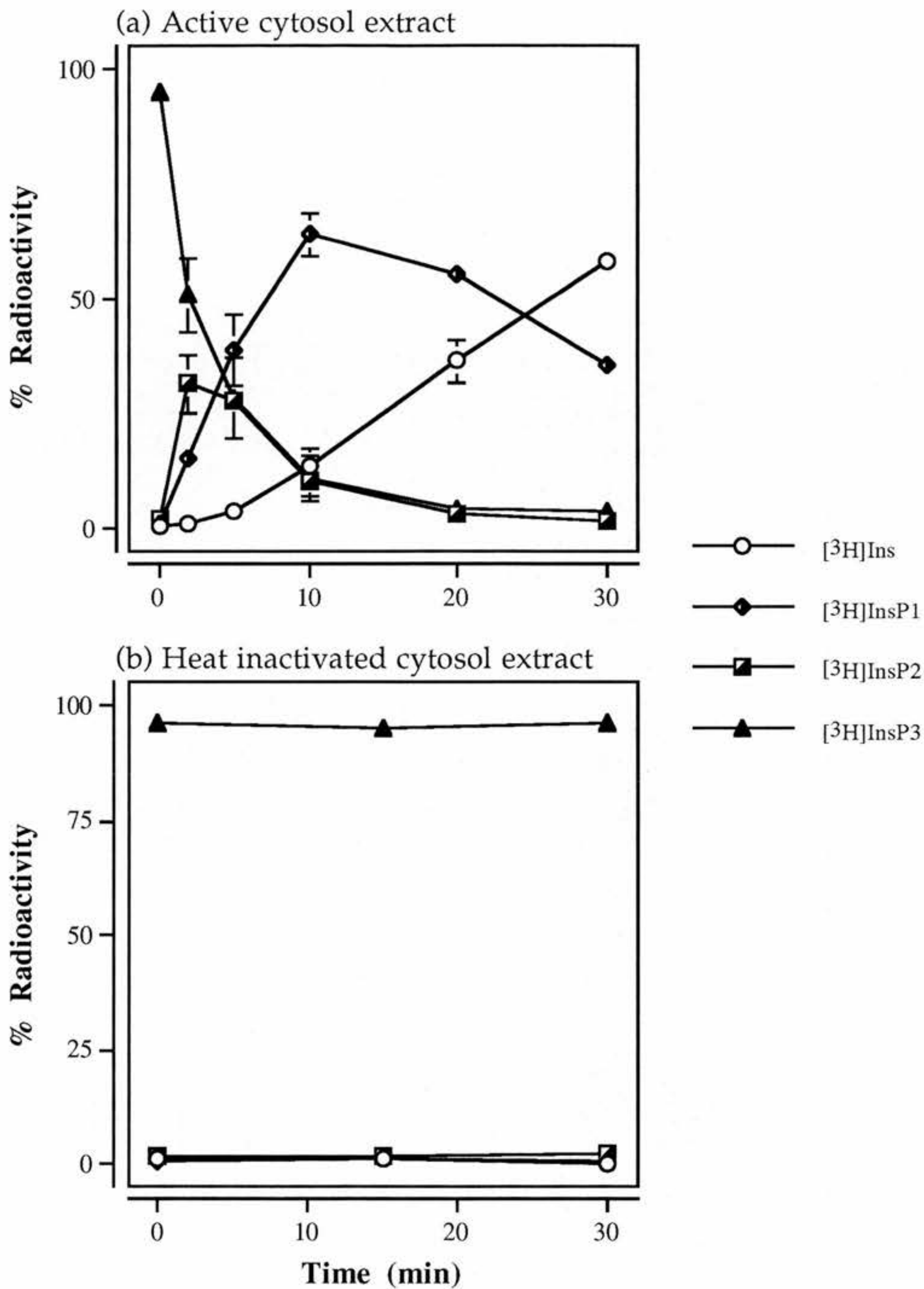

Figure 3.2.1.1 Metabolism of [^{3}H]inositol 1,4,5-trisphosphate by bovine tracheal smooth muscle cytosol extract

[^{3}H]Ins(1,4,5)P_3 (10,000 DPM) was incubated with 20 µl BTSM cytosol extract (2.8.1) in a final volume of 100 µl as described in section 2.8.3. Reactions were terminated with 100 µl TCA at the time-points indicated and the [^{3}H]InsP fractions separated by Dowex AG 1-X8 anion exchange chromatography (2.6.2). Results represent the mean ± SEM of three separate experiments, each performed in duplicate, using three separate cytosol extract preparations.

$[^3H]InsP_1$ to free $[^3H]$inositol. However, these data do not give any information as to the individual $[^3H]$InsPP isomers formed during the reaction. In order to determine the isomeric composition of the $[^3H]$InsPPs generated similar experiments were carried out in which the incubations were terminated after 10 min (the time at which the accumulation of $[^3H]InsP_1$ is maximal), and the individual $[^3H]$InsPP isomers separated and quantified by H.P.L.C.. Figure 3.2.1.2 shows a typical H.P.L.C. trace from one of these experiments and the relative accumulations of each of the $[^3H]$InsPPs is summarised in table 3.2.1. $[^3H]Ins(1,4,5)P_3$ was not degraded by dephosphorylation to less polar $[^3H]$InsPPs following incubation with the heat-inactivated cytosol extract. It is unlikely that any further phosphorylation could have taken place with either the heat-inactivated or active cytosol extracts since there was no added ATP in the system and any endogenous ATP was substantially diluted during the cytosol extract preparation. The trace amounts of $[^3H]Ins(2,4,5)P_3$ and $[^3H]InsP_4$ detected in the samples represent minor impurities in the $[^3H]Ins(1,4,5)P_3$ standard used. When $[^3H]Ins(1,4,5)P_3$ was incubated with active cytosol extract it was degraded solely to $[^3H]Ins(1,4)P_2$ and $[^3H]$Ins4P, as is consistent with sequential actions of the $Ins(1,4,5)P_3$ 5-phosphatase and inositol polyphosphate 1-phosphatase on $[^3H]Ins(1,4,5)P_3$ and $[^3H]Ins(1,4)P_2$ respectively.

3.2.2 Degradation of $[^3H]$inositol 1,3,4,5-tetrakisphosphate by bovine tracheal smooth muscle cytosol extract

The degradation of $[^3H]Ins(1,3,4,5)P_3$ by BTSM cytosol extract was monitored in an identical fashion to that of $[^3H]Ins(1,4,5)P_3$. Reactions were performed in an 'intracellular-like' buffer and $[^3H]$InsPs formed over a 30 min time-course were initially separated and quantified by

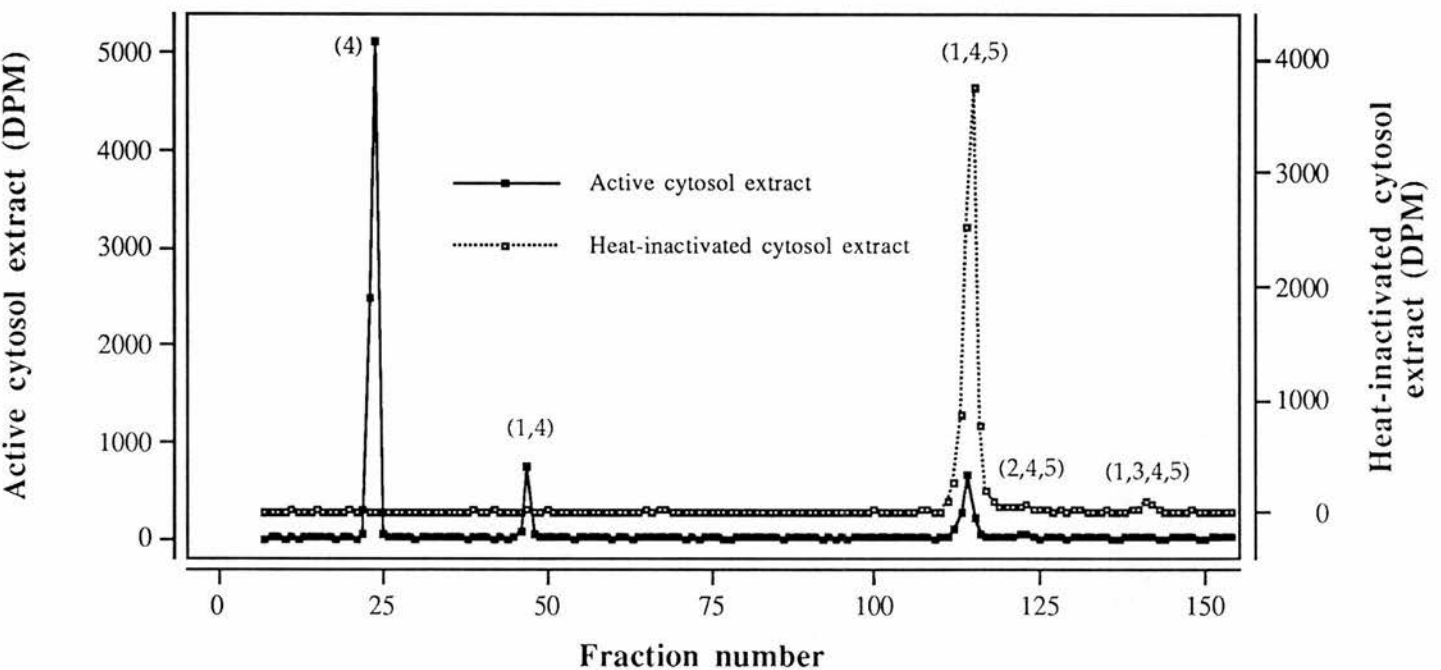

Figure 3.2.1.2 H.P.L.C. profile of [^{3}H]inositol 1,4,5-trisphosphate dephosphorylation metabolites

[^{3}H]Ins(1,4,5)P_3 (20,000 DPM) was incubated with 20 µl BTSM cytosol extract (2.8.1) in a final volume of 100 µl as detailed in section 2.8.3. Reactions were terminated after 10 min with 100 µl 1 M TCA and the individual [^{3}H]InsPP isomers in neutralised extracts separated, using H.P.L.C. (2.6.3) , and quantified by liquid scintillation counting.

$[^3H]Ins(x)PP$	% Total Radioactivity Inactive cytosol extract (n=2)	Active cytosol extract (n=4)
1/3	0.12 ± 0.12	0.21 ± 0.04
4	0.06 ± 0.06	81.35 ± 4.42
1,3	0.09 ± 0.09	0.08 ± 0.01
1,4	0.22 ± 0.10	5.81 ± 2.13
3,4	0.09 ± 0.03	0.18 ± 0.06
4,5	0.23 ± 0.06	0.08 ± 0.03
1,3,4	0.07 ± 0.07	0.14 ± 0.07
1,4,5	92.99 ± 1.42	8.77 ± 3.13
2,4,5	1.97 ± 0.40	0.96 ± 0.14
1,3,4,5	3.71 ± 1.66	2.23 ± 2.07

Table 3.2.1 $[^3H]$Inositol 1,4,5-trisphosphate dephosphorylation metabolites $[^3H]Ins(1,4,5)P_3$ (10-15,000 DPM) was incubated with 20 µl BTSM cytosol extract (2.8.1) in a final volume of 100 µl as detailed in section 2.8.3. Reactions were terminated after 10 min with 100 µl 1 M TCA and the individual $[^3H]$InsPP isomers in neutralised extracts separated, using H.P.L.C. (2.6.3), and quantified by liquid scintillation counting. Results are expressed as the percentage of the total retrieved radioactivity associated with the $[^3H]$InsPs (12,431 ± 1,321 DPM, n=6) and represent the mean ± SEM of n separate experiments, using two or three separate cytosol extract preparations.

Dowex AG 1-X8 chromatography (see figure 3.2.2.1). As expected, incubation of $[^3H]Ins(1,3,4,5)P_4$ with cytosol extract resulted in a rapid decline in the levels of $[^3H]InsP_4$ ($t_{1/2}$ = 1.25 min), accompanied by a transient accumulation of $[^3H]InsP_3$ (maximal at 5 min), a secondary increase in the level of $[^3H]InsP_2$ and delayed accumulations of $[^3H]InsP_1$ and $[^3H]$inositol. These results suggest that $[^3H]Ins(1,3,4,5)P_4$ is degraded

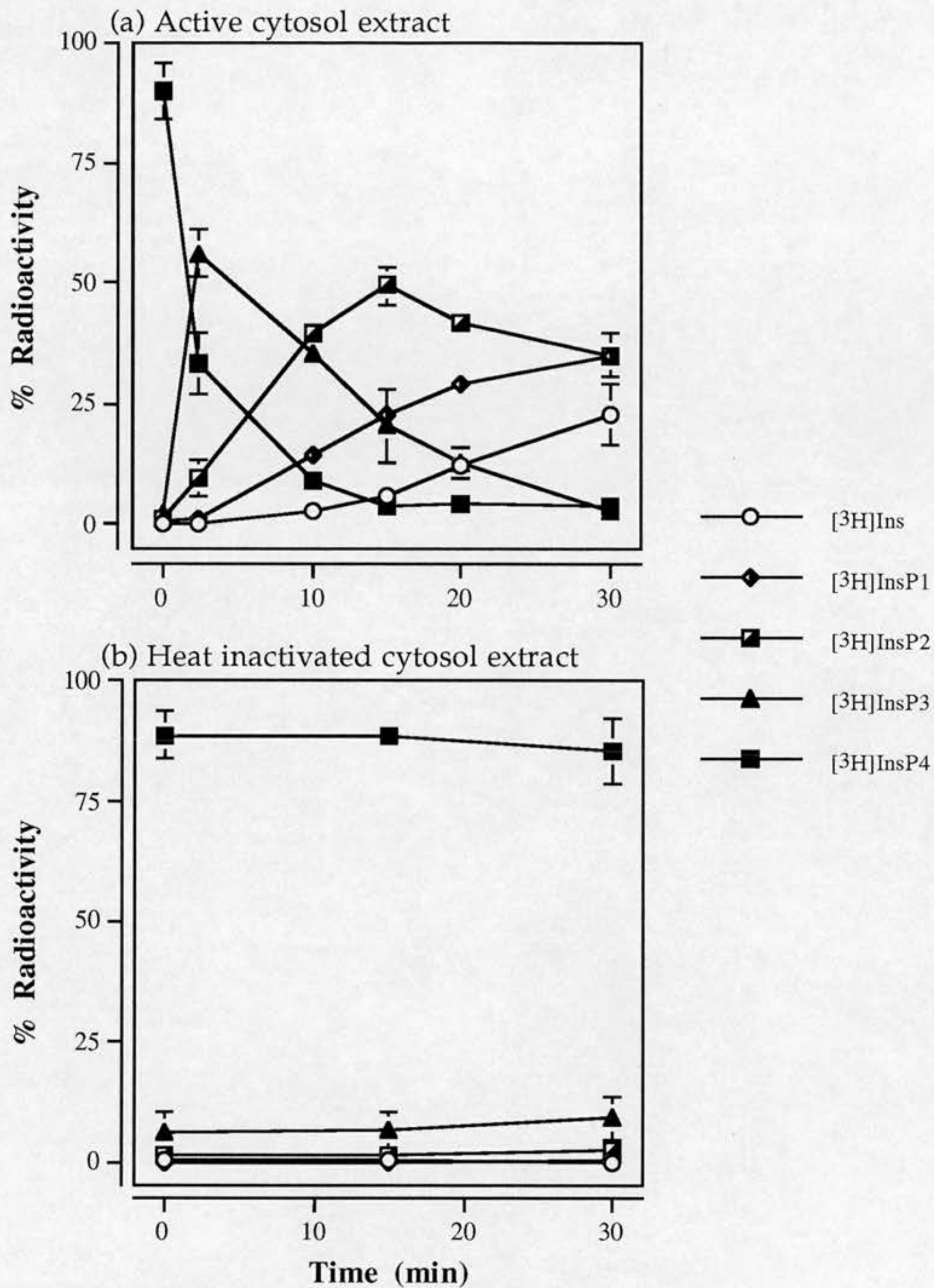

Figure 3.2.2.1 Metabolism of [^{3}H]inositol 1,3,4,5-tetrakisphosphate by bovine tracheal smooth muscle cytosol extract

[^{3}H]Ins(1,3,4,5)P_4 (10,000 DPM) was incubated with 20 μl cytosol extract (2.8.1) in a final volume of 100 μl as described in section 2.8.3. Reactions were terminated with 100 μl 1 M TCA and the [^{3}H]InsP fractions separated by Dowex AG 1-X8 anion exchange chromatography (2.6.2). Results represent the mean ± SEM of three or four experiments, each performed in duplicate, using three separate cytosol extract preparations.

to free [^{3}H]inositol by BTSM cytosol extract via a series of sequential dephosphorylation reactions.

H.P.L.C. separation of the [^{3}H]InsPP isomers formed following incubation with BTSM cytosol extract for 30 min (the time at which the greatest accumulation of [^{3}H]InsP$_1$ was observed and which therefore affords optimal conditions for identification of the isomeric composition of [^{3}H]InsP$_1$) indicates the presence of the 3-kinase pathway metabolites [^{3}H]Ins(1,3,4)P$_3$, [^{3}H]Ins(3,4)P$_2$, and [^{3}H]Ins1/3P (see figure 3.2.2.2 and table 3.2.2). However the other common 3-kinase pathway metabolite [^{3}H]Ins(1,3)P$_2$ could not be detected. The activity of the InsPP 1-phosphatase which hydrolyses Ins(1,3,4)P$_3$ to Ins(3,4)P$_2$ exhibits a sigmoidal dependence on Mg^{2+} ions with half maximal stimulation occurring at 0.3 mM [Mg^{2+}] (Inhorn and Majerus, 1987). Since the incubation buffer utilised in these experiments contains 2 mM $MgCl_2$ it is likely to favour the 1-phosphatase route of Ins(1,3,4)P$_3$ metabolism. In addition the 1-phosphatase from calf brain has a slightly higher affinity for Ins(1,3,4)P$_3$ (K_m ~ 20 μM, Inhorn and Majerus, 1987) than the InsPP 4-phosphatase responsible for the formation of Ins(1,3)P$_2$ (K_m = 40 μM, Bansal *et al.*, 1990).

Under the conditions outlined, [^{3}H]Ins(1,3,4,5)P$_4$ metabolism results in a significant accumulation of [^{3}H]Ins(1,4)P$_2$ and [^{3}H]Ins4P, compounds which cannot be accounted for by metabolism via any of the recognised '3-kinase pathway' enzymes. Similar data were consistently observed in a series of five experiments utilising two separate BTSM cytosol extract preparations, suggesting the presence of an active 3-phosphatase in the cytosol extract which, under the conditions specified, may attack either [^{3}H]Ins(1,3,4,5)P$_4$ or [^{3}H]Ins(1,3,4)P$_3$. It is tempting to suggest that the latter compound is the 3-phosphatase substrate in this system as no

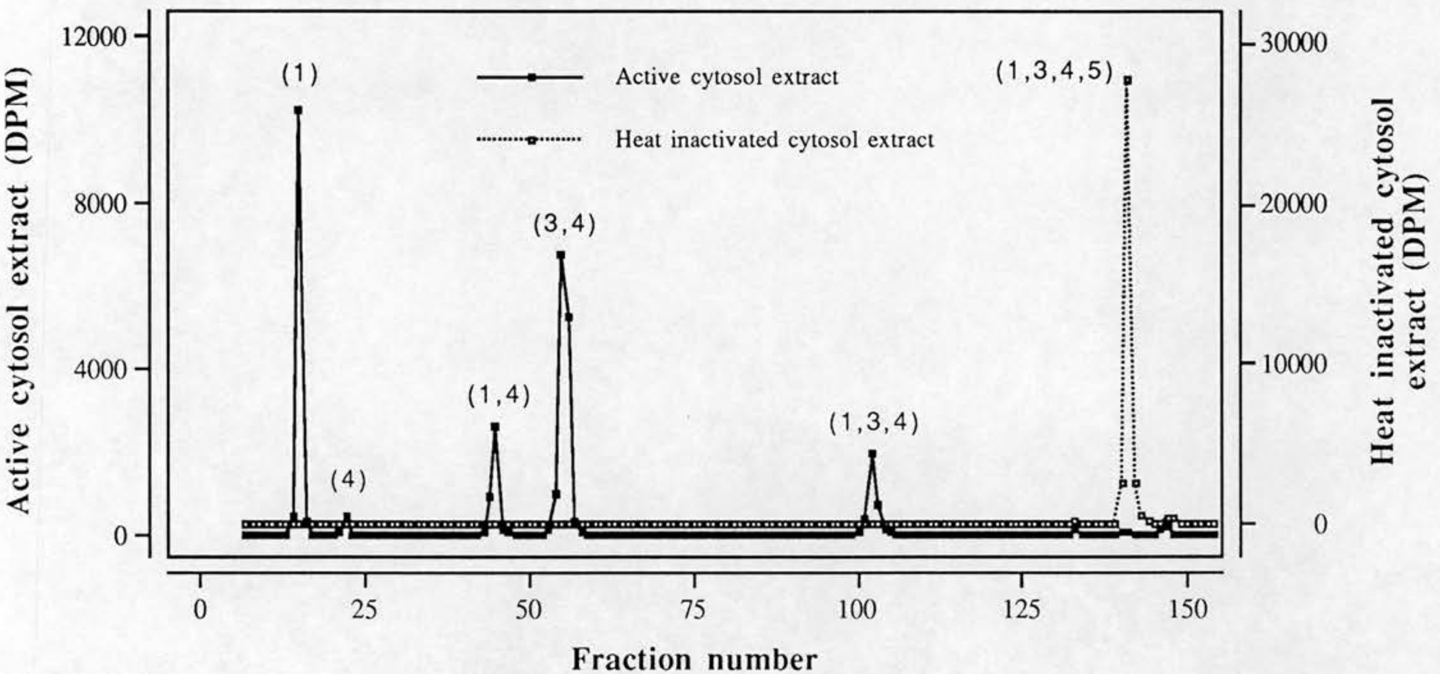

Figure 3.2.2.2 H.P.L.C. profile of [^{3}H]inositol 1,3,4,5-tetrakisphosphate dephosphorylation metabolites
40,000 or 60,000 DPM [^{3}H]Ins(1,3,4,5)P_4 was incubated with 20 µl of heat-inactivated or active BTSM cytosol extract (2.8.1) respectively, in a final volume of 100 µl, as detailed in section 2.8.3. Reactions wre terminated after 30 min with 100 µl 1 M TCA and the individual [^{3}H]InsPP isomers in neutralised extracts separated, using H.P.L.C. (2,6,3), and quantified by liquid scintillation counting.

$[^3H]Ins(x)PP$	% Total Radioactivity Inactive cytosol extract n=2	Active cytosol extract n=5
1/3	0.00 ± 0.00	30.63 ± 7.79
4	0.00 ± 0.00	1.35 ± 0.35
1,3	0.00 ± 0.00	0.00 ± 0.00
1,4	0.00 ± 0.00	13.28 ± 1.16
3,4	0.00 ± 0.00	40.47 ± 4.43
4,5	0.00 ± 0.00	0.23 ± 0.02
1,3,4	0.73 ± 0.62	10.80 ± 1.89
1,4,5	0.34 ± 0.12	0.20 ± 0.03
2,4,5	0.00 ± 0.00	0.17 ± 0.06
1,3,4,5	97.12 ± 0.42	0.60 ± 0.09

Table 3.2.2 $[^3H]Ins(1,3,4,5)P_4$ dephosphorylation metabolites
$[^3H]Ins(1,3,4,5)P_3$ (40-60,000 DPM) was incubated with 20 µl BTSM cytosol extract (2.8.1) in a final volume of 100 µl as detailed in section 2.8.3. Reactions were terminated after 30 min with 100 µl 1 M TCA and the individual $[^3H]$InsPP isomers in neutralised extracts separated using H.P.L.C. (2.6.3), and quantified by liquid scintillation counting. Results are expressed as the percentage of the total radioactivity retrieved (29,462 ± 1,738, n=5) and represent the mean ± SEM of n separate experiments, using two or three separate cytosol extract preparations.

$[^3H]Ins(1,4,5)P_3$ could be detected in the samples, however such a reaction has not been described in any other cell type. The incubation buffer used also supports 5-phosphatase activity and hence a more likely conclusion is that any $[^3H]Ins(1,4,5)P_3$ formed is rapidly dephosphorylated to $[^3H]Ins(1,4)P_2$ as described in section 3.2.1.

3.2.3 Degradation of [^{3}H]inositol 1,4,5-trisphosphate and [^{3}H]inositol 1,3,4,5-tetrakisphosphate by bovine tracheal smooth muscle homogenate

The above *in vitro* experiments indicate that a 5-phosphatase pathway operates in BTSM which may facilitate the sequential dephosphorylation of Ins(1,4,5)P_3 to free inositol via Ins(1,4)P_2 and Ins4P. Ins(1,3,4,5)P_4 may be metabolised by a 3-kinase to Ins(1,3,4)P_3, but may also be acted on by a 3-phosphatase enzyme. A similar 3-phosphatase activity has been identified previously in a variety of cell types including rat liver (Hodgson and Shears, 1990) and parotid gland (Hughes and Shears, 1990), in which the total activity in cytosolic and membrane preparations is greater than in that associated with the parent homogenate. It is possible that this enzyme may be less active *in vivo* as a result of being bound or sequestered within a subcellular compartment. Indeed, in liver, the 3-phosphatase has been shown to be compartmentalised within the endoplasmic reticulum (Ali *et al.*, 1993) and is evenly distributed between the 'rough' and 'smooth' subfractions (Craxton *et al.*, 1995). The Ins(1,3,4,5)P_4 3-phosphatase from turkey erythrocytes however, is localised to the inner face of the plasma membrane (Estrada-Garcia *et al.*, 1991). Craxton *et al.* (1995) have shown that these two activities are structurally distinct since the latter enzyme is not recognised by a polyclonal antibody directed against the hepatic enzyme. It is also possible that *in vivo* the 3-phosphatase may be subject to regulation by agents that are separated from it or diluted in the preparation of cell free extracts. In view of this uncertainty and the fact that these possible alterations of regulation could equally well affect any of the enzymes under study, further experiments were conducted to ascertain the pattern of metabolism of both [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 by BTSM homogenates.

Table 3.2.3.1 summarises the data obtained when 60,000 DPM of either $[^3H]Ins(1,4,5)P_3$ or $[^3H]Ins(1,3,4,5)P_4$ were incubated for 10 or 30 minutes respectively with BTSM homogenates and the resulting $[^3H]InsP$ products separated and quantified by Dowex anion exchange chromatography. Neither $[^3H]Ins(1,4,5)P_3$ nor $[^3H]Ins(1,3,4,5)P_4$ were hydrolysed by the heat-inactivated homogenate preparation. The active homogenate resulted in the degradation of $[^3H]Ins(1,4,5)P_3$ to $[^3H]InsP_2$, $[^3H]InsP_1$ and $[^3H]$inositol, with the majority of the radioactivity (81 %) residing in the $[^3H]InsP_1$ fraction. H.P.L.C. analysis of samples obtained from identical incubations showed this monophosphate fraction to be comprised of $[^3H]Ins4P$ alone (see figure 3.2.3.1). $[^3H]Ins(1,3,4,5)P_4$ was degraded by treatment with the BTSM homogenate preparation to $[^3H]InsP_2$, $[^3H]InsP_1$, and $[^3H]$inositol (see table 3.2.3.1). Any $[^3H]InsP_3$ that may have been formed must itself have been hydrolysed after a 30 min incubation period. H.P.L.C. analysis of identical samples showed that the $[^3H]InsPs$ formed under the specified conditions were comprised of $[^3H]Ins(1,3,4)P_3$, $[^3H]Ins(1,3)P_2$, $[^3H]Ins(3,4)P_2$ and $[^3H]Ins1/3P$ alone (see figure 3.2.3.2 and table 3.2.3.2). X and Y represent impurities in the $[^3H]Ins(1,3,4,5)P_4$ standard. Hence BTSM homogenates contain enzymes capable of metabolising $[^3H]Ins(1,3,4,5)P_4$ to all of its well characterised dephosphorylation products, but unlike BTSM cytosol extracts do not contain any detectable 3-phosphatase activity. This latter result is entirely consistent with previous observations of $Ins(1,3,4,5)P_4$ 3-phosphatase activity in other tissues. Such enzyme activity has only been detected in permeabilised cell (Oberdisse *et al.*, 1990), broken cell (e.g. Foster *et al.*, 1994; Hoer and Oberdisse, 1991; Hughes and Shears, 1990; Nogimori *et al.*, 1991) or electroporated cell (Cullen *et al.*, 1989) preparations and, as mentioned above, the total activity detected in isolated cellular fractions of rat liver and parotid

$[^3H]$InsP fraction	% Radioactivity in each $[^3H]$InsP fraction			
	Active enzyme		Heat inactivated enzyme	
	$[^3H]Ins(1,4,5)P_3$	$[^3H]Ins(1,3,4,5)P_4$	$[^3H]Ins(1,4,5)P_3$	$[^3H]Ins(1,3,4,5)P_4$
$[^3H]$Ins	14.21	15.19	0.00	0.00
$[^3H]InsP_1$	81.39	51.80	0.00	0.00
$[^3H]InsP_2$	2.75	31.55	0.00	0.00
$[^3H]InsP_3$	1.66	0.00	100.00	0.00
$[^3H]InsP_4$	0.00	1.48	0.00	100.00

Table 3.2.3.1. $[^3H]$Inositol phosphates formed following dephosphorylation of $[^3H]$Inositol 1,4,5-trisphosphate and $[^3H]$inositol 1,3,4,5-tetrakisphosphate with bovine tracheal smooth muscle homogenate

BTSM homogenate (20 µl) (2.8.2) was incubated with 60,000 DPM of either $[^3H]Ins(1,4,5)P_3$ or $[^3H]Ins(1,3,4,5)P_4$ in a final volume of 100 µl as detailed in section 2.8.3. Reactions were terminated after 10 or 30 min for $[^3H]Ins(1,4,5)P_3$ and $[^3H]Ins(1,3,4,5)P_4$ incubations respectively. $[^3H]$InsPs were separated from neutralised TCA extracts (2.4.2) using Dowex AG 1-X8 anion exchange chromatography (2.6.2) and quantified by liquid scintillation counting. Results represent data from one experiment.

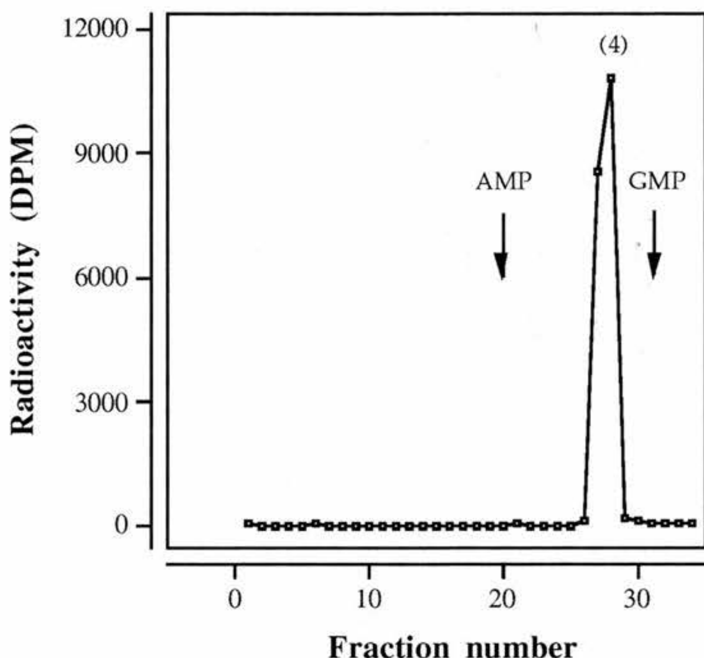

Figure 3.2.3.1 Typical H.P.L.C. profile (monophosphate fraction) following incubation of [^{3}H]inositol 1,4,5-trisphosphate with active bovine tracheal smooth muscle homogenate

[^{3}H]Ins(1,4,5)P_3 (40,000 DPM) was incubated with 20 µl active BTSM homogenate (2.8.2) in a final volume of 100 µl as detailed in section 2.8.3. The reaction was terminated after 10 min by addition of 100 µl 1 M TCA. The individual [^{3}H]InsP_1 isomers were separated and quantified by H.P.L.C. as described in section 2.6.3, except that the following gradient was used: After sample injection the column was washed for 10 min with dH_2O, and the [^{3}H]InsP_1 isomers eluted with a linear gradient 0 - 0.07 M $NH_4H_2PO_4$ (adjusted to pH 3.7 with H_3PO_4). Radioactivity in the column eluate was monitored by liquid scintillation counting.

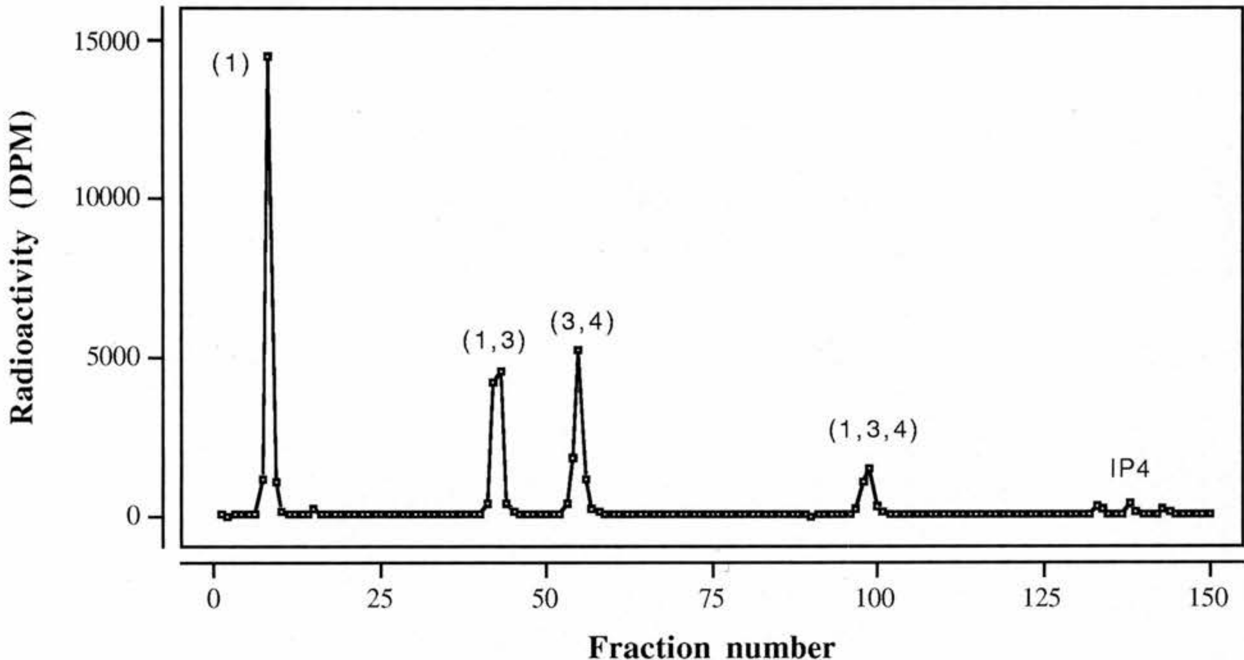

Figure 3.2.3.2 Typical H.P.L.C. profile following incubation of [^{3}H]inositol 1,3,4,5-tetrakisphosphate with active bovine tracheal smooth muscle homogenate

[^{3}H]Ins(1,3,4,5)P_4 (60,000 DPM) was incubated with 20 µl active BTSM homogenate (2.8.2) in a final volume of 100 µl as detailed in section 2.8.3. The reaction was terminated after 30 min by addition of 100 µl 1 M TCA and the individual [^{3}H]InsPP isomers separated by H.P.L.C. (see seciton 2.6.3). Radioactivity in the column eluate was determined by liquid scintillation counting,

$[^3H]Ins(x)PP$	Radioactivity (DPM)		% Total radioactivity		% Radioacitvity in $[^3H]InsP$ fraction	
1/3	16,861	16,258	41.22	38.69	98.29	98.21
4	293	296	0.72	0.70	1.71	1.79
1,3	9539	9940	23.32	23.66	50.76	49.93
1,4	56	88	0.14	0.21	0.30	0.44
3,4	9129	9723	22.32	23.14	48.58	48.84
4,5	68	155	0.17	0.37	0.36	0.78
1,3,4	3277	3878	8.01	9.23	96.30	97.05
1,4,5	53	46	0.13	0.11	1.56	1.15
2,4,5	73	72	0.18	0.17	2.15	1.80
X	624	581	1.53	1.38	40.13	37.15
IP_4	536	616	1.31	1.47	34.47	39.39
Y	395	367	0.97	0.87	25.40	23.47

Table 3.2.3.2 $[^3H]$Inositol polyphosphate isomers formed on hydrolyisis of $[^3H]$inositol 1,3,4,5-tetrakisphosphate with bovine tracheal smooth muscle homogenate

$[^3H]Ins(1,3,4,5)P_4$ (60,000 DPM) was incubated with 20 µl BTSM homogenate (2.8.2) in a final volume of 100 µl as detailed in section 2.8.3. Reactions were terminated after 30 min by the addition of 100 µl 1 M TCA. The individual $[^3H]InsP$ isomers in neutralised TCA extracts (2.4.2) were separated by H.P.L.C. (2.6.3) and quantified by liquid scintillation counting. Results represent the duplicate data from a single experiment. Total retrieved radioactivity = 41,462 ± 558.

glands was found to be greater than that of the parent homogenate, indicating that the enzyme may be intimately regulated or even non-functional *in vivo*.

Since the first description of the Ins(1,3,4,5)P_4 3-phosphatase it has become apparent that Ins(1,3,4,5,6)P_5 and InsP_6 (phytic acid) act as extremely potent inhibitors of this enzyme (Hoer and Oberdisse, 1991; Hughes and Shears, 1990; Nogimori *et al.*, 1991). The latter authors have demonstrated that these highly phosphorylated InsPs can inhibit the purified 3-phosphatase from rat liver with K_i values of 25 and 0.5 nM respectively (similar values were also obtained in pig brain, Hoer and Oberdisse, 1991), which closely resemble their K_m values when used as substrates for the enzyme. The affinity of InsP_6 for the 3-phosphatase is the highest yet defined for any enzyme involved in inositol phosphate metabolism. In addition, the 3-phosphatase inhibitory activity of liver co-elutes with standard InsP_6 on Cellufine GCL-90 size exclusion gel filtration and is depleted by treatment with phytase (Ali *et al.*, 1993). These data suggest that InsP_6, and possibly Ins(1,3,4,5,6)P_5 are the true substrates for the 3-phosphatase *in vivo*, and given the high intracellular concentration of these compounds in mammalian cells (see Berridge and Irvine, 1989; Shears, 1992) dephosphorylation of Ins(1,3,4,5)P_4 by this enzyme *in vivo* is likely to be negligible. Large amounts of InsP_6 have been found associated with rat cerebellar membranes in the presence of trace amounts of trivalent cations (Poyner *et al.*, 1993). A similar metal ion-dependent association of InsP_6 with cell membranes may explain the presence of Ins(1,3,4,5)P_4 3-phosphatase activity detected in BTSM cytosol extracts but not homogenates in the current study.

Although the cell free experiments described above were not designed to examine whether or not a 3-kinase activity exists in BTSM, they do indicate that all of the other enzymes required for formation of exclusive 3-kinase and 5-phosphatase pathway metabolites from Ins(1,4,5)P_3 are present. Chilvers *et al.* (1990a) have identified Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 in CCh-stimulated BTSM slices indicating that a 3-kinase enzyme is present in this tissue. Hence it is likely that both the 3-kinase and 5-phosphatase are important for the regulation of Ins(1,4,5)P_3 levels in BTSM. The data obtained using cell free BTSM extracts imply that in the absence of 3-phosphatase activity (as would be expected in the intact cell model), the two pathways do indeed yield mutually exclusive products.

3.3 ASSESSMENT OF THE ROLE OF PHOSPHATIDYLINOSITOL AND PHOSPHATIDYLINOSITOL 4-PHOSPHATE HYDROLYSIS IN INOSITOL POLYPHOSPHATE ACCUMULATION IN BOVINE TRACHEAL SMOOTH MUSCLE

It has been shown that a number of different isozymes of PIC exist in tissues which can be distinguished by their biochemical properties, immunological analysis and their primary protein structure (for reviews see Crooke and Bennett, 1989; Meldrum *et al.*, 1991; Rhee *et al.*, 1989, 1991). Each of these isozymes are capable of hydrolysing all three of the major phosphoinositides (i.e. PtdIns, PtdIns4P and PtdIns(4,5)P_2) *in vitro* (e.g. Hiramatsu *et al.*, 1992; Wilson *et al.*, 1984), however the substrate specificity of each of these enzymes in the intact cell is unclear. From studies carried out *in vitro* it seems that in general, PIC will hydrolyse the polyphosphoinositides at low Ca^{2+} concentrations whereas hydrolysis of

PtdIns requires a higher Ca^{2+} concentration (e.g. Griendling *et al.*, 1991). Hence it has been suggested that at later time-points of the agonist-stimulated response, PIC may utilise PtdIns and PtdIns4P as substrates in addition to PtdIns(4,5)P_2. An increase in $[Ca^{2+}]_i$ may also activate PIC directly since elevation of $[Ca^{2+}]_{free}$ from 2.7 nM to 617 nM has been reported to enhance basal InsP accumulation (1.6-fold) in digitonin-permeabilised canine tracheal smooth muscle cells (Yang *et al.*, 1993). It has been widely reported that agents which increase $[Ca^{2+}]_i$, such as calcium ionophores and membrane depolarising concentrations of K^+ ions, result both in an accumulation of [^{3}H]InsPs in [^{3}H]Ins prelabelled neuronal and synaptosomal preparations (Baird and Nahorski, 1989, 1990; Brammer and Weaver; 1989; del Rio *et al.*, 1994; Fisher *et al.*, 1989) and an increase in Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 mass in rat cerebral cortex slices (Challiss and Nahorski, 1991). These studies noted a preferential accumulation of [^{3}H]InsP_2 over that of [^{3}H]InsP_3 following application of depolarising agents and calcium ionophores even in the presence of the 5-phosphatase inhibitor 2,3-DPG (Fisher *et al.*, 1988), suggesting that in contrast to agonist activation of PIC, PtdInsP rather than PtdInsP_2 may be the predominant substrate for such agonist-independent PIC activation. The majority of these studies have been conducted in excitable tissue, in particular neuronal cells; however phosphoinositide hydrolysis in response to calcium ionophores and depolarising agents has also been observed in guinea-pig visceral smooth muscle preparations (Best and Bolton, 1986; Jafferji and Michell, 1976; Sasaguri and Watson, 1988; Watson and Downes, 1983) and guinea-pig trachea (Kardasz *et al.*, 1987). Some of the responses in these smooth muscle preparations have subsequently been shown to be neuronal in origin (Watson *et al.*, 1990), or secondary to endogenous neurotransmitter release from neuronal

tissue contained within the preparation (Akhtar and Abdel-Latif, 1984; Watson and Downes, 1983). If PIC is able to hydrolyse the lower phosphoinositides in significant quantities in BTSM, the resulting formation of Ins1P and Ins(1,4)P_2 isomers (indistinguishable from those formed by Ins(1,4,5)P_3 metabolism) would preclude the assessment of Ins(1,4,5)P_3 3-kinase and 5-phosphatase metabolites. To address whether or not a Ca^{2+}-mediated activation of PIC occurred in BTSM slices, [^{3}H]InsPs accumulating over a 30 min period in the presence of lithium were quantified following incubation with depolarising concentrations of KCl or the calcium ionophore ionomycin, and compared with basal and CCh-stimulated values.

Receptor-stimulated activation of phospholipase D (PLD) is common to a large number of agonists which also activate PIC, and may occur secondary to PKC activation (see Billah, 1993). In guinea-pig tracheal smooth muscle cells PLD can be activated following stimulation with either bradykinin or the protein kinase C activator phorbol myristate acetate (PMA) (Pyne and Pyne, 1993). This PLD response is inhibited both by the PKC inhibitor staurosporine or by prior down regulation of PKC. Phosphatidylcholine appears to be the preferred substrate for PLD, however in some systems PLD may also hydrolyse phosphatidylethanolamine (Kiss, 1992) or PtdIns (Huang *et al.*, 1992). Hence a PKC-stimulated PLD activity directed against the phosphoinositides could occur in BTSM following muscarinic cholinoceptor stimulation, resulting in a non-direct receptor mediated accumulation of inositol phosphates. In order to ascertain whether or not such an activation of PLD contributes significantly to the CCh-stimulated accumulation of [^{3}H]InsPs in BTSM additional incubations

were carried out in the presence of the PKC-stimulating phorbol ester phorbol dibutyrate (PDBu).

3.3.1 Effect of potassium ions, ionomycin and phorbol dibutyrate on [^{3}H]inositol phosphate accumulation in bovine tracheal smooth muscle

[^{3}H]Inositol pre-labelled BTSM slices were incubated for 30 min with 100 μM CCh, 80 mM KCl, 5 μM ionomycin, 100 nM PDBu or vehicle and the resulting [^{3}H]InsPs separated from neutralised TCA extracts using Dowex AG 1-X8 resin (see figure 3.3.1). Although not statistically significant, there was a trend for KCl to inhibit both basal and CCh-stimulated [^{3}H]InsP accumulation (17 % and 23 % respectively). This is in agreement with further KCl experiments carried out in collaboration with E.R. Chilvers, R.A.J. Challiss and G.J. Offer (see Chilvers *et al.*, 1994b). Moreover, these authors report that application of 80 mM KCl results in a decrease in both basal and CCh-stimulated Ins(1,4,5)P_3 mass after a five second incubation (the time at which Ins(1,4,5)P_3 mass is maximal). Biden *et al.* (1993) demonstrated that CCh-stimulated Ins1P accumulation is directly proportional to the calculated membrane potential in rat pancreatic islets, an effect which is not mediated by voltage-gated Ca^{2+} channels. A similar regulation of phosphoinositide hydrolysis by membrane-potential in BTSM could account for the decrease in [^{3}H]InsP accumulation observed in the presence of a high K^+ concentration. Alternatively this effect may result from a Ca^{2+}-mediated inhibition of PtdIns synthesis as described in rabbit vas deferens (Egawa, *et al.*, 1981) and insect salivary glands (Berridge and Fain, 1979). In most mammalian systems however, PtdIns synthesis is enhanced by hormonal stimulation. The concentration of K^+ ions in these experiments was chosen specifically because it has been shown to initiate a prompt contractile response in

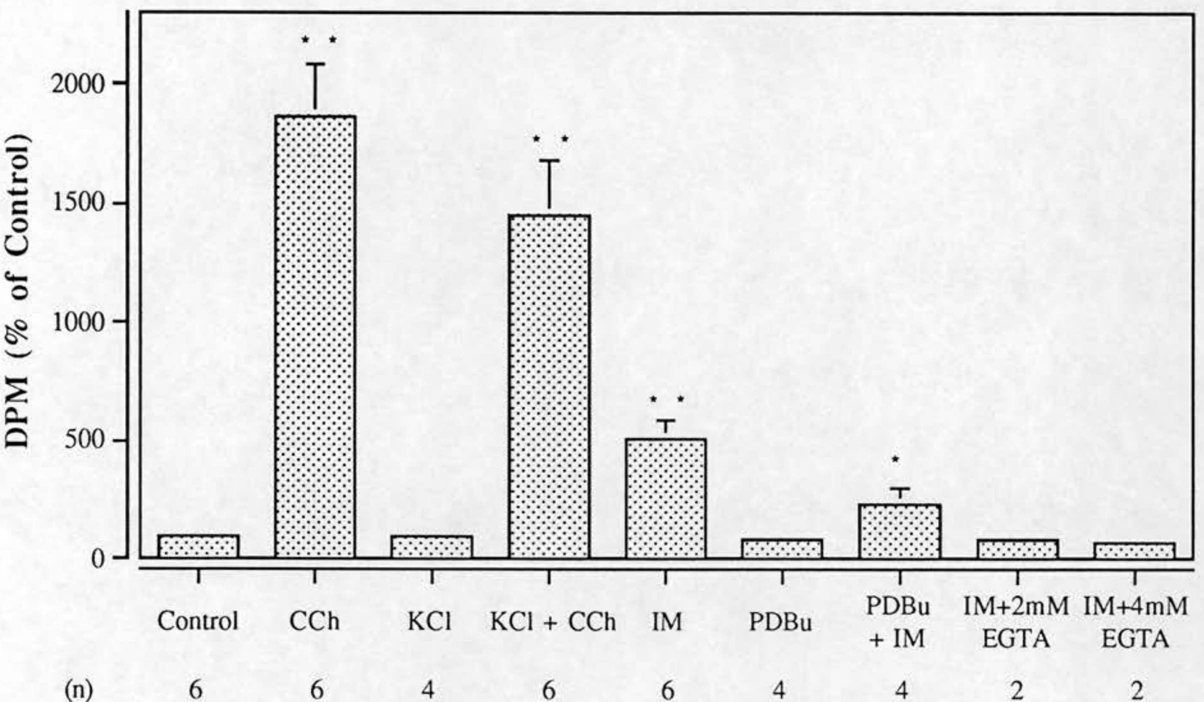

Figure 3.3.1 Effect of potassium ions, ionomycin and phorbol dibutyrate on [^{3}H]inositol phosphate accumulation in bovine tracheal smooth muscle

[^{3}H]Inositol pre-labelled BTSM slices (2.3.2) were incubated in 24 well tissue culture plates for 30 min with 100 μM CCh, 80 mM KCl, 5 μM ionomycin (IM), 100 nM PDBu or vehicle in a final volume of 1 ml. For dual additions KCl, PDBu and EGTA were added 30 sec prior to CCh or ionomycin. Reactions were terminated by the addition of 300 μl 3 M TCA. Total [^{3}H]InsPs were separated from neutralised extracts (2.4.2) by Dowex AG 1-X8 anion-exchange chromatograpy (2.6.1) and quantified by liquid scintillation counting. Results represent the mean ± SEM of n separate experiments, each performed in triplicate. **/* Represents $p < 0.01/0.05$ for comparisons with control (student's two-tailed, unpaired t-test).

BTSM, associated with both membrane depolarisation and an elevation in $[Ca^{2+}]_i$ secondary to Ca^{2+} influx - an effect that can be greatly inhibited by the Ca^{2+} channel antagonist nimodipine (Takuwa *et al.*, 1987). No reduction in the concentration of Na^+ ions was performed in the experiments involving high K^+ concentrations since in guinea-pig visceral smooth muscle cell types this manoeuvre alone can induce [^{3}H]InsP formation (Best and Bolton, 1986; Sasaguri and Watson, 1988), possibly via stimulation of a Na^+/Ca^{2+} exchange mechanism. However Chilvers *et al.* (1994b) describe control experiments in which addition of 80 mM NaCl fails to reduce Ins(1,4,5)P_3 mass (as observed with 80 mM KCl addition), indicating that the results observed in their experiments are not simply due to an osmotic effect of the high KCl solutions.

By contrast, incubation of [^{3}H]inositol pre-labelled BTSM slices for 30 min with 5 μM ionomycin (see figure 3.3.1) did result in a significant accumulation of [^{3}H]InsPs (27 ± 2 % of that seen with 100 μM CCh). It would seem therefore that depolarisation of the plasma membrane with 80 mM KCl, which results in an influx of extracellular Ca^{2+} is unable to stimulate PIC activity in this tissue, but that increasing $[Ca^{2+}]_i$ with 5 μM ionomycin does enhance the activity of this enzyme. Application of a calcium ionophore would be expected to result in a much greater increase in $[Ca^{2+}]_i$ than would be achieved by K^+ depolarisation since it facilitates free flow of Ca^{2+} across the plasma membrane (and also possibly intracellular membranes), unlike a high KCl concentration which is likely to produce a more physiological increase in Ca^{2+} via the opening of voltage-operated Ca^{2+} channels. In order to restrict the rise in $[Ca^{2+}]_i$ with ionomycin to physiologically relevant levels, further incubations were performed in which 2 mM or 4 mM EGTA was added to the system (resulting in the reduction of $[Ca^{2+}]_{free}$ in the medium from 1.8 mM to 6

μM or 0.8 μM respectively[1]) 30 sec prior to ionomycin addition. Under such conditions the ionomycin response was completely ablated. Hence it is possible to augment $[Ca^{2+}]_i$ artificially to levels that can sustain agonist-independent hydrolysis of the $[^3H]$phosphoinositides, but this can only be achieved at unphysiologically high $[Ca^{2+}]_i$ since when $[Ca^{2+}]_{free}$ is reduced to 6 μM or 0.8 μM no significant accumulation of $[^3H]$InsPs can be detected.

The effect of the PKC-activating phorbol ester PDBu on $[^3H]$InsP accumulation was also monitored in order to ascertain whether or not a PLD-mediated breakdown of the phosphoinositides secondary to PKC activation occurs in this tissue. PDBu (100 nM) had no significant effect on the basal accumulation of $[^3H]$InsPs and failed to augment the ionomycin effect. It is unlikely therefore that PKC-mediated PLD activity could contribute significantly to the accumulation of $[^3H]$InsPs in BTSM since no detectable increase in $[^3H]$InsP levels was observed after a 30 min incubation in the presence of PDBu.

These data indicate that there is no significant contribution to $[^3H]$InsP accumulation in BTSM slices from either PLD-mediated phosphoinositde hydrolysis, or breakdown of PtdIns(4,5)P_2 or the lower phosphoinositides by PIC secondary to physiologically relevant increases in $[Ca^{2+}]_i$.

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[1][The $Ca^{2+}]_{free}$ under identical conditions had been determined in the study by Chilvers *et al.*, (1994) using a Ca^{2+}-sensitive electrode according to the method of Wojcikiewicz *et al.* (1990).

3.4 MUSCARINIC CHOLINOCEPTOR-STIMULATED ACCUMULATION OF [^{3}H]INOSITOL POLYPHOSPHATE ISOMERS IN BOVINE TRACHEAL SMOOTH MUSCLE

It is well established that lithium ions inhibit inositol monophosphatase in an uncompetitive manner (see Nahorski *et al.*, 1991), thus in the presence of an ample concentration of its substrate ($InsP_1$) lithium ions potently inhibit this enzyme, and provide an effective block of $InsP_1$ hydrolysis. BTSM exhibits a low level of basal inositol phospholipid hydrolysis (see table 3.4.1), and therefore by introducing lithium ions into the system 10 min prior to agonist/vehicle addition, a sufficiently high concentration of $InsP_1$ can be attained to facilitate inhibition of the inositol monophosphatase enzyme, thereby trapping any [^{3}H]InsPs formed within the cytosolic fraction of the cell. The effective trapping of [^{3}H]InsPs in the cytoplasm is demonstrated by their linear CCh-stimulated accumulation in this system (figure 2.3.1.2a). The use of this protocol should thus enable accurate quantification of the [^{3}H]InsPPs formed in these experiments. Since the 3-kinase and 5-phosphatase pathways yield mutually exclusive products in this tissue (see section 3.2), and there would appear to be no significant contribution to [^{3}H]InsPP accumulation from PIC action on the lower [^{3}H]phosphoinositides or PLD-mediated breakdown of the [^{3}H]inositol phosphates (see section 3.3) it can be concluded that an appropriate experimental system has been devised for the study of the flux of [^{3}H]Ins(1,4,5)P_3 metabolism through the 3-kinase and 5-phosphatase enzymes.

The only additional requirement of these studies is that agonist stimulation of BTSM should result only in accumulation of [^{3}H]InsPP isomers that can be accounted for by the 3-kinase and 5-phosphatase pathways. As detailed in section 3.4.1 below, this pre-requisite was also

fulfilled except for the quantitatively very minor accumulations of [^{3}H]Ins(4,5)P_2 and [^{3}H]Ins(2,4,5)P_3 (see table 3.4.1) which would have no significant bearing on the calculations of flux of [^{3}H]Ins(1,4,5)P_3 through the 3-kinase and 5-phosphatase pathways. The use of a short-term [^{3}H]inositol labelling strategy was a deliberate ploy to ensure that the results were not complicated by the labelling of additional InsPPs.

3.4.1 Accumulation of [^{3}H]inositol polyphosphates following 30 minute carbachol stimulation of BTSM slices

[^{3}H]Inositol pre-labelled BTSM slices were incubated with 100 μM CCh or vehicle in the presence or absence of 5 mM LiCl for 30 min and the resulting individual [^{3}H]InsPP isomers separated and quantified using H.P.L.C.. The results of these experiments are summarised in table 3.4.1. An accumulation of [^{3}H]InsPs was observed over the 30 min incubation, even under basal conditions in the absence of lithium ions. When lithium was added into the system a 3.3-fold increase in basal [^{3}H]InsP accumulation could be detected. CCh caused a dramatic (33-fold) increase in [^{3}H]InsP accumulation which was also markedly enhanced (6.7-fold) by the inclusion of lithium ions. Several [^{3}H]InsPP isomers were isolated from the BTSM slices and were identified on the basis of co-elution with commercial and generated standards, and by comparison with other inositol polyphosphate H.P.L.C. separation studies (e.g. Batty *et al.*, 1989; Wong *et al.*, 1992) as illustrated in the materials and methods section (2.6.3).

Two dominant peaks of radioactivity were detected in the [^{3}H]InsP_1 fraction under all conditions specified which co-eluted exactly with [^{3}H]Ins1/3P and [^{3}H]Ins4P standards. [^{3}H]Ins1P and [^{3}H]Ins3P are enantiomers and as such cannot be resolved by the H.P.L.C. technique

Ins(x)PP	% Total Radioactivity in Fraction			
	- Li^+		+ Li^+	
	Control (n=4)	CCh (n=8)	Control (n=9)	CCh (n=9)
1/3	25.69 ± 1.02	16.73 ± 0.78	25.21 ± 2.08	21.87 ± 1.30
4	13.49 ± 2.38	30.74 ± 3.55	30.51 ± 2.31	59.97 ± 1.68
1,3	2.35 ± 0.12	5.83 ± 0.77	3.22 ± 1.06	1.27 ± 0.24
1,4	18.63 ± 2.24	36.01 ± 2.57	16.18 ± 3.18	13.89 ± 2.11
3,4	5.00 ± 0.31	2.33 ± 0.33	3.79 ± 0.70	0.32 ± 0.03
4,5	0.56 ± 0.28	0.73 ± 0.05	0.96 ± 0.82	1.27 ± 0.06
1,3,4	3.72 ± 0.86	4.94 ± 0.46	3.27 ± 0.70	1.05 ± 0.14
1,4,5	10.50 ± 0.76	1.29 ± 0.14	6.46 ± 0.57	0.17 ± 0.02
2,4,5	0.96 ± 0.36	0.05 ± 0.01	0.34 ± 0.09	0.01 ± 0.001
$InsP_4$	14.95 ± 0.71	1.64 ± 0.10	10.04 ± 1.08	0.28 ± 0.03
3-kinase metabolites	61.68 ± 0.36	32.03 ± 1.82	49.70 ± 3.14	25.13 ± 1.34
5-phosphatase metabolites	38.33 ± 0.36	67.97 ± 1.82	50.30 ± 3.14	74.87 ± 1.34
Total DPM/50 µl slices	1,398 ± 142	45,856 ± 7,281	4,550 ± 957	306,468 ± 35,797

Table 3.4.1 Carbachol-stimulated accumulation of [^{3}H]inositol polyphosphates in bovine tracheal smooth muscle slices. [^{3}H]Inositol pre-labelled BTSM slices (2.3.1) were incubated for 30 min at 37 °C in the presence or absence of 5 mM LiCl and 100 µM CCh. [^{3}H]InsPPs in pooled triplicate TCA extracts (2.4.2) were separated using a Partisphere 5 SAX H.P.L.C. column (2.6.3) and quantified by liquid scintillation counting. Results are expressed as the percentage of total retrieved radioactivity associated with each [^{3}H]InsPP isomer, or as the percentage of metabolised [^{3}H]Ins(1,4,5)P_3 present as 3-kinase or 5-phosphatase pathway metabolites, and represent the mean ± SEM of n separate experiments.

employed here. $[^3H]Ins1/3P$ represented the dominant $[^3H]InsP_1$ isomer present under basal conditions. The accumulation of both monophosphates was enhanced following prolonged CCh stimulation with $[^3H]Ins4P$ constituting the major $[^3H]InsP_1$ under these conditions (64 ± 3 % and 73 ± 1 % of the $[^3H]InsP_1$ pool in the absence and presence of lithium respectively). In addition, a further very minor $[^3H]InsP_1$ peak could be detected in some of the basal samples which has been tentatively identified as $[^3H]Ins2P$ since it co-eluted with a generated standard for this compound (see section 2.6.3), however it is also possible that it represents $[^3H]Ins5P$, low concentrations of which have been detected in brain (Ackermann *et al.*, 1987). The accumulation of this isomer was negligible (if detectable at all) under all conditions assayed.

Four peaks of radioactivity were detected in the $[^3H]InsP_2$ fraction, two co-eluting with standard or generated $[^3H]Ins(1,4)P_2$ and $[^3H]Ins(4,5)P_2$ (see section 2.6.3), the other two being identified as $[^3H]Ins(1,3)P_2$ and $[^3H]Ins(3,4)P_2$ by comparison with a similar study by Batty *et al.* (1989) in rat cerebral cortex slices. $[^3H]Ins(1,4)P_2$ was the major bisphosphate in all samples assayed.

Three $[^3H]InsP_3$ peaks were observed - two of which co-eluted with standard and generated $[^3H]Ins(1,4,5)P_3$ and $[^3H]Ins(2,4,5)P_3$ (see section 2.6.3), the third identified as $[^3H]Ins(1,3,4)P_3$ since it is the only known trisphosphate isomer to elute prior to $Ins(1,4,5)P_3$ (Batty *et al.*, 1989; Wong *et al.*, 1992). $[^3H]Ins(1,4,5)P_3$ was the major $[^3H]InsP_3$ present in basal samples whereas $[^3H]Ins(1,3,4)P_3$ was by far the main constituent under CCh-stimulated conditions. Accumulation of $[^3H]Ins(2,4,5)P_3$ was negligible under all conditions assayed. This isomer may represent an acid-hydrolysis product of trace amounts of $[^3H]Ins(1{:}2c,4,5)P_3$, which has been reported to accumulate in a variety of tissues including thrombin-

stimulated platelets (Ishii *et al.*, 1986) and vasopressin-stimulated WRK1 rat mammary tumour cells (Wong *et al.*, 1988).

Although the H.P.L.C. gradient used in these studies does not allow good separation of $[^3H]InsP_4$ isomers, it is probable from previous data on CCh-stimulated increases in $Ins(1,3,4,5)P_4$ in this model (Chilvers *et al.*, 1991b) that the agonist-stimulated increase in $[^3H]InsP_4$ accumulation represents an increase in the $[^3H]Ins(1,3,4,5)P_4$ isomer. In addition no late-running $[^3H]InsP_4$ peak or shoulder to the $[^3H]InsP_4$ peak was observed suggesting that there is no significant accumulation of $[^3H]Ins(1,3,4,6)P_4$ under the described conditions.

The presence of 5 mM LiCl in the control incubations resulted in a minor increase in the accumulation of both $[^3H]Ins1/3P$ and $[^3H]Ins4P$. Under CCh-stimulated conditions the presence of lithium resulted in a 9-fold and 13-fold increase in $[^3H]Ins1/3P$ and $[^3H]Ins4P$ accumulation respectively, and a 2.6-fold increase in $[^3H]Ins(1,4)P_2$. Lithium had a more minor effect on $[^3H]Ins(1,3)P_2$, $[^3H]Ins(3,4)P_2$ and $[^3H]Ins(1,3,4)P_3$ accumulation. These results are in keeping with the known effects of lithium as a potent uncompetitive inhibitor of the inositol monophosphatase responsible for converting each of the inositol monophosphate isoforms into free inositol, and a weaker inhibitor of the inositol polyphosphate 1-phosphatase which converts $Ins(1,4)P_2$ and $Ins(1,3,4)P_3$ into Ins4P and $Ins(3,4)P_2$ respectively. Lithium ions therefore result in a marked accumulation of the immediate substrates of these enzymes, and a lesser accumulation of their upstream precursors. In addition, lithium resulted in a 10-fold increase in $[^3H]Ins(4,5)P_2$ in CCh-stimulated BTSM slices, suggesting the presence of at least one further lithium-sensitive metabolising enzyme. The lithium-sensitive, agonist-

stimulated accumulation of this novel bisphosphate isomer is discussed in further detail in chapter six.

It is possible therefore to calculate the relative proportions of [^{3}H]Ins(1,4,5)P_3 that had been metabolised by the 3-kinase and 5-phosphatase pathways in these experiments by totalling the radioactivity associated with each of the respective pathway metabolites (i.e. the 5-phosphatase pathway metabolites are quantified by totalling the radioactivity in the [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins4P fractions, whereas the 3-kinase pathway is represented by the total radioactivity associated with [^{3}H]Ins(1,3,4,5)P_4, [^{3}H]Ins(1,3,4)P_3, [^{3}H]Ins(1,3)P_2, [^{3}H]Ins(3,4)P_2, and [^{3}H]Ins1/3P). The only [^{3}H]inositol polyphosphate isomers excluded from these calculations were [^{3}H]Ins2P, [^{3}H]Ins(2,4,5)P_3 and [^{3}H]Ins(4,5)P_2, as the source of these isomers is uncertain, and their accumulation following CCh stimulation was very minor (accumulation of the two former isomers was negligible under all conditions assayed, while [^{3}H]Ins(4,5)P_2 represented only 1 % of the total inositol polyphosphate pool after 30 min in the presence of Li^+). Using this approach, the relative contributions of these two enzyme pathways to [^{3}H]Ins(1,4,5)P_3 metabolism has been assessed for each of the experimental incubations described above.

When lithium ions were included in the system the contribution to [^{3}H]Ins(1,4,5)P_3 metabolism by the 5-phosphatase pathway increased from 38 % to 50 % in basal samples, and from 68 % to 75 % in 30 min CCh-stimulated samples. The inclusion of lithium ions probably gives a more accurate portrayal of the activity of these two enzyme pathways since the hydrolysis of [^{3}H]InsP_1 isomers to [^{3}H]Ins, and therefore their omission from the calculations, would be negligible under these conditions. In the presence of lithium ions, [^{3}H]Ins(1,4,5)P_3 formed under basal, unstimulated conditions would appear to be metabolised in equal

proportions by the 3-kinase and 5-phosphatase over the 30 min incubation period. Because of the small accumulation of [^{3}H]InsPP isomers under basal conditions however, the calculation is much less certain. In the presence of a maximally effective concentration of CCh (100 μM) the 5-phosphatase enzyme clearly dominates, accounting for 75 % of the total [^{3}H]Ins(1,4,5)P_3 metabolism.

In other tissues it has been shown that Ins1/3P and Ins4P share a common enzyme for their degradation to free inositol and that this enzyme has a similar affinity (K_m = 0.1-0.2 mM) for Ins1/3P and Ins4P (Gee *et al.*, 1988; Hallcher and Sherman, 1980). If a similar monophosphatase is responsible for the degradation of these isomers in BTSM then steady-state [^{3}H]InsPP accumulation should still reflect the metabolism of [^{3}H]Ins(1,4,5)P_3 through the 3-kinase and 5-phosphatase pathways in the absence of lithium.

3.4.2 Accumulation of [^{3}H]inositol polyphosphates over a 30 minute time-course of carbachol stimulation

The accumulation of [^{3}H]InsPP isomers in [^{3}H]inositol pre-labelled BTSM slices in response to CCh stimulation in the presence of 5 mM LiCl was assessed over a 30 min time-course. Table 3.4.2 summarises the results obtained from these experiments, and the accumulation of [^{3}H]InsPPs over the first 5 min following agonist stimulation is illustrated in figure 3.4.2.1. Agonist stimulation resulted in a rapid (< 5 sec) rise in the accumulation of [^{3}H]Ins(1,4,5)P_3 (figure 3.4.2.1c), consistent with it being the primary product of muscarinic receptor-stimulated [^{3}H]PtdIns(4,5)P_2 hydrolysis. In the continued presence of agonist the accumulation of this isomer returned to basal levels within 1 min with only a minor increase observed at much later time-points following CCh stimulation. It is

$[^3H]Ins(x)PP$	% Total Radioactivity in Fraction					
	0 sec	5 sec	30 sec	1 min	5 min	30 min
1/3	16.67 ± 7.08	21.79 ± 1.35	9.43 ± 2.76	8.90 ± 2.00	12.78 ± 0.71	26.57 ± 3.20
4	29.63 ± 7.41	31.17 ± 3.85	22.22 ± 5.77	27.05 ± 3.24	48.63 ± 0.89	50.47 ± 8.67
1,3	3.55 ± 1.37	0.98 ± 0.50	0.95 ± 0.49	2.19 ± 0.28	2.35 ± 0.31	0.73 ± 0.37
1,4	27.08 ± 11.86	37.17 ± 6.56	59.86 ± 7.88	55.65 ± 5.11	31.73 ± 0.29	19.67 ± 5.32
3,4	2.85 ± 0.95	1.53 ± 0.23	0.90 ± 0.09	0.90 ± 0.09	0.63 ± 0.03	0.30 ± 0.03
4,5	0.13 ± 0.07	0.10 ± 0.08	0.07 ± 0.03	0.28 ± 0.01	0.28 ± 0.01	1.11 ± 0.09
1,3,4	4.83 ± 1.03	3.13 ± 0.38	3.7 ± 0.32	3.52 ± 0.39	2.49 ± 0.04	0.79 ± 0.07
1,4,5	5.51 ± 1.87	6.33 ± 1.81	1.15 ± 0.18	0.51 ± 0.09	0.39 ± 0.01	0.14 ± 0.01
2,4,5	0.39 ± 0.10	0.17 ± 0.08	0.07 ± 0.02	0.04 ± 0.003	0.03 ± 0.01	0.01 ± 0.00
$InsP_4$	8.49 ± 3.09	4.41 ± 1.32	1.23 ± 0.19	1.00 ± 0.15	0.67 ± 0.02	0.21 ± 0.00
3-kinase metabolites	37.87 ± 8.44	26.63 ± 5.14	16.48 ± 2.34	16.66 ± 1.91	18.87 ± 0.57	28.97 ± 3.50
5-phosphatase metabolites	62.14 ± 8.44	73.37 ± 5.14	83.52 ± 2.34	83.34 ± 1.91	81.13 ± 0.57	71.03 ± 3.50
DPM/50 µl slices	4809 ± 1861	8672 ± 3420	26626 ± 5541	30841 ± 7583	63529 ± 10165	337179 ± 14219

Table 3.4.2 Time-course of carbachol-stimulated accumulation of $[^3H]$inositol polyphosphates in bovine tracheal smooth muscle slices

$[^3H]$Inositol pre-labelled BTSM slices (2.3.1) were stimulated in the presence of 5 mM LiCl with 100 µM CCh for the times indicated. $[^3H]$InsPPs in pooled triplicate TCA extracts (2.4.2) were separated using a Partisphere 5 SAX H.P.L.C. column and quantified by liquid scintillation counting. Results are expressed as the percentage of total retrieved radioactivity associated with each $[^3H]$InsPP isomer, or as the percentage of metabolised $[^3H]Ins(1,4,5)P_3$ present as 3-kinase or 5-phosphatase metabolites and represent the mean ± SEM of three separate experiments.

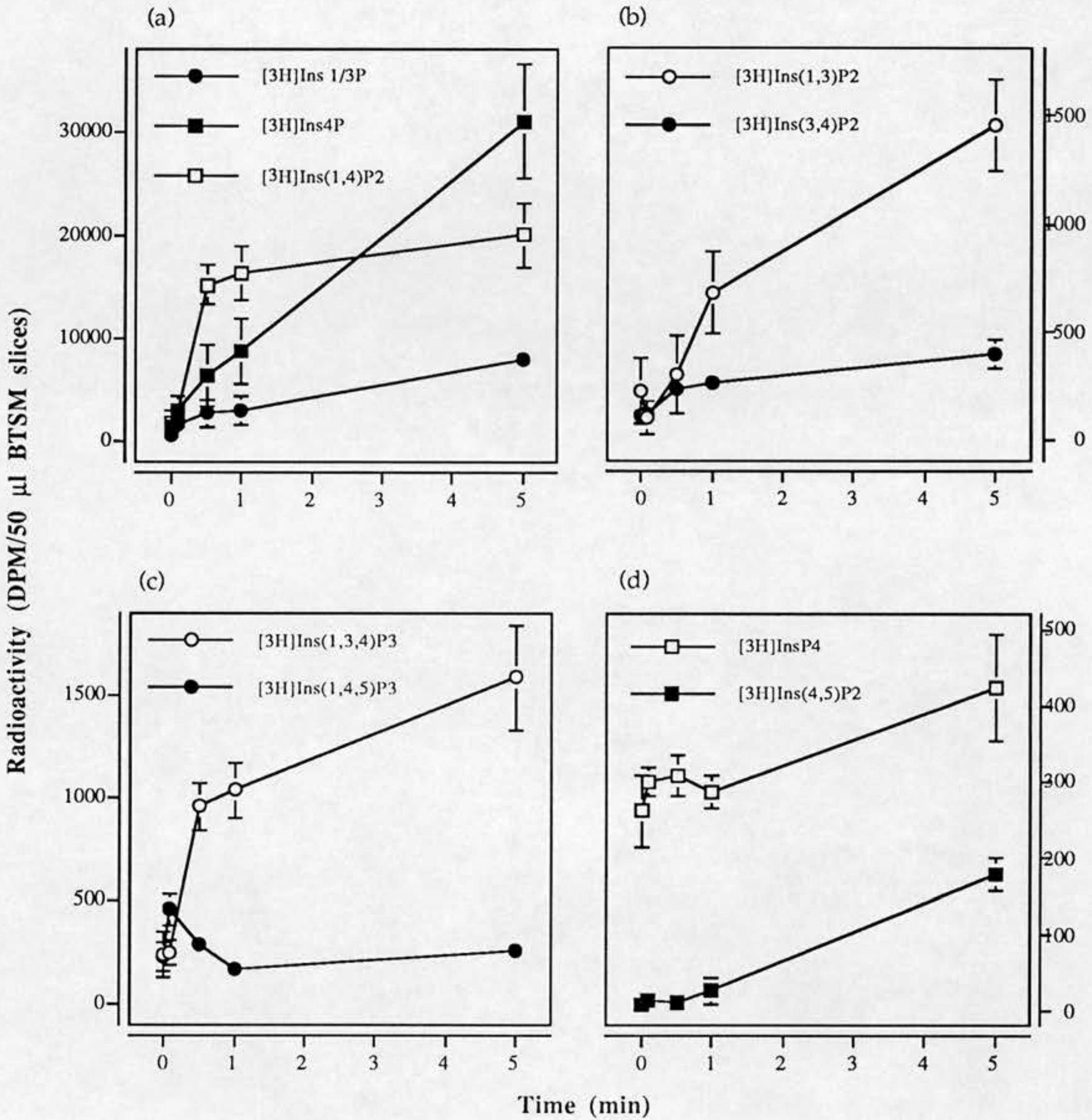

Figure 3.4.2.1 Pattern of [^{3}H]inostiol polyphosphate accumulation over five minutes following carbachol-stimulation of bovine tracheal smooth muscle slices

[^{3}H]Inositol pre-labelled BTSM slices (2.3.1) were incubated with 100 µM CCh in the presence of 5 mM LiCl in a final volume of 300 µl. Reactions were terminated at the times indicated by addition of 300 µl 1 M TCA. Individual [^{3}H]InsPPs were separated from poled triplicate neutralised extracts (2.4.2) by H.P.L.C. (2.6.3) and the radioactivity associated with each isomer quantified by liquid scitntillation counting. Results represent the mean ± SEM of three separate experiments.

possible that this delayed and quantitatively very minor increase in the '[^{3}H]Ins(1,4,5)P_3 peak' was due to the accumulation of non-resolving [^{3}H]InsP_3 isomers such as [^{3}H]Ins(1,4,6)P_3 which has previously been identified in avian erythrocytes (Stephens and Downes, 1990; Stephens *et al.*, 1989) and its enantiomer [^{3}H]Ins(3,4,6)P_3 identified in the slime mould *Dictyostelium discoideum* (Stephens and Irvine, 1990). Indeed Wong *et al.* (1992) identified an additional minor component in their 'Ins(1,4,5)P_3 peak' which increased following stimulation with vasopressin. They provide evidence to indicate that this compound was Ins(1,4,6)P_3.

A rapid elevation in the levels of [^{3}H]Ins(1,4)P_2, the product of Ins(1,4,5)P_3 5-phosphatase, was seen upon CCh stimulation (see figure 3.4.2.1a), which plateaued after 1 min (the time at which [^{3}H]Ins(1,4,5)P_3 radioactivity had returned to basal values). [^{3}H]Ins(1,4)P_2 was the major [^{3}H]inositol bisphosphate present in all samples assayed. The other 5-phosphatase pathway metabolite [^{3}H]Ins4P showed a linear accumulation in response to CCh, and was the dominant [^{3}H]InsP_1 isomer at all time-points measured.

[^{3}H]Inositol tetrakisphosphate accumulated only to a minor degree over the agonist stimulation period (figure 3.4.2.1d), and in agreement with Chilvers *et al.* (1990a) only increased to significant levels after 5 min. It would seem that at early time-points any [^{3}H]Ins(1,3,4,5)P_4 formed is rapidly dephosphorylated to [^{3}H]Ins(1,3,4)P_3 since large increases in this isomer can be detected within 30 sec following agonist addition (figure 3.4.2.1c). The rate of increase in this [^{3}H]inositol trisphosphate slows after 1 min. In agreement with Chilvers *et al.* (1990a), [^{3}H]Ins(1,3,4)P_3 was the major trisphosphate isomer in BTSM slices stimulated for 30 sec or more with 100 μM CCh, accounting for > 80 % of the trisphosphate fraction after 1 min of stimulation with this agonist. The other major 3-kinase

pathway metabolites ([^{3}H]Ins(1,3)P_2, [^{3}H]Ins(3,4)P_2 and [^{3}H]Ins1/3P) exhibited a delayed accumulation in response to CCh, only increasing to significant levels after 1 and 5 min for the bisphosphates and monophosphates respectively (figures 3.4.2.1a and b).

[^{3}H]Ins(2,4,5)P_3 and [^{3}H]Ins2P levels were negligible under all conditions assayed and hence are not illustrated in figure 3.4.2.1. Interestingly, [^{3}H]Ins(4,5)P_2 showed a very delayed accumulation in response to CCh stimulation (figure 3.4.2.1d) suggesting an indirect formation of this bisphosphate isomer. Further studies designed to address the possible routes of formation and metabolism of this isomer are detailed in chapter six.

The flux of the inositol headgroup through the Ins(1,4,5)P_3 3-kinase and 5-phosphatase regulated pathways following CCh stimulation was determined in an identical fashion to that described in section 3.4.1 and is shown in table 3.4.2. Although the initial contribution from the two pathways (at 0 sec) appears to vary slightly from that documented for basal conditions over 30 min (see section 3.4.1), the difference between these values is not statistically significant. The 5-phosphatase was the dominant Ins(1,4,5)P_3 metabolising pathway at all time-points following CCh stimulation, however it would seem that the 3-kinase becomes increasingly important after 1-5 min. A detailed picture of the flux of the inositol headgroup through these two enzymes was obtained when the 3-kinase and 5-phosphatase metabolites were quantified over time-intervals (as opposed to the cumulative response). The result of this analysis is summarised in figure 3.4.2.2. The 5-phosphatase pathway was the dominant route for [^{3}H]Ins(1,4,5)P_3 metabolism at all time-intervals measured, accounting for over 85 % of its metabolism over the first 5 sec following agonist addition. At later time-points of the agonist-stimulated

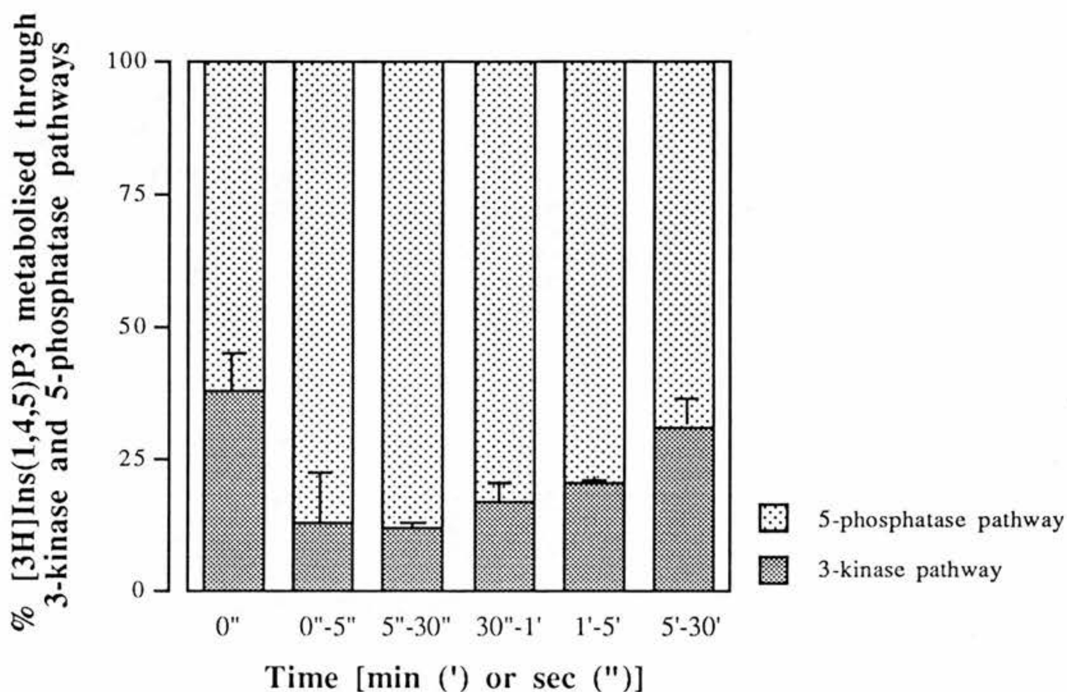

Figure 3.4.2.2 Flux of [^{3}H]inositol 1,4,5-trisphosphate through the 3-kinase and 5-phosphatase pathways following carbachol-stimulation

[^{3}H]Inositol pre-labelled BTSM slices (2.3.1) were incubated with 100 μM CCh in the presence of 5 mM LiCl in a final volume of 300 μl. Reactions were terminated after 0 sec, 5 sec, 30 sec, 1 min, 5 min and 30 min by the addition of 300 μl 1M TCA and the individual [^{3}H]InsPP isomers present in pooled triplicate neutralised extracts (2.4.2) separated and quantified by H.P.L.C. (2.6.3). The individual [^{3}H]InsPPs accumulating over the time-intervals shown were calculated, and the radioactivity associated with the [^{3}H]Ins(1,4,5)P_3 3-kinase and 5-phosphatase metabolites over these time-intervals determined. Data represents the mean ± SEM of three separate experiments.

response however, the contribution from the 3-kinase pathway became increasingly important.

3.5 DISCUSSION

The experiments described in this chapter were designed to characterise, in detail, the accumulation of [^{3}H]inositol polyphosphates following agonist stimulation of BTSM and to assess the routes of metabolism of Ins(1,4,5)P_3. While the dominant route of metabolism, both at early and late time-points after agonist addition, appears to be via the 5-phosphatase pathway, the 3-kinase pathway plays an increasing role at later time-points following agonist addition. A second bifurcation point in inositol polyphosphate metabolism is seen with the breakdown of Ins(1,3,4)P_3 since this compound can be converted either to Ins(1,3)P_2 or to Ins(3,4)P_2. Following prolonged CCh stimulation (30 min), a greater accumulation of [^{3}H]Ins(1,3)P_2 over [^{3}H]Ins(3,4)P_2 was detected (see table 3.4.1) both in the absence and presence of lithium (2.5- and 4-fold respectively). This is in agreement with Batty *et al.* (1989) who detected an accumulation of [^{3}H]Ins(1,3)P_2 to levels three times that of [^{3}H]Ins(3,4)P_2 following CCh stimulation (30 min) of rat cerebral cortex slices and with Wreggett and Irvine (1993) who found Ins(1,3)P_2 to be the predominant Ins(1,3,4)P_3 metabolite in thrombin- and histamine-stimulated human umbilical vein endothelial cells. The latter two studies were carried out in the presence of lithium ions which inhibit the Ins(1,4)P_2/Ins(1,3,4)P_3 1-phosphatase and would therefore favour metabolism of Ins(1,3,4)P_3 via the 4-phosphatase. It is also possible that the greater proportion of [^{3}H]Ins(1,3)P_2 produced following CCh stimulation in the absence of lithium ions in the present study results

from [^{3}H]Ins(1,3,4)P_3 competing with the much larger concentration of [^{3}H]Ins(1,4)P_2 for the Ins(1,3,4)P_3/Ins(1,4)P_2 1-phosphatase (Inhorn and Majerus, 1987, 1988). Indeed, under basal conditions, when the 3-kinase pathway contributes substantially to Ins(1,4,5)P_3 metabolism, proportionally more [^{3}H]Ins(3,4)P_2 is produced compared to [^{3}H]Ins(1,3)P_2 (see table 3.4.1).

Despite its continued formation [^{3}H]Ins(1,4,5)P_3 accumulates only transiently following CCh stimulation (Chilvers *et al.*, 1991a). The underlying reason for this apparent tight metabolic control of Ins(1,4,5)P_3 accumulation in BTSM remains uncertain, and contrasts to the situation pertaining in a number of other tissues including rat cerebral cortex slices where Ins(1,4,5)P_3 mass remains elevated for a prolonged period following agonist stimulation (Challiss *et al.*, 1988). It is possible that some of the more recently described functions of Ins(1,4,5)P_3, such as its effect on tyrosine phosphatase activity (Stader and Hofer, 1992) underlie a need for differential regulation of Ins(1,4,5)P_3 metabolism between tissues. Hence it may be more important to rapidly curtail the Ins(1,4,5)P_3 response in BTSM, a process which would require stringent regulation of the enzymes responsible for the metabolism of this second messenger.

The demonstration that the 5-phosphatase and 3-kinase pathways yield mutually exclusive products was verified in part by examining [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 metabolism in BTSM homogenates. Given evidence suggesting little, if any, hydrolysis of the PtdIns and PtdIns4P, or PLD-stimulated phosphoinositide hydrolysis in the production of InsPs in this tissue, it would seem that the individual InsPP isomers detected in these experiments are derived predominantly from the sequential dephosphorylation of Ins(1,4,5)P_3 and its primary metabolite Ins(1,3,4,5)P_4. The short-term labelling protocol employed in

these studies has been extensively validated and provides steady-state phosphoinositide labelling and prevents agonist-stimulated changes in the specific radioactivity of $[^3H]PtdIns(4,5)P_2$ (Chilvers *et al.*, 1989a). This approach results in very little, if any, labelling of higher inositol phosphates (i.e. $Ins(1,3,4,5,6)P_5$ and $InsP_6$) and hence avoids many of the problems inherent in attempting to follow $Ins(1,4,5)P_3$ metabolism in tissues labelled to isotopic equilibrium over many days. This BTSM model therefore provides a good preparation for the study of $Ins(1,4,5)P_3$ metabolism, facilitating the analysis of the relative contributions of the 3-kinase and 5-phosphatase pathways.

Although the 3-kinase enzyme contributes significantly to $Ins(1,4,5)P_3$ breakdown under the conditions specified, the 5-phosphatase pathway appears to dominate especially at early time-points after agonist stimulation when the accumulation of $Ins(1,4,5)P_3$ is still elevated over control values. Of interest was the finding that the 3-kinase pathway becomes an increasingly important route for $Ins(1,4,5)P_3$ metabolism at later time-points following agonist stimulation. This could relate to a Ca^{2+}/calmodulin-mediated activation of this enzyme as suggested in insulin secreting RINm5F cells (Biden and Wollheim, 1986) and later substantiated in a variety of tissues including rabbit tracheal smooth muscle (Rosenberg *et al.*, 1991), pig aortic smooth muscle (Yamaguchi *et al.*, 1987), rat aortic smooth muscle (Rossier *et al.*, 1987) and rat heart (Renard and Poggioli, 1987). Increases in the concentration of this ion following CCh stimulation occur as a result of both its release from intracellular stores and an increase in Ca^{2+} influx (Takuwa *et al.*, 1987). In most cases studied the 5-phosphatase is unaffected by physiological increases in $[Ca^{2+}]_i$ (e.g. Biden *et al.*, 1986), however notable exceptions are the 5-phosphatases present in porcine coronary artery smooth muscle,

which is enhanced by 0.1-1 μM free Ca^{2+} concentrations (Sasaguri *et al.*, 1985), and that found in bovine iris sphincter smooth muscle which is inhibited by $[Ca^{2+}]_{free}$ concentrations greater than 1 μM (Wang *et al.*, 1994).

There are also reports that PKC may regulate the 3-kinase and 5-phosphatase enzymes: PKC-mediated phosphorylation results in a 3-fold stimulation of the Type Ia 5-phosphatase enzyme from human platelets (Connolly *et al.*, 1986). This effect is substantiated by data in thrombin-stimulated platelets where inhibition of PKC with staurosporine results in a decrease in 5-phosphatase activity (King and Rittenhouse, 1989). To date however, PKC-regulation of 5-phosphatase activity has not been described in any other cell type, although indirect evidence for such regulation was obtained in C62B glioma cells in which pre-treatment with the PKC-inhibitor sphingosine resulted in an enhanced ACh-stimulated $InsP_3$ accumulation, accompanied by a concurrent decrease in $InsP_2$ accumulation (Brooks *et al.*, 1987). By contrast, the conversion of $Ins(1,4,5)P_3$ to $Ins(1,4)P_2$ in RINm5F cells decreased slightly in the presence of the PKC activating phorbol ester phorbol myristate acetate (PMA) (Biden *et al.*, 1988b).

The activity of the $Ins(1,4,5)P_3$ 3-kinases from rat brain (Sim *et al.*, 1990) and human platelets (Lin *et al.*, 1990) are inhibited by PKC-dependent serine phosphorylation, an effect mediated by a dramatic reduction in the V_{max} of the enzyme (Sim *et al.*, 1990). In contrast with this data Imboden and Pattison (1987) reported a PKC-mediated activation of the 3-kinase from a clonal T cell line and Biden *et al.*, (1988a and b) noted a slight enhancement of 3-kinase activity in the presence of PMA in both insulin-secreting RINm5F cells and rat hepatocytes. Furthermore the V_{max} of the 3-kinase in saponin-permeabilised platelets was increased 1.8-fold in the

presence of PDBu (King and Rittenhouse, 1989). The 3-kinases cloned from rat brain (Takazawa *et al.*, 1990b) and human hippocampus (Takazawa *et al.*, 1991a) contain putative PKC-dependent serine and threonine phosphorylation sites.

The K_m values of the Ins(1,4,5)P_3 5-phosphatase and 3-kinase enzymes have been calculated in rabbit ASM as 95 μM and 5 μM respectively (Rosenberg *et al.*, 1991). Indeed, in all tissues studied so far the 3-kinase has a greater affinity for Ins(1,4,5)P_3 than the 5-phosphatase. It would therefore be anticipated that at low concentrations of Ins(1,4,5)P_3 this second messenger would be metabolised preferentially by the 3-kinase enzyme. It is important to note however that Ins(1,4,5)P_3 may be compartmentalised within the cell (Horstman *et al.*, 1988; Challiss *et al.*, 1990) or produced preferentially at a specific cellular locus, and therefore may be present locally in concentrations above that expected from our data. This consideration may be of particular relevance in BTSM where mass measurements suggest a relatively high resting Ins(1,4,5)P_3 concentration of 3-4 μM (Challiss *et al.*, 1990), as compared with the 'normal' range of 0.1-0.2 μM (see Shears, 1991).

Studies now performed in a variety of other tissues suggest that the flux of Ins(1,4,5)P_3 via the 3-kinase and 5-phosphatase pathways may vary considerably, not only with regard to the differential routing during the lifetime of the agonist-stimulated response but also with respect to the tissue, agonist and species under study: Batty and colleagues (1992) have shown in rat cerebral cortex stimulated with a maximally effective concentration of CCh that Ins(1,4,5)P_3 is metabolised by the 3-kinase and 5-phosphatase enzymes in roughly equal proportions; Wreggett and Irvine (1993) have demonstrated that the 3-kinase pathway is more active in human umbilical vein endothelial cells stimulated with histamine

than with thrombin at concentrations which give a comparable level of total InsPs; Pirotton *et al.* (1991) have shown that ATP stimulation of vascular endothelial cells results in a rapid and transient routing of Ins(1,4,5)P_3 metabolism almost exclusively through the 5-phosphatase pathway at early time-points of stimulation followed by a small, but sustained increase in 3-kinase routing. In light of these studies it is very possible that the metabolic fate of Ins(1,4,5)P_3 accumulation in response to other PIC-coupled agonists, or even sub-maximal concentrations of CCh, may differ to that described in this study. Such possibilities are examined in chapter five.

It is now clear that at least three different isoforms of both the Ins(1,4,5)P_3 5-phosphatase and 3-kinase exist, with the possibility of further active forms of the latter enzyme being produced by limited proteolysis (see section 1.5.1). If these 3-kinase and 5-phosphatase isoforms vary in their kinetics of Ins(1,4,5)P_3 metabolism their differential distribution or activation may account for the variations in Ins(1,4,5)P_3 accumulation seen in many tissues. Indeed the Type Ib (Hodgkin *et al.*, 1994; Mitchell *et al.*, 1989) and Type II (Erneux *et al.*, 1989; Hansbro *et al.*, 1994; Hansen *et al.*, 1991) 5-phosphatases generally exhibit a lower affinity for Ins(1,4,5)P_3 than their Type Ia counterparts. In addition, a differential ability of PKC to phosphorylate these 5-phosphatase isoforms has been detected in platelets where PKC phosphorylates the Type Ia (Conolly *et al.*, 1986) but not the Type Ib (Mitchell *et al.*, 1989) enzyme. Consistent with these observations, several putative PKC phosphorylation sites have been identified on the Type Ia receptors cloned from dog thyroid (Verjans *et al.*, 1994) and human placenta (Laxminarayan *et al.*, 1994) but not on the type Ib enzymes cloned from human erythroleukaemic and megakaryocytic cells (Ross *et al.*, 1991).

PIC-linked receptor agonists may also activate a host of different signalling pathways secondary to PtdIns(4,5)P_2 hydrolysis which may interact with the phosphoinositide cycle, regulating both the formation and metabolism of Ins(1,4,5)P_3. For example, PKA (stimulated following activation of adenylyl cyclase and formation of cAMP) may be an important regulator of 3-kinase activity since cloning of the rat brain 3-kinase has revealed a consensus sequence for PKA phosphorylation on serine (Takazawa *et al.*, 1990b), and reconstitution experiments conducted by Sim *et al.* (1990) demonstrate a near doubling of the V_{max} following cAMP-dependent serine phosphorylation of this enzyme.

Further studies aimed at identification and characterisation of the possible modes of regulation of the 3-kinase and 5-phosphatase enzymes which could thereby dictate the flux of Ins(1,4,5)P_3 through these two metabolic pathways are detailed in chapters four and five.

The regulation of Ins(1,4,5)P_3 metabolism is clearly an important control point with respect to Ins(1,4,5)P_3-mediated Ca^{2+} release, but may also play a crucial role in cell functioning by determining the cytosolic concentrations of other inositol polyphosphates, in particular Ins(1,3,4,5)P_4. The 5-phosphatase pathway is generally thought of as being merely an inactivating pathway with Ins(1,4)P_2 having no Ca^{2+} mobilising activity. The 3-kinase pathway on the other hand may, through the generation of Ins(1,3,4,5)P_4 and a host of other InsPPs with putative functional roles, be very important to Ca^{2+}-signalling (see section 1.5.2).

CHAPTER FOUR
CHARACTERISATION OF THE INOSITOL 1,4,5-TRISPHOSPHATE 3-KINASE AND 5-PHOSPHATASE ENZYMES

4.1 INTRODUCTION

As detailed in chapter three, Ins(1,4,5)P_3 is metabolised via two major enzyme pathways, a 5-phosphatase route to Ins(1,4,)P_2 and a 3-kinase route to Ins(1,3,4,5)P_4. The activity of these two enzymes therefore plays a critical role in determining the levels and patterns of Ins(1,4,5)P_3 accumulation following receptor stimulation. While the Ins(1,4,5)P_3 3-kinase and 5-phosphatase enzymes have been studied in a variety of tissues, very little data exists regarding the activity of these enzymes in ASM. Large variations in the K_m and V_{max} values of these enzymes have been observed in different tissue types (see Shears, 1991) which, although in part may reflect the differing assay conditions employed in these studies, more likely reflects a differential regulation of these enzymes (e.g. by Ca^{2+}, PKC or PKA) or the presence of more than one isozyme. Characterisation of the Ins(1,4,5)P_3 3-kinase and 5-phosphatase in BTSM is therefore an essential component in our understanding of the factors influencing of Ins(1,4,5)P_3 metabolism.

Only two previous studies have examined the kinetics of these Ins(1,4,5)P_3 metabolising enzymes in ASM. Chilvers (1991) determined Ins(1,4,5)P_3 3-kinase K_m (1.9 μM) and V_{max} (385 pmol min^{-1} mg $protein^{-1}$) values in soluble BTSM extracts. These values differ slightly from those obtained in soluble extracts of rabbit tracheal smooth muscle (Rosenberg *et al.*, 1991) where apparent K_m and V_{max} values of 5 μM and 137.8 pmol min^{-1} mg $protein^{-1}$ respectively were calculated. In common with most

other determinations of 3-kinase activity, both of these studies were performed in the absence of calpain inhibitors. This consideration is likely to be of importance since Lee and co-workers (1990) have now shown that the rat brain Ins(1,4,5)P_3 3-kinase may, in common with a number of other calmodulin-binding proteins, be a substrate for calpain, a Ca^{2+}-activated neutral cysteine-proteinase. The presence of six putative calpain cleavage sites ('PEST' regions) in the amino acid sequence predicted from a rat brain 3-kinase cDNA clone (Choi *et al.*, 1990) support this observation. It is therefore highly likely that the 3-kinase may be a substrate for calpain both *in vivo* and *in vitro* and that this proteinase may regulate the activity of the 3-kinase during an agonist-stimulated response. Of interest is the accumulating evidence to suggest that excessive activation of calpain could play a key role in the pathology of a variety of disorders (see Wang and Yuen, 1994).

Studies performed in the absence of calpain inhibitors have provided strong evidence to support a role for Ca^{2+} ions in stimulating 3-kinase activity. This effect is mediated by the close association of the 3-kinase with calmodulin (Daniel *et al.*, 1988; Johanson *et al.*, 1988; Kimura *et al.*, 1987; Takazawa *et al.*, 1989; Yamaguchi, *et al.*, 1987) and results in an increase in the V_{max} of the enzyme (Biden and Wollheim, 1986; Renard and Poggioli, 1987; Rosenberg *et al.*, 1991; Takazawa *et al.*, 1989). To date however, the effect of Ca^{2+} ions on a 3-kinase activity prepared in the presence of calpain inhibitors has not been addressed.

The effect of Ca^{2+} ions on Ins(1,4,5)P_3 5-phosphatase activity is far more controversial. Most studies have indicated that this enzyme is unaffected by physiological changes in the concentration of Ca^{2+} ions (e.g. Biden and Wollheim, 1986; Kennedy *et al.*, 1990; Mitchell *et al.*, 1989; Shears *et al.*, 1987a and b). Other studies however, including those conducted on

smooth muscle preparations, suggest that this enzyme may be either inhibited (Wang *et al.*, 1994, bovine iris smooth muscle) or stimulated (Sasaguri *et al.*, 1985, porcine coronary artery) by Ca^{2+} ions. In view of the evidence indicating a major, early activation of the enzymes responsible for Ins(1,4,5)P_3 breakdown in agonist-stimulated BTSM, the potential for Ca^{2+} to exert a direct effect on 3-kinase or 5-phosphatase is of considerable interest. The characterisation of the 5-phosphatase and 3-kinase activities is particularly important in BTSM since this tissue appears to have an extremely high (1-4 μM) basal Ins(1,4,5)P_3 mass concentration (Challiss *et al.*, 1990; Chilvers *et al.*, 1989b) implying that a 3-kinase enzyme with a typical affinity for Ins(1,4,5)P_3 (K_m = 0.2-2 μM) may be fully active even under resting conditions.

It is apparent therefore that the enzyme kinetics of both the 3-kinase and 5-phosphatase warrant further study and experiments were undertaken to characterise the two enzymes in BTSM and, in particular, to assess the effect of Ca^{2+} ions on their activities.

4.2 CHARACTERISATION OF THE SOLUBLE INOSITOL 1,4,5-TRISPHOSPHATE 3-KINASE ACTIVITY IN BOVINE TRACHEAL SMOOTH MUSCLE

Previous studies on the Ins(1,4,5)P_3 3-kinase from a variety of tissues e.g. RINm5F cells (Biden and Wollheim, 1986), turkey erythrocytes (Morris *et al.*, 1987), rat brain (Johanson *et al.*, 1988; Moon *et al.*, 1989), guinea-pig peritoneal macrophages (Kimura *et al.*, 1987), Rat-1 fibroblast cells (Johnson *et al.*, 1989) and pig aortic smooth muscle (Yamaguchi *et al.*, 1988) indicate that this enzyme is predominantly cytosolic. The latter study showed that 94 % of the total cellular 3-kinase activity could be

located in the cytosolic fraction of the cell. BTSM cytosol was therefore utilised as a source of 3-kinase activity in the following 3-kinase enzyme studies. Calpain inhibitors were included throughout both the enzyme preparation procedures and the enzyme activity assays.

4.2.1 Determination of the optimal conditions for the study of inositol 1,4,5-trisphosphate 3-kinase activity

Preliminary experiments were designed to establish conditions suitable for defining the basic kinetic properties of the 3-kinase enzyme (i.e. a linear rate of conversion of $[^3H]Ins(1,4,5)P_3$ to $[^3H]Ins(1,3,4,5)P_4$ with minimal hydrolysis of substrate or product) and were conducted in the absence of competing unlabelled $Ins(1,4,5)P_3$. BTSM cytosol extracts (see section 2.9.1) were incubated at various dilutions with $[^3H]Ins(1,4,5)P_3$ for 0-20 min in the presence of 1 μM Ca^{2+} under conditions which inhibit 5-phosphatase activity (see section 2.9.2). The conversion of $[^3H]Ins(1,4,5)P_3$ was assessed by monitoring the production of $[^3H]InsP_4$, using AG 1-X8 Dowex columns to separate total $[^3H]InsP_3$ from $[^3H]InsP_4$. Of the various dilutions of cytosol extract chosen (25, 100 and 250-fold dilutions), the 25-fold dilution (final protein concentration = 0.329 ± 0.009 mg/ml) gave the most appropriate results (see figure 4.2.1.). Under these conditions a linear conversion of $[^3H]Ins(1,4,5)P_3$ was observed over 10 min. Since a large proportion (54 %) of the $[^3H]Ins(1,4,5)P_3$ had been metabolised by 10 min a 5 min incubation period was chosen for all subsequent experiments.

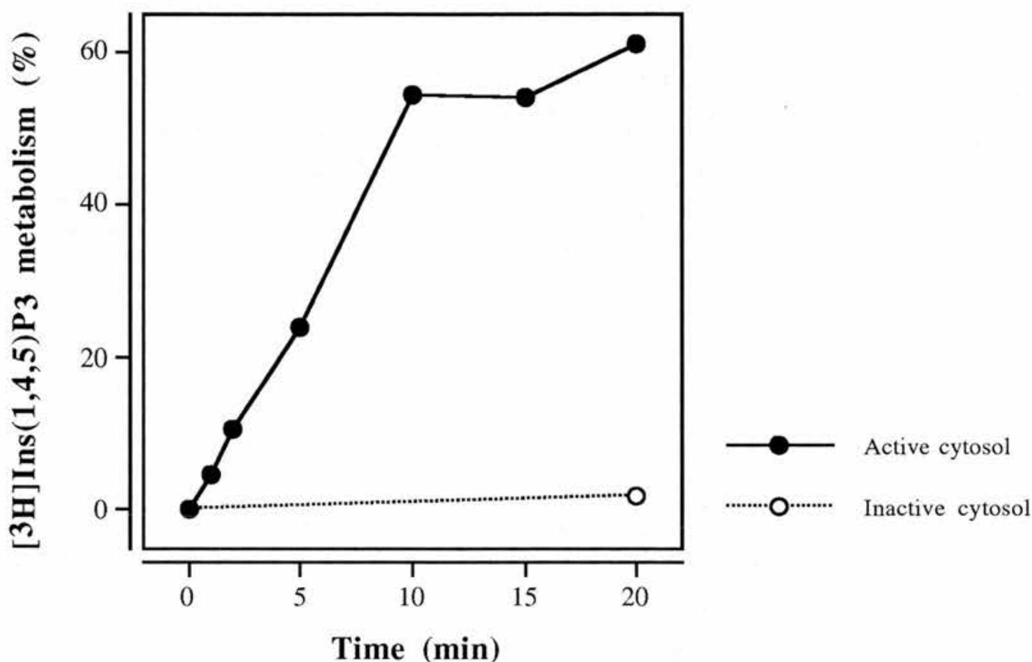

Figure 4.2.1 Metabolism of [^{3}H]Ins(1,4,5)P_3 with bovine tracheal smooth muscle cytosol extract

[^{3}H]Ins(1,4,5)P_3 (12,000 DPM) was incubated with a 25-fold dilution of BTSM cytosol extract (2.9.1) at 37 °C in a final volume of 200 µl (2.9.2). Reactions were terminated at the times indicated with 200 µl 1 M TCA and [^{3}H]InsP fractions separated from neutralised TCA extracts (2.4.2) by Dowex AG 1-X8 anion-exchange chromatography (2.6.2). The increase in radioactive label associated with the [^{3}H]InsP_4 fraction was used as an index of [^{3}H]Ins(1,4,5)P_3 metabolism by the Ins(1,4,5)P_3 3-kinase and is expressed as a percentage of the total retrieved radioactivity. Data is from a single experiment.

4.2.2 Effect of calcium ions on the cytosolic bovine tracheal smooth muscle inositol 1,4,5-trisphosphate 3-kinase

In order to assess the effect of Ca^{2+} ions on the metabolism of Ins(1,4,5)P_3 by the soluble BTSM 3-kinase, incubations were carried out as described in section 4.2.1 in the presence of 'zero', 1 μM and 1 mM $[Ca^{2+}]_{free}$. All cytosol extract preparations (diluted 25-fold) exhibited linear metabolism of [^{3}H]Ins(1,4,5)P_3 over 5 min with the three $[Ca^{2+}]_{free}$ concentrations tested (see figure 4.2.2.1). Data obtained in the presence of 1 μM Ca^{2+} was not greatly different from that obtained in the absence of Ca^{2+} (22.6 % metabolism and 18.33 % metabolism after 5 min respectively), however addition of 1 mM Ca^{2+} resulted in a stimulation of enzyme activity (43.09 % metabolism after 5 min). The isomeric composition of the [^{3}H]Ins(1,4,5)P_3 metabolites formed during the reaction was determined using H.P.L.C.; the results are shown in figure 4.2.2.2 and table 4.2.2.

Of some interest in these studies was the finding that even in the presence of 5 mM 2,3-DPG, 10 mM ATP and 20 mM $MgCl_2$ (all inhibitors of the Ins(1,4,5)P_3 5-phosphatase) a small proportion (~ 5-6 %) of the [^{3}H]Ins(1,4,5)P_3 substrate was broken down to [^{3}H]Ins(1,4)P_2. Despite this, negligible amounts of the [^{3}H]InsP_4 formed were metabolised ([^{3}H]Ins(1,3,4)P_3 and its downstream dephosphorylation metabolites accounted for less than 2 % of the total retrieved radioactivity even in the presence of 1 mM Ca^{2+} when [^{3}H]InsP_4 accumulation is maximal). Accurate determinations of 3-kinase activity could still be calculated therefore if the formation of [^{3}H]InsP_4 was used as a measure of enzyme activity rather than removal of [^{3}H]InsP_3.

The data obtained in the previous two sections (4.2.1 and 4.2.2) indicate that linear conversion of [^{3}H]Ins(1,4,5)P_3 is obtained both in the absence of

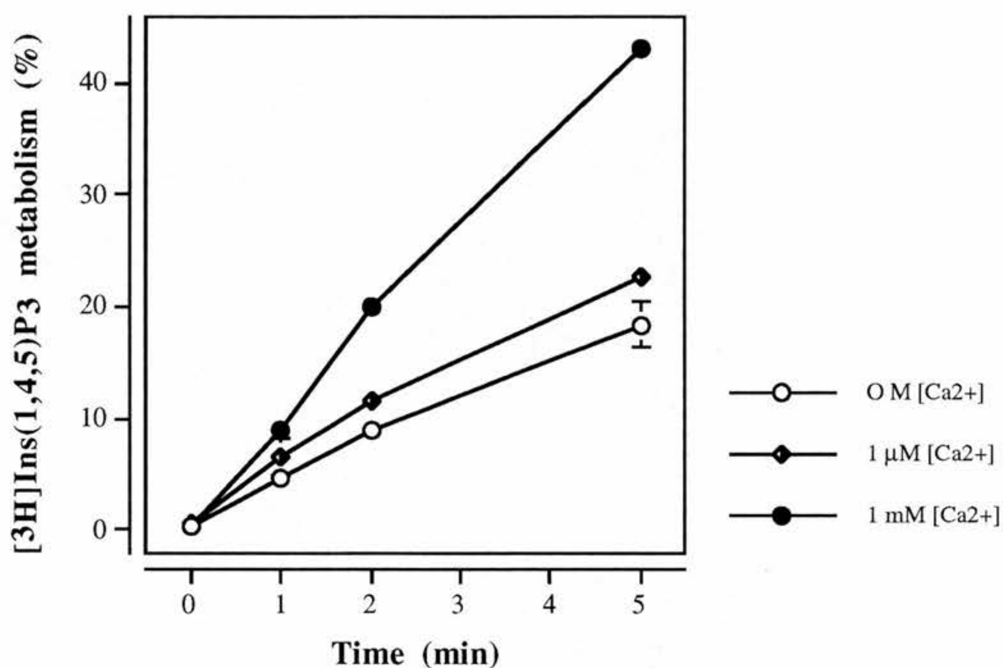

Figure 4.2.2.1 Effect of calcium ions on inositol (1,4,5)P_3 3-kinase activity
[^{3}H]Ins(1,4,5)P_3 (12,000 DPM) was incubated for the times indicated with a 25-fold dilution of BTSM cytosol extract (2.9.1) at 37 °C in the presence of 'zero', 1 μM and 1 mM $[Ca^{2+}]_{free}$ (2.9.2). Reactions were terminated with 1 M TCA after 5 min and the [^{3}H]InsP fractions in neutralised extracts (2.4.2) separated using Dowex AG 1-X8 anion-exchange chromatography (2.6.2). The increase in radioactive label associated with the [^{3}H]InsP_4 fraction was used as an index of metabolism by the Ins(1,4,5)P_3 3-kinase and is expressed as a percentage of the total retrieved radioactivity. Data represents mean ± SEM of duplicate determinations from a single experiment.

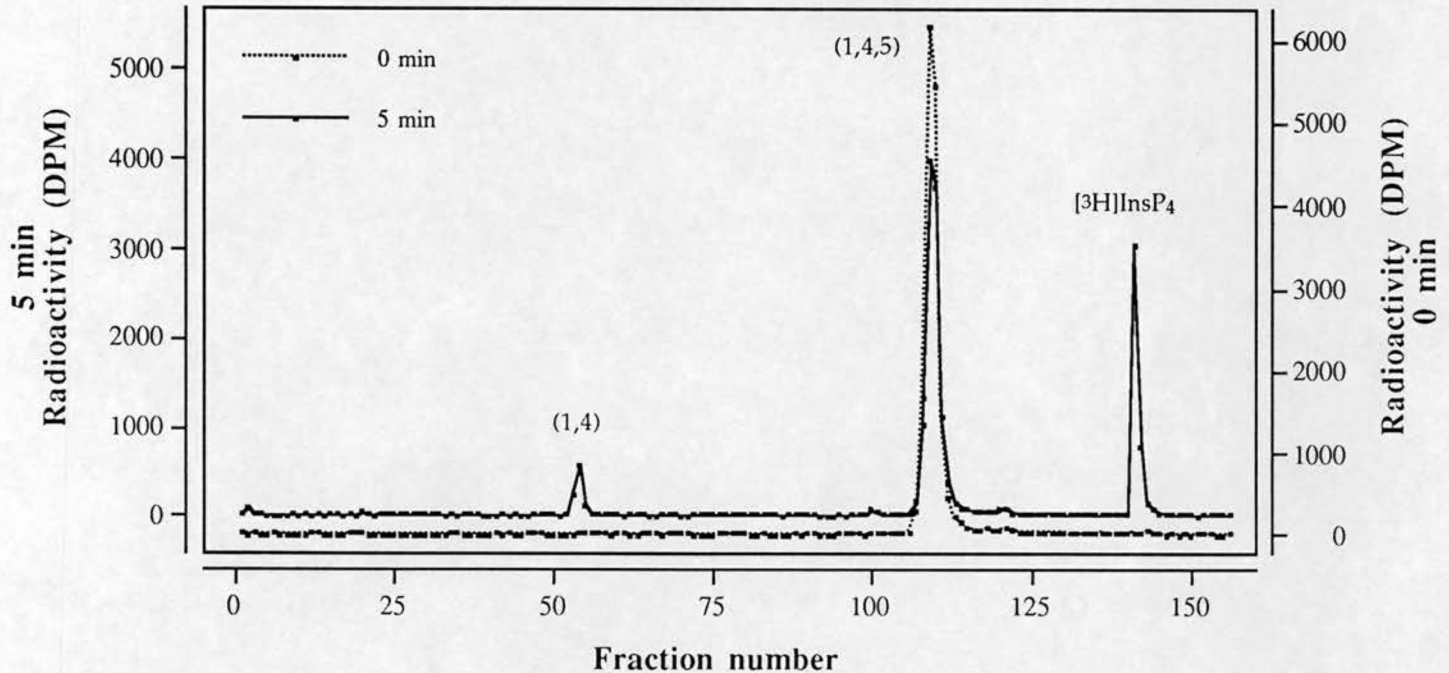

Figure 4.2.2.2 H.P.L.C. separation of individual [^{3}H]inositol polyphosphate isomers formed during inositol 1,4,5-trisphosphate 3-kinase assay

[^{3}H]Ins(1,4,5)P_3 (20,000 DPM) was incubated at 37 °C for 0 or 5 min with a 25-fold dilution of BTSM cytosol extract (2.9.1) in the presence of 1 μM $[Ca^{2+}]_{free}$ (2.9.2). Reactions were terminatedwith 200 μl 1 M TCA and the individual [^{3}H]InsPP isomers separated from neutralised extracts (2.4.2) by H.P.L.C. (2.6.3). The radioactivity associated with each [^{3}H]InsPP isomer was quantified by liquid scintillation counting.

$[^3H]Ins(x)PP$	% Total retrieved radioavtivity					
	Ca^{2+}-free		1 μM $[Ca^{2+}]$		1 mM $[Ca^{2+}]$	
	0 min	5 min	0 min	5 min	0 min	5 min
1/3	0.00	0.36	0.00	0.03	0.06	0.14
4	0.18	0.13	0.00	0.22	0.14	0.41
1,3	0.04	0.06	0.00	0.05	0.00	0.07
1,4	0.01	5.37	0.04	6.01	0.06	4.79
3,4	0.05	0.03	0.10	0.00	0.02	0.02
4,5	0.01	0.00	0.07	0.00	0.10	0.00
1,3,4	0.05	0.54	0.23	0.62	0.06	1.57
1,4,5	98.07	68.58	97.26	66.39	97.60	39.15
2,4,5	1.41	1.05	1.69	1.34	1.63	0.93
$[^3H]InsP_4$	0.19	23.88	0.61	25.34	0.33	52.91
Total retrieved radioactivity (DPM)	15,503	15,398	16,363	15,878	16,172	16,205

Table 4.2.2 $[^3H]$inositol polyphosphate isomers formed during $[^3H]$inositol 1,4,5-trisphosphate 3-kinase incubations
$[^3H]Ins(1,4,5)P_3$ (20,000 DPM) was incubated at 37 °C for 0 or 5 min with a 25-fold dilution of cytosol extract (2.9.1) in the presence of 'zero', 1μM or 1 mM $[Ca^{2+}]_{free}$ (2.9.2). Reactions were terminated with 200 μl 1 M TCA and $[^3H]$InsPP isomers separated from neutralised extracts (2.4.2) by H.P.L.C. (2.6.3). The radioactivity associated with each $[^3H]$InsPP isomer was quantified by liquid scintillation counting and is expressed as a percentage of the total radioactivity retrieved from each sample. Data was obtained from a single experiment.

Ca^{2+} ions and in the presence of either 1 μM or 1 mM $[Ca^{2+}]_{free}$ for up to 5 min. Under these conditions there is minimal breakdown of [^{3}H]Ins(1,3,4,5)P_4. Hence a 5 min incubation with a 25-fold dilution of cytosol extract provides appropriate conditions for the kinetic analysis of the soluble BTSM Ins(1,4,5)P_3 3-kinase.

4.2.3 Determination of kinetic parameters of soluble inositol 1,4,5-trisphosphate 3-kinase

[^{3}H]Ins(1,4,5)P_3 was incubated at 37 °C with a 25-fold dilution of cytosol extract in the presence of 0.1-5 μM competing unlabelled Ins(1,4,5)P_3 for 5 min, in the presence of 'zero', 1 μM and 1 mM Ca^{2+}. The initial reaction velocity (V_0) was calculated for each substrate concentration ([S]) and is illustrated on an Eadie-Hofstee (V_0 vs V_0/[S]) plot in figure 4.2.3. A computer-generated line of best fit was drawn through the data points for each Ca^{2+} concentration to facilitate accurate calculation of the K_m and V_{max} values of the enzyme (y axis intercept = V_{max}; gradient = -K_m) and these parameters are listed in table 4.2.3. Although the data indicate a trend towards an increase in the V_{max} of the 3-kinase when the $[Ca^{2+}]_{free}$ is raised from 0 to 1 mM this effect is not statistically significant (two-tailed, unpaired student's t-test). There was no statistically significant difference in either the K_m or V_{max} values of the soluble BTSM 3-kinase obtained in the absence of Ca^{2+} as compared to those obtained in the presence of either 1 μM or 1 mM $[Ca^{2+}]_{free}$.

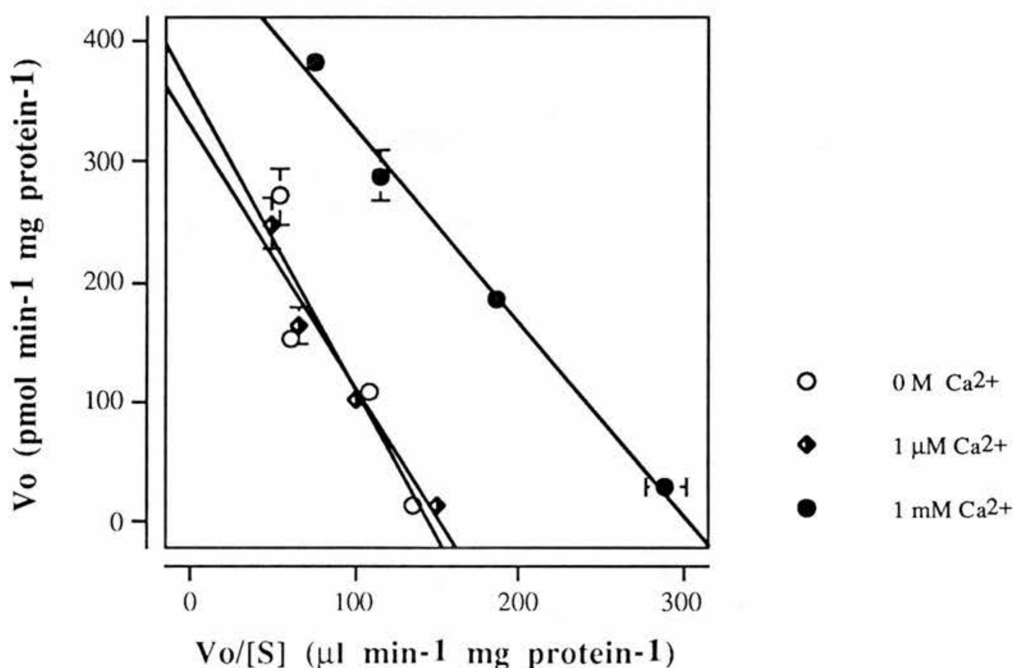

Figure 4.2.3 Eadie-Hofstee analysis of BTSM Ins(1,4,5)P_3 3-kinase activity [^{3}H]Ins(1,4,5)P_3 (12,000 DPM) was incubated at 37 °C with a 25-fold dilution of BTSM cytosol extract (2.9.1) in the presence of varying concentrations (0.1-5 μM) of competing unlabelled Ins(1,4,5)P_3 (2.9.2). Reactions were terminated after 5 min with 200 μl 1 M TCA and [^{3}H]InsP fractions separated from neutralised extracts (2.4.2) using Dowex AG 1-X8 anion-exchamge chromatography (2.6.2). The increase in radioactive label associated with [^{3}H]InsP_4 was used as an index of metabolism by the Ins(1,4,5)P_3 3-kinase and the reaction velocity calculated for each substrate concentration. Results represent the mean ± SEM of three or four separate experiments, each performed in duplicate, using three separate BTSM cytosol extract preparations.

$[Ca^{2+}]_{free}$	K_m (μM)	V_{max} (pmol min^{-1} mg $protein^{-1}$)
0	2.49 ± 0.45	361.92 ± 55.52
1 μM	2.07 ± 0.24	322.83 ± 41.53
1 mM	1.60 ± 0.04	488.53 ± 16.89

Table 4.2.3 Effect of calcium ions on the K_m and V_{max} values of the soluble bovine tracheal smooth muscle inositol 1,4,5-trisphosphate 3-kinase activity

$[^3H]Ins(1,4,5)P_3$ (12,000 DPM) and unlabelled $Ins(1,4,5)P_3$ (0.1-5 μM) were incubated with BTSM cytosol extract (2.9.1) (diluted 25-fold) at 37 °C for 5 min in a final volume of 200 μl (see section 2.9.2). Reactions were terminated with 200 μl 1 M TCA and [³H]InsP fractions were separated from neutralised TCA extracts (2.4.2) using Dowex AG 1-X8 anion-exchange chromatography (2.6.2). The increase in radioactive label associated with the $[^3H]InsP_4$ fraction was used as an index of $[^3H]Ins(1,4,5)P_3$ 3-kinase activity. K_m and V_{max} values were determined using Eadie-Hofstee plots. Results represent the mean ± SEM for three or four separate experiments, each performed in duplicate, using three different BTSM cytosol extract preparations.

4.3 CHARACTERISATION OF THE SOLUBLE AND PARTICULATE INOSITOL 1,4,5-TRISPHOSPHATE 5-PHOSPHATASE IN BOVINE TRACHEAL SMOOTH MUSCLE

In the majority of tissues studied to date 5-phosphatase enzyme activity has been found to be predominantly associated with particulate fractions of cells, e.g. in bovine brain (Erneux *et al.*, 1989), rat brain (Hansen *et al.*, 1987; Moon *et al.*, 1989), rat liver (Joseph *et al*, 1985; Seyfred *et al.*, 1984; Shears *et al.*, 1987a, 1988; Takimoto *et al.*, 1989), turkey and human erythrocytes (Morris *et al.*, 1987; Shears *et al.*, 1987b) and guinea-pig

macrophages (Kukita *et al.*, 1986). There are however, some notable exceptions: the 5-phosphatases from human platelets (Connolly *et al.*, 1985), rabbit peritoneal neutrophils (Kennedy *et al.*, 1990) and porcine skeletal muscle (Hansbro *et al.*, 1994) are all predominantly soluble while the activity in porcine coronary artery (Sasaguri *et al.*, 1985) and bovine iris smooth muscle (Wang *et al.*, 1994) appear to be fairly evenly distributed between the soluble and particulate fractions of the cell. Given this diversity of 5-phosphatase enzyme distribution, the BTSM Ins(1,4,5)P_3 5-phosphatase activity was studied in both a cytosolic and a detergent-solubilised membrane fraction. Nonidet P40 was the detergent chosen to solubilise the membrane-associated BTSM 5-phosphatase since it has been utilised successfully in the solubilisation of 5-phosphatases from bovine testes (Hodgkin *et al.*, 1991) and rat liver microsomes (Takimoto *et al.*, 1989).

4.3.1 Determination of optimal conditions for the study of inositol 1,4,5-trisphosphate 5-phosphatase activity

Preliminary experiments were conducted using both cytosolic and solubilised membrane preparations in order to determine conditions in which linear metabolism of Ins(1,4,5)P_3 could be achieved. BTSM cell extracts were incubated at various dilutions with [^{3}H]Ins(1,4,5)P_3 for 0-20 min in the absence of added ATP to prevent phosphorylation (see section 2.10.2) and the conversion of [^{3}H]Ins(1,4,5)P_3 to [^{3}H]Ins(1,4)P_2 was monitored by measuring the increase in label into [^{3}H]Ins and [^{3}H]InsP_{1-2} (separated by Dowex AG 1-X8 anion-exchange chromatography). A 50-fold dilution of the cytosol extract preparation (final protein concentration = 0.167 ± 0.017 mg/ml) provided linear metabolism for approximately 10 min, while a 75-fold dilution of the solubilised

membrane extract (final protein concentration = 0.078 ± 0.004 mg/ml) was required for linear metabolism over 5 min (figure 4.3.1). Since a 5 min incubation of $[^3H]Ins(1,4,5)P_3$ with the membrane extract resulted in a high percentage (63.85 ± 0.85 %) of substrate being metabolised, the incubation period was reduced to 2 min for subsequent experiments.

4.3.2 Effect of calcium ions on the soluble and membrane-associated inositol 1,4,5-trisphosphate 5-phosphatase activities

In order to address the effect of Ca^{2+} ions on the metabolism of $[^3H]Ins(1,4,5)P_3$ by the soluble and membrane-associated BTSM 5-phosphatases, incubations were carried out as described in section 4.3.1 in the presence of varying $[Ca^{2+}]_{free}$ (0-1 mM). The results are illustrated in figure 4.3.2. It would appear that Ca^{2+} ions have very little effect over a physiologically relevant range, however a statistically significant increase in both the soluble and membrane-associated 5-phosphatase activity was observed in the presence of 1 mM $[Ca^{2+}]_{free}$.

4.3.3 Determinations of kinetic parameters of soluble and membrane-associated inositol 1,4,5-trisphosphate 5-phosphatase

$[^3H]Ins(1,4,5)P_3$ was incubated at 37 °C with either BTSM cytosol or membrane extract under conditions which exhibit linear substrate metabolism (see section 4.3.1), in the presence of competing unlabelled $Ins(1,4,5)P_3$ and varying $[Ca^{2+}]_{free}$ ('zero', 1 μM and 1 mM). The results are illustrated as Eadie-Hofstee plots (figure 4.3.3) and the calculated K_m and V_{max} values for the soluble and particulate 5-phosphatase activities are shown in table 4.3.3. The K_m values determined for both the soluble and particulate enzymes were very similar whereas the V_{max} value was much greater for the particulate enzyme. Increasing $[Ca^{2+}]_{free}$ from 'zero' to 1 μM did not affect the K_m or V_{max} values of either the soluble or

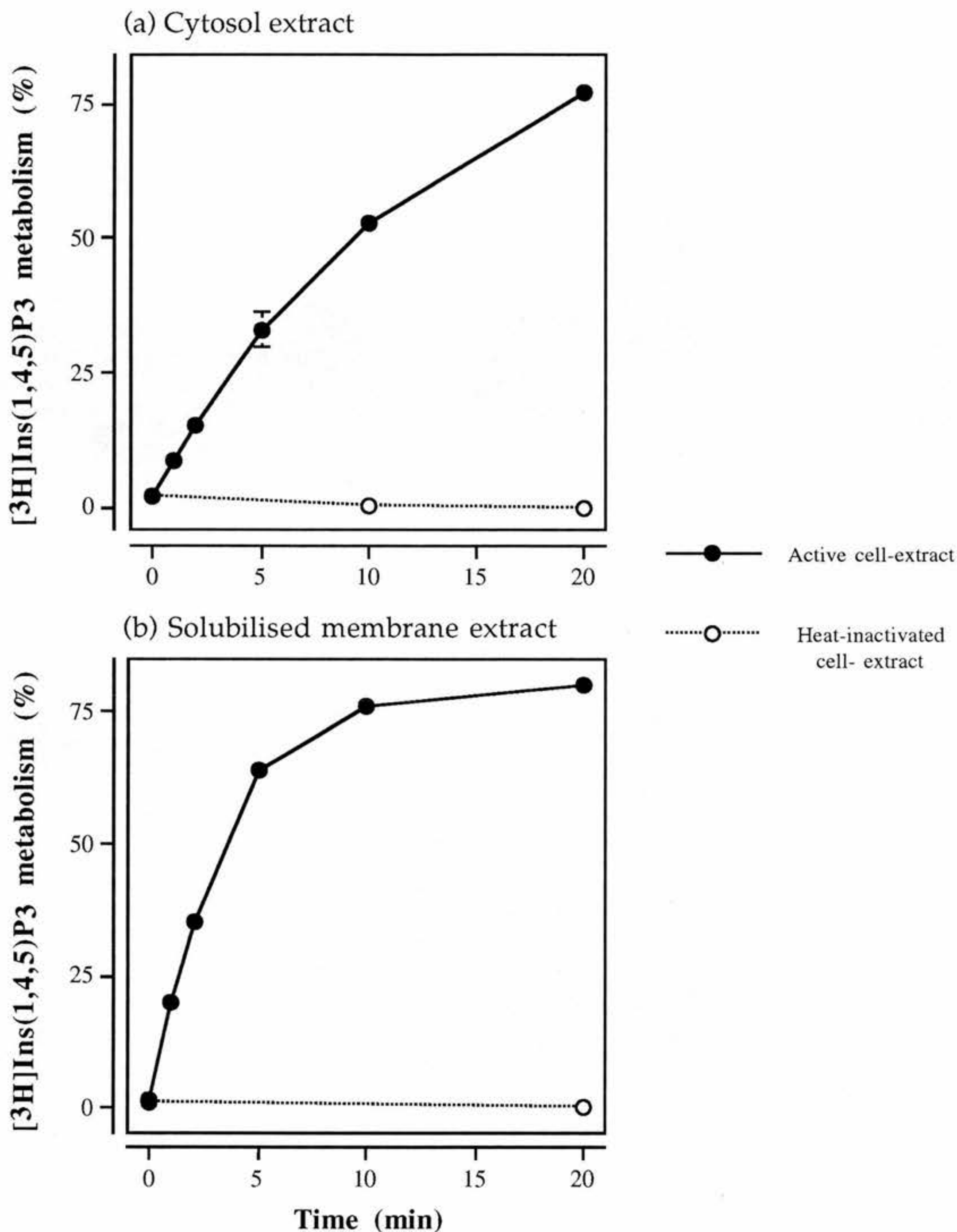

Figure 4.3.1 [^{3}H]Ins(1,4,5)P_3 metabolism by BTSM 5-phosphatase
[^{3}H]Ins(1,4,5)P_3 (12,000 DPM) was incubated at 37 °C with a 50-fold or 75-fold dilution of BTSM cytosol or membrane extract (2.10.1) respectively (2.10.2). Reactions were terminated at the times indicated and the [^{3}H]InsP fractions in neutralised extracts (2.4.2) separated by Dowex chromatography (2.6.2). Data represents the mean ± SEM of a single experiment performed in triplicate. Where not shown, error bars lie within symbols.

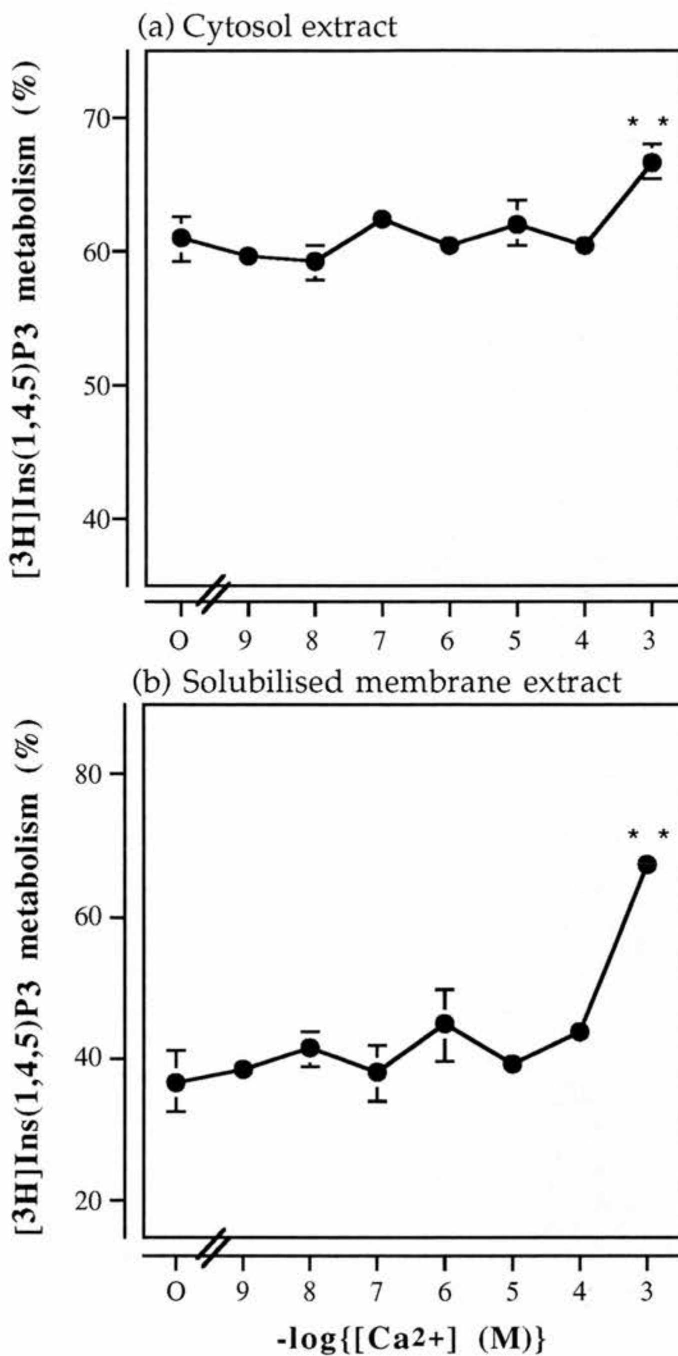

Figure 4.3.2 Effect of Ca^{2+} ions on the BTSM $Ins(1,4,5)P_3$ 5-phosphatase
$[^3H]Ins(1,4,5)P_3$ (12,000 DPM) was incubated at 37 °C with a 50- or 75-fold dilution of BTSM cytosol or membrane extract (2.10.1) respectively (2.10.2). Reactions were terminated after (a) 10 or (b) 2 min and $[^3H]$InsP fractions in neutralised TCA extracts (2.4.2) separated by Dowex chromatography (2.6.2). Data represent the mean ± SEM of (a) three or (b) two experiments, each performed in triplicate. Two or three separate solubilised membrane or cytosol extracts were used respectively.
* * Denotes $p < 0.01$ for comparisons with data obtained in Ca^{2+}-free buffer (two-tailed, unpaired students' t-test).

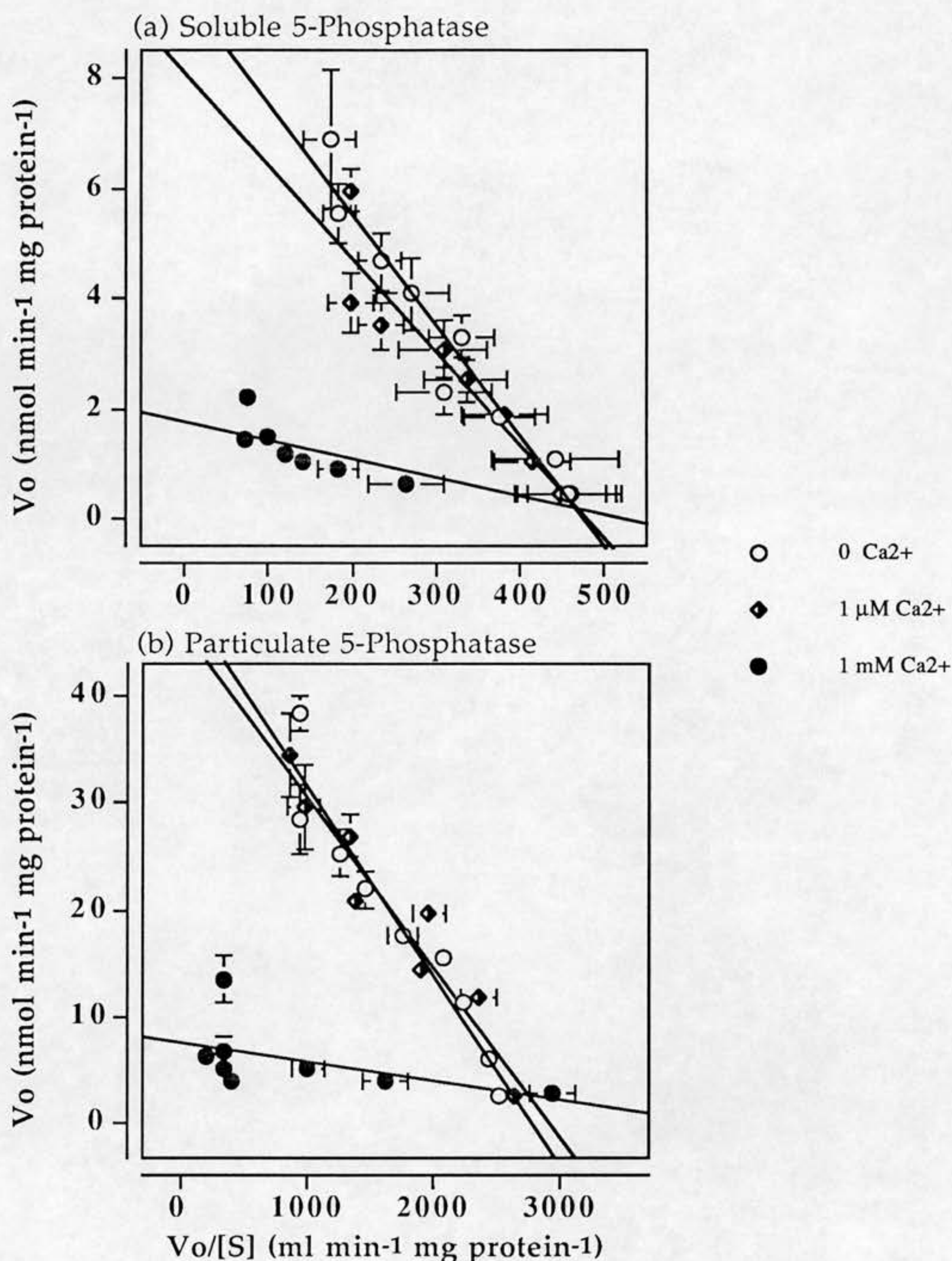

Figure 4.3.3 Eadie-Hofstee analysis of BTSM 5-phosphatase activities
$[^3H]Ins(1,4,5)P_3$ (12,000 DPM) was incubated at 37 °C with 50- or 75-fold dilutions of BTSM cytosol or membrane extract (2.10.1) respectively in the presence of 1-40 μM competing unlabelled $Ins(1,4,5)P_3$ (2.10.3). Reactions were terminated after (a) 10 or (b) 2 min and $[^3H]InsP$ fractions in TCA extracts (2.4.2) separated by Dowex chromatography (2.6.2). Data represents the mean ± SEM of three separate experiments, each performed in duplicate, using three separate cytosol and membrane extract preparations

$[Ca^{2+}]_{free}$	Soluble enzyme K_m (μM)	Soluble enzyme V_{max} (nmol min^{-1} mg $protein^{-1}$)	Particulate enzyme K_m (μM)	Particulate enzyme V_{max} (nmol min^{-1} mg $protein^{-1}$)
0	18.7 ± 1.9	9.05 ± 1.07	17.7 ± 2.2	50.02 ± 6.09
1 μM	16.7 ± 1.5	8.04 ± 1.47	14.7 ± 1.9	44.86 ± 4.70
1 mM	3.3 ± 1.2 *	2.10 ± 0.15 *	1.3 ± 0.7 *	6.97 ± 1.07 *

Table 4.3.3 Effect of calcium ions on the K_m and V_{max} values of the soluble and particulate bovine tracheal smooth muscle inositol 1,4,5-trisphosphate 5-phosphatase activities

[^{3}H]Ins(1,4,5)P_3 (12,000 DPM) was incubated with 50- or 75-fold dilutions of BTSM cytosol or membrane extract (2.10.1) respectively in the presence of 1-40 μM competing unlabelled Ins(1,4,5)P_3 (2.10.3). Reactions were terminated after (a) 10 or (b) 2 min and the [^{3}H]InsP fractions in TCA extracts (2.4.2) separated by Dowex AG 1-X8 anion-exchange chromatography (2.6.2). Increases in radioactivity associated with [^{3}H]Ins and [^{3}H]InsP_{1-2} were used as an index of 5-phosphatase acitivty. K_m and V_{max} values were determined using Eadie-Hofstee plots. Data represents the mean ± SEM from three separate experiments, each performed in duplicate, using three separate BTSM cytosol or membrane extracts. * Denotes $p < 0.05$ for comparisons with data obtained in Ca^{2+}-free buffer (two-tailed, paired student's t-test).

particulate 5-phosphatase. However, Eadie-Hofstee analysis demonstrated a statistically significant decrease in both the K_m and V_{max} of the enzymes in the presence of 1 mM Ca^{2+}. It is unclear if this reflects a real regulation of 5-phosphatase activity by Ca^{2+} since the Eadie-Hofstee plot deviates from a straight-line under these conditions (indicating non-standard Michaelis-Menten kinetics). These latter results may reflect a degree of co-operativity exhibited by the 5-phosphatase in the presence of 1 mM Ca^{2+} or may simply be due to indirect effects of the unphysiologically high Ca^{2+} concentration used.

4.4 DISCUSSION

The kinetic parameters of the Ins(1,4,5)P_3 3-kinase an 5-phosphatase enzymes have been determined in detail in cell free extracts of BTSM. Ca^{2+} ions exerted very little effect on the 3-kinase and 5-phosphatase activities over a physiologically relevant concentration range, although both enzymes appeared to be stimulated in the presence of a much higher (1 mM) Ca^{2+} concentration. The enzymes were subsequently characterised further in order to obtain K_m and V_{max} values for Ins(1,4,5)P_3 metabolism at 'zero', 1 μM and 1 mM $[Ca^{2+}]_{free}$ concentrations.

The 3-kinase is generally understood to be a Ca^{2+}-regulated enzyme with increases in $[Ca^{2+}]$ causing an increase in the V_{max} of the enzyme via its interaction with calmodulin. Activation appears to occur across a narrow range of Ca^{2+} concentrations (Biden *et al.*, 1988a; Imboden and Pattison, 1987; Morris *et al.*, 1987; Ryu *et al.*, 1987), a property that is a common feature of calmodulin-stimulated enzymes. However, maximum stimulation of the 3-kinase by Ca^{2+} is generally only 2-3 fold, provided

$[Ca^{2+}]_{free}$ is at least 1 μM (Balla *et al.*, 1988; Biden *et al.*, 1988a; Biden and Wollheim, 1986; Johanson *et al.*, 1988; Kimura *et al.*, 1987; Morris *et al.*, 1987; Renard and Poggioli, 1987) and hence the physiological significance of Ca^{2+}-mediated regulation may need to be questioned. The data obtained from the experiments described in this chapter, performed in the presence of calpain inhibitors, argue that the intact soluble BTSM 3-kinase kinetic parameters are unaffected by increases in $[Ca^{2+}]$ over a physiological range. An increase in V_{max} (from 362 to 489 pmol min^{-1} mg $protein^{-1}$) was observed in the presence of 1 mM $[Ca^{2+}]_{free}$ but found to be statistically insignificant. Experiments conducted in pig aortic smooth muscle (Yamaguchi *et al.*, 1987), guinea-pig peritoneal macrophages (Kimura *et al.*, 1987), human platelets (Daniel *et al.*, 1988), insulin-secreting RINm5F cells (Biden *et al.*, 1987) and rat brain (Johanson *et al.*, 1988) indicate that the purified 3-kinase is only regulated by Ca^{2+} ions in the presence of added exogenous calmodulin. The experiments described here however, utilised a crude BTSM cytosol extract as a source of 3-kinase activity; the enzyme would therefore be expected to have access to endogenous BTSM calmodulin.

It is possible that calpain acts as a regulator of the 3-kinase *in vivo* by selective proteolysis of the enzyme. Even when cell-extracts are prepared in the presence of a battery of protease inhibitors many contain a number of differently sized peptides which exhibit 3-kinase activity (Choi *et al.*, 1990; Lee *et al.*, 1990; Lin *et al.*, 1990; Takazawa *et al.*, 1989, 1991a). Indeed Lee *et al.*, (1990) identified several calpain proteolysis products of the rat brain 3-kinase which surprisingly retained catalytic activity; moreover, the specific activity of the 40 KDa enzyme obtained in this study was slightly higher than that of the parent 53 KDa enzyme. In summary therefore, when prepared in the presence of calpain-inhibitors, BTSM

cytosol extract contains a 3-kinase activity which does not appear to be modulated by Ca^{2+}. Clearly this does not preclude a regulation of the 3-kinase by Ca^{2+} ions *in vivo*: calpain may be activated during the agonist-stimulated response and may partially degrade the 3-kinase to form smaller proteins which retain enzyme activity but have altered regulatory properties; alternatively Ca^{2+} may stimulate other enzymes (e.g. PKC) which could in turn regulate 3-kinase activity.

The K_m calculated for the soluble BTSM 3-kinase was 2.49 ± 0.45 μM. This figure is very comparable to that obtained in the same tissue by Chilvers (1991). K_m values determined for the 3-kinase from other sources are shown in table 4.4.1. In the majority of tissues a K_m value of between 0.2 and 2 μM was observed; the K_m for the BTSM 3-kinase hence lies at the top of this range and therefore this enzyme can be considered to have a relatively low affinity for Ins(1,4,5)P_3. In a few of the tissues studied however, a higher range of K_m values, between 5 and 11 μM, were determined. It is possible that these variations could represent alternative isozymes. The high resting concentration of Ins(1,4,5)P_3 in BTSM may explain the relatively low affinity of the BTSM 3-kinase for Ins(1,4,5)P_3, as this may be required in order to prevent its constitutive activation.

It is not generally possible to compare V_{max} values from different studies, since this parameter is dependent on the specific activity of the enzyme and therefore the purity of the enzyme preparation. The BTSM cytosol extract utilised in the present study however, was prepared according to a similar protocol used by Chilvers (1991), and hence the V_{max} value obtained by this author (385 pmol min^{-1} mg $protein^{-1}$) can be readily compared to that described here (362 ± 56 pmol min^{-1} mg $protein^{-1}$).

Source of 3-kinase activity	K_m for $Ins(1,4,5)P_3$ (μM)	Reference
rabbit tracheal smooth muscle	5.0	Rosenberg *et al.*, 1991
porcine aortic smooth muscle	0.4	Yamguchi *et al.*, 1988
rat heart	5.61	Renard and Poggioli, 1987
porcine skeletal muscle	0.42	Foster *et al.*, 1994
bovine brain	0.7	Ryu *et al.*, 1987
bovine brain	1-2	Takazawa *et al.*, 1989
rat brain	1.3 *	Sim *et al.*, 1990
rat brain	11	Takazawa *et al.*, 1990a
rat brain	8.8	Takazawa *et al.*, 1990b
rat brain	0.21	Johanson *et al.*, 1988
rat brain	0.6	Irvine *et al.*, 1986
human hippocampus	1.6	Takazawa *et al.*, 1991b
human platelets	0.76	Lin *et al.*, 1990
bovine adrenal cortex	0.4	Balla *et al.*, 1988
human Jurkat cells	0.2	Imboden and Pattison, 1987
RINm5F cells	1.5	Biden *et al.*, 1986

Table 4.4.1 Affinity of 3-kinase enzymes for inositol 1,4,5-trisphosphate

* This study utilised calpain inhibitors during the enzyme purification procedure.

The kinetic parameters of both the soluble and particle-associated BTSM 5-phosphatases were found to be unaffected by physiologically relevant Ca^{2+} concentrations, however the K_m and V_{max} values were both dramatically decreased in the presence of 1 mM $[Ca^{2+}]_{free}$. These results are in accordance with data obtained in human platelets (Mitchell *et al.*, 1989), rabbit peritoneal neutrophils (Kennedy *et al.*, 1990), human placenta (Laxminarayan *et al.*, 1993), and rat liver (Takimoto *et al.*, 1989) where no effect of Ca^{2+} was seen over a physiological Ca^{2+} range but gradual inhibition was observed at higher Ca^{2+} concentrations. The stimulation of the BTSM Ins(1,4,5)P_3 5-phosphatase activity noted at 1 mM $[Ca^{2+}]_{free}$ in the absence of added competing unlabelled Ins(1,4,5)P_3 (see section 4.3.2) probably reflects the increased affinity of this enzyme for Ins(1,4,5)P_3 (i.e. the lowered K_m), since an extremely low concentration of substrate (~1-1.5 nM) is available to the enzyme under these conditions and the V_{max} is therefore highly unlikely to be attained.

The K_m values obtained for Ins(1,4,5)P_3 dephosphorylation by both the soluble and particle-associated 5-phosphatase enzymes are very similar suggesting that these two enzyme activities may represent the same protein. These results are reminiscent of previous data in which the kinetic and physical properties of the Type Ia soluble 5-phosphatase and particulate 5-phosphatase activities were found to be remarkably similar (Erneux *et al.*, 1989; Takimoto *et al.*, 1989). It is likely therefore that the BTSM 5-phosphatase activities identified here in BTSM are of a Type Ia classification (i.e. hydrolyse both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4). It is clear that an enzyme exists in BTSM capable of converting Ins(1,3,4,5)P_4 to Ins(1,3,4)P_3 (see chapter 3); whether this 5-phosphatase activity is the same as that characterised in the present chapter remains to be

determined. It would be interesting to carry out further kinetic experiments to characterise the Ins(1,3,4,5)P_4 5-phosphatase.

The much higher V_{max} value of the Ins(1,4,5)P_3 5-phosphatase in BTSM membrane extracts than in cytosol extracts is indicative of a concentration of the enzyme in the particulate fraction. In agreement, the 5-phosphatase has been found to be predominantly particulate in most systems studied to date, and in liver has been localised to the cytoplasmic face of the plasma membrane (Seyfred *et al.*, 1984; Shears *et al.*, 1987b, 1988; Joseph *et al.*, 1985).

The K_m values determined for the 5-phosphatase from various sources are shown in table 4.4.2; the affinity of the enzyme for Ins(1,4,5)P_3 is highly variable with K_m values ranging from 1 to 95 μM. Although the K_m for Ins(1,4,5)P_3 metabolism determined in BTSM (~12-20 μM) is in close agreement with those observed in rat liver membranes, human erythrocyte membranes, human platelet cytosol, rabbit peritoneal neutrophil cytosol and rat pancreatic islets, it is dramatically lower than determined in rabbit tracheal smooth muscle and slightly lower than that of the bovine iris smooth muscle 5-phosphatase. Where Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 metabolism by the 5-phosphatase have been studied in the same tissue, the affinity of the enzyme for Ins(1,3,4,5)P_3 is always much higher than for Ins(1,4,5)P_3 (with the exception of soluble Type II isozymes, classified as such because they do not readily metabolise Ins(1,3,4,5)P_4); the V_{max} values however, are much higher for Ins(1,4,5)P_3, generally 10-15 fold higher, with the notable exception of the soluble type I enzyme from porcine skeletal muscle which has a 28-fold greater V_{max} for Ins(1,4,5)P_3 than for Ins(1,3,4,5)P_4.

Source of 5-phosphatase activity	K_m ($InsP_3$) (μM)	K_m ($InsP_4$) (μM)	V_{max} ($InsP_3$):V_{max} ($InsP_4$) ratio	Reference
rabbit tracheal smooth muscle	95.3			Rosenberg *et al.*, 1991
bovine iris smooth muscle	28.6			Wang *et al.*, 1994
porcine skeletal muscle: soluble Type Ia	8.9	1.1	28	Hansbro *et al.*, 1994
soluble Type II	71.4			Hansbro *et al.*, 1994
particulate	46.3	1.9		Foster *et al.*, 1994
bovine brain: soluble Type Ia	11	1	11.1	Erneux *et al.*, 1989
soluble Type II	72			Erneux *et al.*, 1989
particulate	10	0.9	11	Erneux *et al.*, 1989
rat brain: soluble Type Ia	6	0.8	11.5	Takimoto *et al.*, 1989
soluble Type II	8	130	0.02	Takimoto *et al.*, 1989
rat brain: soluble Type Ia	3.0	0.8	12.2	Hansen *et al.*, 1987
soluble Type II	18.3	> 150		Hansen *et al.*, 1987
human frontal cortex	65			De Smedt *et al.*, 1994
canine thyroid	28.3	3.4	11.4	Verjans *et al.*, 1994
rat liver membrane fractions	~15		10-15	Shears *et al.*, 1988
rat liver (particulate fraction)	5	0.8	11.1	Takimoto *et al.*, 1989
rat liver: plasma membrane	1.4			Joseph *et al.*, 1985
cytosol	1.0			Joseph *et al.*, 1985
bovine testis (particulate)	22	1.9		Hodgkin *et al.*, 1994
human erythrocytes (particulate)	14	1.1		Hodgkin *et al.*, 1994
human erythrocyte membranes	25			Downes *et al.*, 1982
human platelets (75 kDa soluble Type Ib)	24	7.5		Mitchell *et al.*, 1989
human platelets (40 kDa soluble)	17			Connolly *et al.*, 1986
human platelets (soluble fraction)	30			Connolly *et al.*, 1985
RINm5F cells (particulate fraction)	~30			Biden and Wollheim, 1986
rabbit peritoneal neutrophils (soluble)	18	(K_i=1.0 μM)		Kennedy *et al.*, 1990
human placental membranes	5	1.2		Laxminarayan *et al.*, 1993
rat pancreatic islets	16			Rana *et al.*, 1985

Table 4.4.2 Kinetic parameters of 5-phosphatase activities from various sources

($InsP_3$ and $InsP_4$ represent Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 respectively).

The $Ins(1,4,5)P_3$ 3-kinase and 5-phosphatase enzymes have been characterised in BTSM cell free extracts. Although the affinity of the BTSM 3-kinase for $Ins(1,4,5)P_3$ is relatively low compared to the enzyme from other tissues, it is still much greater than that of the BTSM 5-phosphatase; the 3-kinase pathway therefore is likely to be the favoured route of $Ins(1,4,5)P_3$ metabolism at low concentrations of this second messenger. The 5-phosphatase however, has a greater capacity to metabolise $Ins(1,4,5)P_3$ than the 3-kinase (as exemplified by their V_{max} values). These kinetic properties of the $Ins(1,4,5)P_3$ metabolising enzymes may explain, at least in part, the observed flux of $Ins(1,4,5)P_3$ through the 3-kinase and 5-phosphatase pathways following agonist stimulation. At early time-points of the agonist-stimulated response the elevated $Ins(1,4,5)P_3$ concentration would be expected to favour metabolism through the 5-phosphatase pathway whereas after 30 sec, when the $Ins(1,4,5)P_3$ concentration has decreased, the 3-kinase enzyme with its higher affinity for $Ins(1,4,5)P_3$ could be activated. Surprisingly both enzymes appear to be relatively unaffected by Ca^{2+} ions over a physiologically relevant range, however it is possible that Ca^{2+} may have an indirect effect on their activity *in vivo* by modulating the activity of other enzymes (e.g. calpain and PKC) which may interact with the 3-kinase and 5-phosphatase.

CHAPTER FIVE
REGULATION OF INOSITOL POLYPHOSPHATE METABOLISM IN BOVINE TRACHEAL SMOOTH MUSCLE

5.1 INTRODUCTION

As discussed in chapters three and four, a host of different regulatory factors could influence the routing of Ins(1,4,5)P_3 metabolism through the 3-kinase and 5-phosphatase pathways. The experiments described in this chapter were designed to address some of the potential mechanisms underlying this regulation. One obvious but important factor which may influence Ins(1,4,5)P_3 metabolism is the concentration of this second messenger itself. The enzyme kinetic experiments detailed in chapter four show that the 3-kinase from BTSM has a much greater affinity for Ins(1,4,5)P_3 than does the 5-phosphatase and hence at low concentrations of Ins(1,4,5)P_3 the 3-kinase pathway would be expected to be the preferred route of removal for this compound. To explore this hypothesis further, experimental conditions were examined which could (or might be predicted to) reduce the level of agonist-stimulated inositol phosphates formed and hence the level of Ins(1,4,5)P_3 accumulation. The effects of stimulation with an alternative PIC-linked agonist aside from CCh, activation of PKC with phorbol dibutyrate (PDBu), and the addition of β_2-adrenoceptor agonists or glucocorticosteroids on the accumulation of [^{3}H]InsPs was examined. The rationale behind these experiments is discussed in more detail in sections 5.2-5. In addition to studying the effects of a reduced Ins(1,4,5)P_3 concentration, the differing assay conditions also enabled the function of other possible regulatory

processes to be addressed concurrently. For example, the use of two different agonists could highlight the potential for receptor-mediated and/or G-protein-mediated regulation of Ins(1,4,5)P_3 metabolism. There is very little data available regarding the effects of PKC activation on [^{3}H]InsP accumulation in ASM, and hence the data obtained in the presence of PDBu is of particular interest, allowing the role of PKC in the regulation of InsP formation and metabolism in this tissue to be examined. In addition treatment with salmeterol, a long-acting β_2-adrenoceptor agonist, provides an important opportunity to explore the relative importance of β_2-adrenoceptor-mediated inhibition of InsP formation to the relaxation of ASM. Furthermore, the use of both β_2-adrenoceptor agonists and the glucocorticosteroid dexamethasone may provide some insight into the potential interaction of other hormones and second messenger pathways with the InsP response in BTSM.

5.2 COMPARISON OF CARBACHOL- AND HISTAMINE-STIMULATED INOSITOL POLYPHOSPHATE ACCUMULATION IN BTSM

Phosphoinositide hydrolysis is a mode of signal transduction utilised by an extensive range of eukaryotic cell types in response to a wide variety of stimuli (see Berridge, 1987; Berridge and Irvine, 1989; Rana and Hokin, 1990). The pattern, regulation and localisation of inositol polyphosphate production is therefore likely to vary considerably in response to different stimuli to allow this common signalling pathway to generate its vast array of discrete effector events. In order to address whether Ins(1,4,5)P_3 metabolism, or the pattern of InsPP accumulation differs following stimulation with different PIC-linked agonists the accumulation of [^{3}H]InsPP in response to a prolonged stimulation with both Hist and CCh

was assessed. These two compounds stimulate InsPP formation through H_1-histaminergic (Barnes *et al.*, 1986) and M_3-muscarinic (Roffel *et al.*, 1990) receptors respectively and the different classes of receptors may well recruit alternative subsets of G-proteins or PIC isozymes which could influence the pattern of inositol polyphosphate formation.

[^{3}H]Inositol labelled BTSM slices (2.3.1) were incubated with a maximally effective concentration of either histamine (1 mM) or CCh (100 μM) for 30 min in the presence of 5 mM LiCl and the accumulating [^{3}H]InsPP isomers isolated and separated using H.P.L.C. (as detailed in section 2.6.3). As shown in table 5.2, stimulation of the BTSM slices with Hist resulted in a reduced accumulation of [^{3}H]InsPs compared to that following CCh stimulation (31 % of the CCh response). This reduction in [^{3}H]InsP formation was associated with a statistically significant ($p < 0.05$, two-tailed, unpaired student's t-test) change in the routing of Ins(1,4,5)P_3 through the 3-kinase pathway (calculated as detailed in chapter three) from 23.77 ± 1.80 % in the presence of CCh to 33.02 ± 2.38 % in the presence of Hist. The proportional increase in 3-kinase activity observed in the presence of Hist (compared to CCh) resulted in an increase in the relative accumulation of [^{3}H]Ins(1,3,4,5)P_4 and hence may have important implications in calcium signalling (see section 1.5.2). In addition the ratio of [^{3}H]Ins(1,4)P_2 to [^{3}H]Ins4P accumulation was greatly reduced in the presence of Histamine compared to CCh. This latter result probably reflects the uncompetitive nature of the inhibition of the InsPP 1-phosphatase by LiCl, i.e. the smaller amount of [^{3}H]Ins(1,4)P_2 formed in the presence of Hist decreases the effectiveness of Li^+ inhibition and results in an increased metabolism through this enzyme.

$[^3H]Ins(x)PP$	% Total radioactivity Control		Histamine		CCh	
	- PDBu (n=3)	+ PDBu (n=3)	- PDBu (n=3)	+ PDBu (n=3)	- PDBu (n=5)	+ PDBu (n=5)
1/3	32.50 ± 2.72	29.21 ± 2.59	30.28 ± 1.98	31.77 ± 3.11	21.46 ± 1.66	24.42 ± 1.13
4	38.01 ± 5.44	23.32 ± 1.97	61.29 ± 3.14	58.24 ± 5.01	60.35 ± 3.13	57.82 ± 2.75
1,3	1.65 ± 0.20	1.67 ± 0.39	1.05 ± 0.12	1.01 ± 0.19	0.94 ± 0.09	1.02 ± 0.11
1,4	10.92 ± 1.64	19.85 ± 2.05	5.26 ± 1.16	5.29 ± 1.24	15.02 ± 1.75	14.44 ± 2.64
3,4	3.20 ± 0.58	3.50 ± 0.67	0.33 ± 0.03	0.62 ± 0.06	0.30 ± 0.02	0.36 ± 0.02
4,5	0.03 ± 0.01	0.18 ± 0.08	0.18 ± 0.07	0.09 ± 0.04	0.94 ± 0.07	0.80 ± 0.13
1,3,4	1.69 ± 0.29	2.38 ± 0.27	0.76 ± 0.22	0.86 ± 0.40	0.62 ± 0.08	0.78 ± 0.13
1,4,5	5.34 ± 0.81	10.03 ± 2.15	0.35 ± 0.09	0.93 ± 0.20	0.17 ± 0.04	0.16 ± 0.03
2,4,5	0.22 ± 0.04	0.80 ± 0.16	0.02 ± 0.01	0.05 ± 0.03	0.01 ± 0.003	0.01 ± 0.004
$InsP_4$	5.57 ± 0.64	7.11 ± 1.27	0.38 ± 0.07	0.98 ± 0.22	0.18 ± 0.01	0.18 ± 0.02
3-kinase metabolites	45.55 ± 2.06	50.41 ± 3.80	33.02 ± 2.38	35.69 ± 3.90	23.77 ± 1.80	27.85 ± 1.74
5-phosphatase metabolites	54.45 ± 2.06	49.59 ± 3.80	66.98 ± 2.38	64.31 ± 3.90	76.23 ± 1.80	72.15 ± 1.74
DPM/50 µl slices	7,405 ± 2,461	4,003 ± 607	130,373 ± 26,960	36,570 ± 6,250	413,901 ± 57,508	365,115 ± 56,159

Table 5.2 Effect of phorbol dibutyrate on carbachol- and histamine-stimulated $[^3H]$inositol polyphosphate accumulation
$[^3H]$Inositol pre-labelled BTSM slices (2.3.1) were pre-incubated for 30 min at 37 °C with 100 nM PDBu or vehicle prior to histamine- (1 mM) or CCh- (100 µM) stimulation (30 min) in the presence of 5 mM LiCl. $[^3H]$InsPPs in pooled triplicate TCA extracts (2.4.2) were separated using a Partisphere 5 SAX H.P.L.C. column (2.6.3) and quantified by liquid scintillation counting. Results represent the mean ± SEM of n separate experiments. $Ins(4,5)P_2$ and $Ins(2,4,5)P_3$ accumulations were not included in the 3-kinase and 5-phosphatase 'routing' calculations as their metabolic origins are uncertain.

5.3 PROTEIN KINASE C-MEDIATED REGULATION OF CARBACHOL- AND HISTAMINE-STIMULATED INOSITOL POLYPHOSPHATE ACCUMULATION IN BOVINE TRACHEAL SMOOTH MUSCLE.

PKC proteins represent a large family of isoenzymes which appear to differ markedly in their tissue expression, substrate specificity, and mode of activation (see Hug and Sarre, 1993). To date ten different PKC isoenzymes have been identified which can be classified into those requiring Ca^{2+} for activation (the Ca^{2+}-dependent or cPKCs) including the α, β_1, β_2 and γ isoforms, the Ca^{2+}-independent or novel PKCs (nPKCs) including the δ, ε, η and θ isoforms, and the atypical (aPKCs) which are not activated by phorbol ester and include the ζ and λ isoforms.

A wealth of evidence has accumulated to suggest that activation of PKC may inhibit receptor-stimulated phosphoinositide hydrolysis. For example activation of cPKC/nPKC with either phorbol esters or stable analogues of DAG has been shown to inhibit the agonist-stimulated inositol phosphate response in a range of cell types including Hist-, CCh- and GTPγS-stimulated astrocytoma cells (Orellana *et al.*, 1985, 1987), angiotensin II-stimulated rat aorta smooth muscle cells (Brock *et al.*, 1985; Pfeilschifter *et al.*, 1989), CCh-stimulated PC12 cells (Vincentini *et al.*, 1985) Hist- and bradykinin-stimulated adrenal chromaffin cells (Boarder and Challiss, 1992), epidermal growth factor-, angiotensin II- and adrenaline-stimulated hepatocytes (Johnson and Garrison, 1987; Lynch *et al.*, 1985) thrombin-stimulated platelets (Rittenhouse and Sasson, 1985) and CCh-stimulated intestinal smooth muscle (Prestwich and Bolton, 1995). Moreover, the addition of purified PKC to astrocytoma cell membrane preparations has been shown to mimic the effects of phorbol myristate acetate (PMA) in decreasing agonist-stimulated $[^3H]InsP_3$ production (Orellana *et al.*, 1987), while inhibition (Boarder and Challiss,

1992; King and Rittenhouse, 1989) or down-regulation (Pfeilschifter *et al.*, 1989) of PKC activity have the opposite effect.

A similar phorbol ester-mediated regulation of phosphoinositide hydrolysis has also been demonstrated in airways smooth muscle where both muscarinic- and histaminergic-stimulated accumulation of InsP is markedly attenuated in the presence of phorbol ester (Baba *et al.*, 1989; Murray *et al.*, 1989; Yang *et al.*, 1994). In order to ascertain whether PKC activation could influence InsPP metabolism in BTSM and whether or not such an effect could discriminate between CCh- and Hist-stimulated responses, $[^3H]$InsPP accumulation was monitored following a 30 min pre-incubation with 0.1 nM - 10 μM PDBu.

5.3.1 Differential effect of phorbol dibutyrate on carbachol- and histamine-stimulated $[^3H]$inositol phosphate accumulation

The effect of a 30 min pre-incubation of BTSM slices with various concentrations of PDBu (0.1 nM - 10 μM) on CCh (100 μM)- and Histamine (1 mM)-stimulated $[^3H]$InsP generation is illustrated in figure 5.3.1. Both the CCh- and Hist-stimulated $[^3H]$InsP responses were inhibited by PDBu treatment in a concentration-dependent manner. The Hist response was decreased by as little as 1 nM PDBu and could be completely abolished (99.1 ± 0.6 % inhibition) with 10 μM PDBu (IC_{50} = 5 nM). The CCh-stimulated $[^3H]$InsP response was far more resistant to inhibition by PDBu pre-treatment with detectable inhibition only being observed at concentrations greater than 10 nM, and a 'maximal' inhibition of 75.7 ± 1.6 % (IC_{50} = 230 nM).

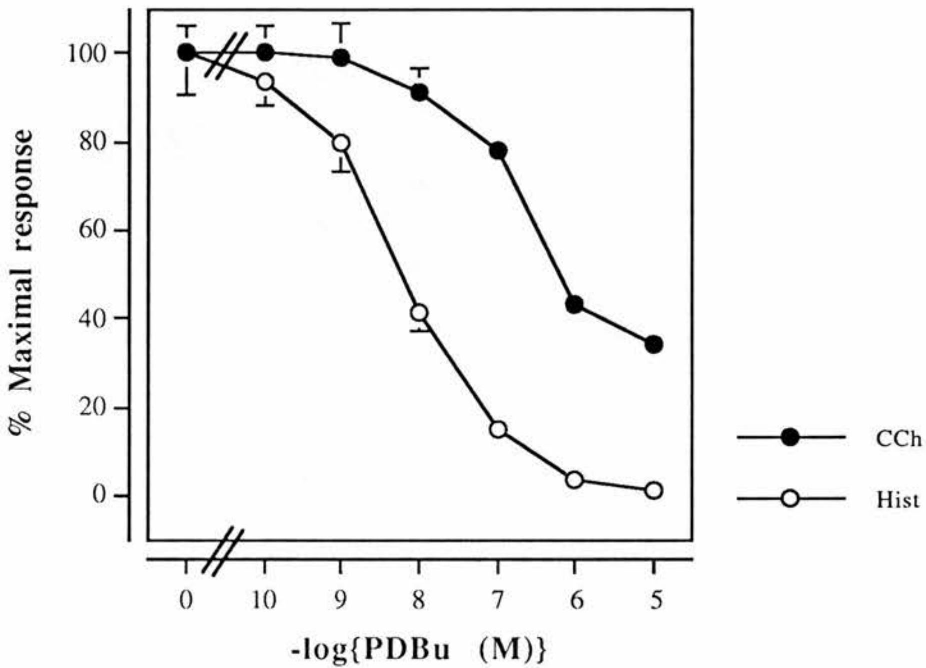

Figure 5.3.1 Phorbol dibutyrate concentration-response curve for carbachol- and histamine-stimulated [^{3}H]inositol polyphosphate accumulation in bovine tracheal smooth muscle.

[^{3}H]Inositol pre-labelled bovine tracheal smooth muscle slices (2.3.1) were pre-incubated for 30 min with 5 mM LiCl and the indicated concentration of PDBu prior to a 30 min stimulation with either 100 µM CCh or 1 mM Hist. Total [^{3}H]InsPs were separated from neutralised TCA extracts (2.4.2) by Dowex AG 1 X-8 anion-exchange chromatography (2.6.1) and quantified by liquid scintillation counting. Results are expressed as the percentage maximal [^{3}H]InsPP response (415434 ± 39930 and 162121 ± 5066 DPM/50 µl BTSM slices for CCh and Hist respectively), and represent the mean ± SEM of three separate experiments, each performed in duplicate.

5.3.2 Effect of phorbol dibutyrate on carbachol- and histamine-stimulated inositol 1,4,5-trisphosphate mass and [^{3}H]inositol 1,4,5-trisphosphate metabolism

Since pre-treatment of BTSM slices with PDBu can inhibit, albeit it to a different extent, the CCh- and Hist-stimulated total [^{3}H]InsP responses, it might be expected that this agent may also decrease the peak concentration of Ins(1,4,5)P_3 accumulation in response to these agonists. This prediction is based on the assumption that PKC activation results in receptor-PIC uncoupling (e.g. Murray *et al.*, 1989; Orellana *et al.*, 1987), unlike the inhibition of agonist-stimulated [^{3}H]InsP accumulation observed with β_2-adrenoceptor stimulation that results in inhibition of the sustained but not immediate phase of PtdIns(4,5)P_2 hydrolysis (Challiss and Boyle, 1994). Such a proposal is also supported by the observation that the response to maximally effective (1 mM) Hist stimulation of BTSM is approximately 30 % of that following maximally effective (100 μM) CCh stimulation for both total [^{3}H]InsP accumulation over 30 min (see table 5.2) and Ins(1,4,5)P_3 mass at 5 sec (Chilvers *et al.*, 1989b). In addition, the concentration-response curves for CCh-stimulated [^{3}H]InsP accumulation (see figure 2.3.1.1; Chilvers *et al.*, 1994b) and Ins(1,4,5)P_3 accumulation (Chilvers *et al.*, 1989b) are near identical. Indeed data obtained by Chilvers (personal communication, see figure 5.3.2.1) show that the peak Ins(1,4,5)P_3 response in CCh-stimulated BTSM slices is reduced by 36.9 ± 2.5 % following a 30 min pre-incubation with 1 μM PDBu. This data correlates closely with the above [^{3}H]InsP accumulation data (figure 5.3.1).

As described earlier, small alterations in the concentration of Ins(1,4,5)P_3 may in itself have a significant influence on the routing of this second messenger through the 3-kinase and 5-phosphatase pathways. In order to

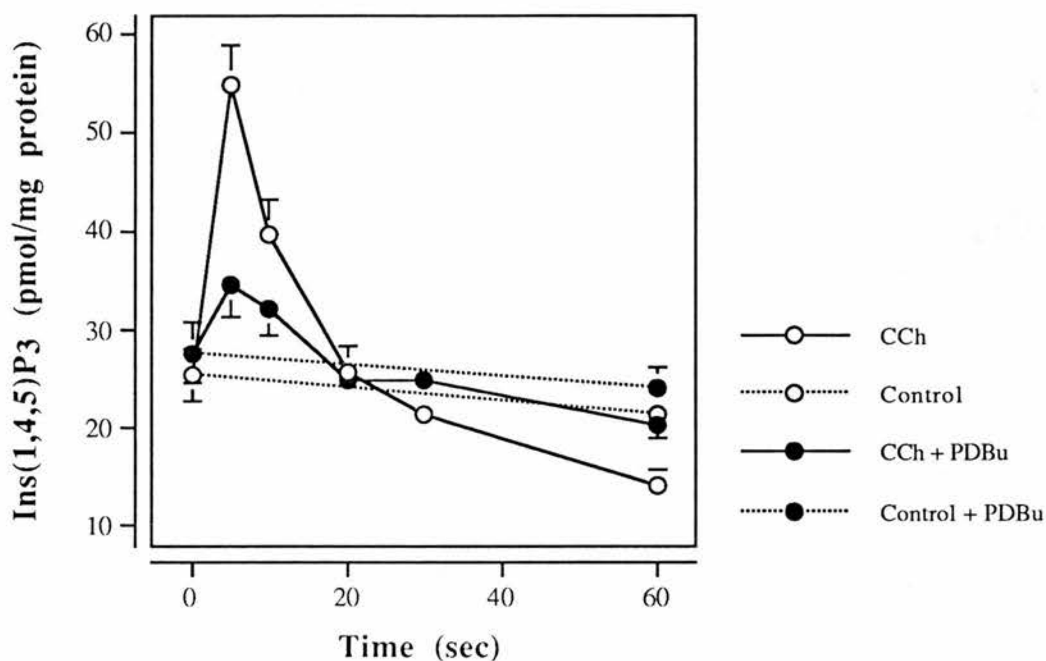

Figure 5.3.2.1 Effect of phorbol dibutyrate on carbachol-stimulated inositol 1,4,5-trisphosphate mass in bovine tracheal smooth muscle

BTSM slices (2.2) were pre-incubated for 30 min in oxygenated KHB in the presence or absence of 1 μM PDBu (2.7.1) prior to further incubation with either CCh (100 μM) or vehicle. Reactions were terminated at the indicated time-points with 1 M TCA and the Ins(1,4,5)P_3 present in neutralised extracts (2.4.2) quantified using a radioreceptor assay (2.7.3). Results represent the mean ± SEM of three separate experiments, each performed in triplicate.

assess the effect PDBu could have on the metabolism of $Ins(1,4,5)P_3$ by the 3-kinase and 5-phosphatase enzymes a concentration of PDBu (100 nM) was chosen that could facilitate a measurable decrease in the CCh-stimulated $[^3H]$InsP response while still permitting a significant Hist-stimulated response. The effect of 100 nM PDBu pre-treatment on the accumulation of the individual $[^3H]$InsPP isomers in response to prolonged CCh- and Hist stimulation is shown in table 5.2, and the flux of $[^3H]Ins(1,4,5)P_3$ through the 3-kinase and 5-phosphatase pathways illustrated in figure 5.3.2.2. PDBu (100 nM) pre-treatment results in a 71.05 ± 3.75 % reduction in the total $[^3H]$InsP response to Hist, and a 30.88 ± 14.15 % and a 12.53 ± 2.17 % reduction of the $[^3H]$InsP response in control and CCh-stimulated slices respectively. It is clear from this data that although there is a trend in all experiments undertaken in the presence of PDBu for more $[^3H]Ins(1,4,5)P_3$ to be metabolised via the 3-kinase route, this does not reach statistical significance. These results exclude any major influence of PKC stimulation on $Ins(1,4,5)P_3$ routing over a 30 min agonist stimulation period.

5.4 β_2-ADRENOCEPTOR AGONIST-MEDIATED REGULATION OF CARBACHOL- AND HISTAMINE-STIMULATED $[^3H]$INOSITOL PHOSPHATE ACCUMULATION IN BOVINE TRACHEAL SMOOTH MUSCLE

It is well documented that agents which elevate the tissue cAMP concentration in ASM including β-adrenoceptor agonists can inhibit spasmogen-induced contraction and initiate relaxation in tissue pre-contracted with a range of agonists (e.g. Ellis *et al.*, 1995; Francis *et al.*, 1988; Gorenne *et al.*, 1995; Torphy *et al.*, 1985, 1988). Tracheal smooth

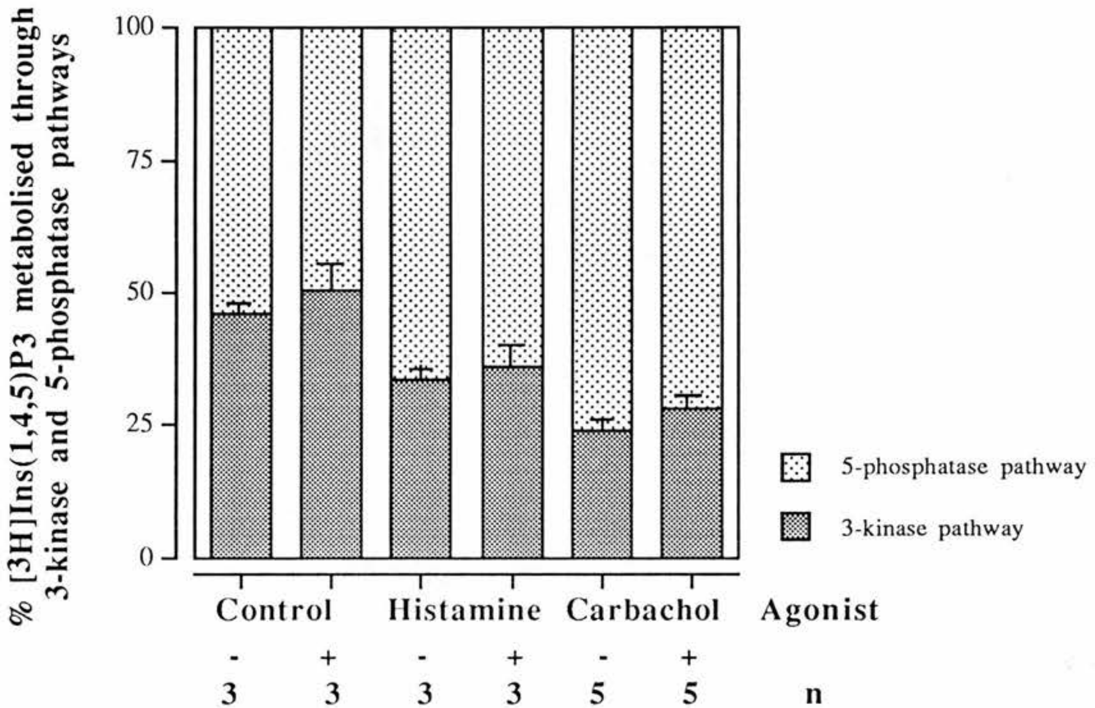

Figure 5.3.2.2 Effect of phorbol dibutyrate on [^{3}H]inositol 1,4,5-trisphosphate routing through the 3-kinase and 5-phosphatase pathways [^{3}H]Inositol pre-labelled BTSM slices (2.3.1) were pre-incubated for 30 min at 37 °C with 100 nM PDBu prior to histamine- (1 mM) or CCh- (100 μM) stimulation (30 min) in the presence of 5 mM LiCl. [^{3}H]InsPPs in pooled triplicate extracts (2.4.2) were separated using a Partisphere 5 SAX H.P.L.C. column (2.6.3) and quantified by liquid scintillation counting. Results are expressed as the proportion of [^{3}H]Ins(1,4,5)P_3 metabolised by the 3-kinase and phosphatase pathways (see section 3.4.1) and represent the mean ± SEM of n separate experiments.

muscle from a number of tissues expresses both β_1- and β_2-adrenoceptors, predominantly β_2, coupled to adenylyl cyclase (Barnes *et al.*, 1983c; Popovich *et al.*, 1984). The β-adrenoceptor agonists salmeterol and salbutamol exhibit very high β_2- versus β_1-selectivity (e.g Dougall *et al.*, 1991). Salmeterol, (a derivative of salbutamol with a long, flexible, lipophilic *N*-substituted side-chain) is a designer β_2-adrenoceptor agonist, specifically formulated to provide long-acting β_2-adrenoceptor stimulation (see Johnson *et al.*, 1993; Jack, 1991 and references therein). By virtue of its lipophilic side-chain, salmeterol is thought to bind non-competitively to an exo-site deep within the hydrophobic core of the receptor protein. This agent does not therefore readily dissociate from the membrane and is capable of producing both persistent, non-desensitising relaxation of ASM *in vitro* (Johnson *et al.*, 1993) and long-lasting (> 12 h) bronchodilatation *in vivo*, (see Jack, 1991) Salmeterol (Salm) and salbutamol (Salb) have been characterised as partial agonists (with respect to adrenaline and isoprenaline) for β-adrenoceptor stimulation of cAMP accumulation (Dougall, *et al.*, 1991), although the potency of Salm for relaxation is similar to that of isoprenaline in guinea-pig trachea and human bronchus (Ball *et al.*, 1991; Bradshaw *et al.*, 1987). The availability of highly selective β_2-adrenoceptor agonists with very different kinetics with respect to the duration of relaxation (both *in vivo* and *in vitro*) affords an opportunity to re-assess the likely relative importance of β-adrenoceptor-mediated inhibition of PtdIns(4,5)P_2 hydrolysis to ASM relaxation.

5.4.1 Comparison between effects of salmeterol and salbutamol on agonist-stimulated [^{3}H]inositol phosphate accumulation in bovine tracheal smooth muscle

To permit a subsequent comparison between the effects of Salm and Salb on the duration of inhibition of Hist-stimulated [^{3}H]InsP accumulation initial experiments were performed to investigate the potency of these two β_2-adrenoceptor agonists on agonist-stimulated [^{3}H]InsP accumulation. BTSM slices were pre-treated for 30 min with either Salm (0.01-100 nM) or Salb (3 nM - 1 μM) prior to stimulation for 30 min with Hist (1 mM) or CCh (100 μM). The CCh-stimulated [^{3}H]InsP response was unaffected by Salm (see figure 5.4.1.1), whereas the Hist-stimulated [^{3}H]InsP response was significantly attenuated by both Salm and Salb (see figures 5.4.1.1 and 5.4.1.2). Salm was more potent (IC_{50} = 0.24 ± 0.9 nM) than Salb (IC_{50} = 10.8 ± 2.9 nM) in inhibiting the Hist-stimulated [^{3}H]InsP response. These potencies correlate well with those obtained in a comparable system by Ellis and co-workers (1995) where IC_{50} values of 1.4 nM and 13.8 nM were obtained for Salm- and Salb-mediated inhibition of Hist-stimulated [^{3}H]InsP accumulation respectively. These authors noted that the maximal inhibitory effect of the two β-adrenoceptor agonists (~ 60 % inhibition) on the [^{3}H]InsP response were not significantly different. While Salm appeared to be more effective in inhibiting Hist-stimulated [^{3}H]InsP accumulation than Salb in the present study (maximal inhibition = 59.8 ± 1.8 % and 49.5 ± 1.8 % for Salm and Salb respectively) this difference did not reach statistical significance and the results are therefore in close agreement with Ellis and co-workers. Similarly, the maximal inhibitory effect of Salb and isoprenaline on Hist-stimulated [^{3}H]InsP formation were not found to differ (66 % and 68 % respectively) in BTSM (Hall and Hill, 1988). The latter study did however observe a

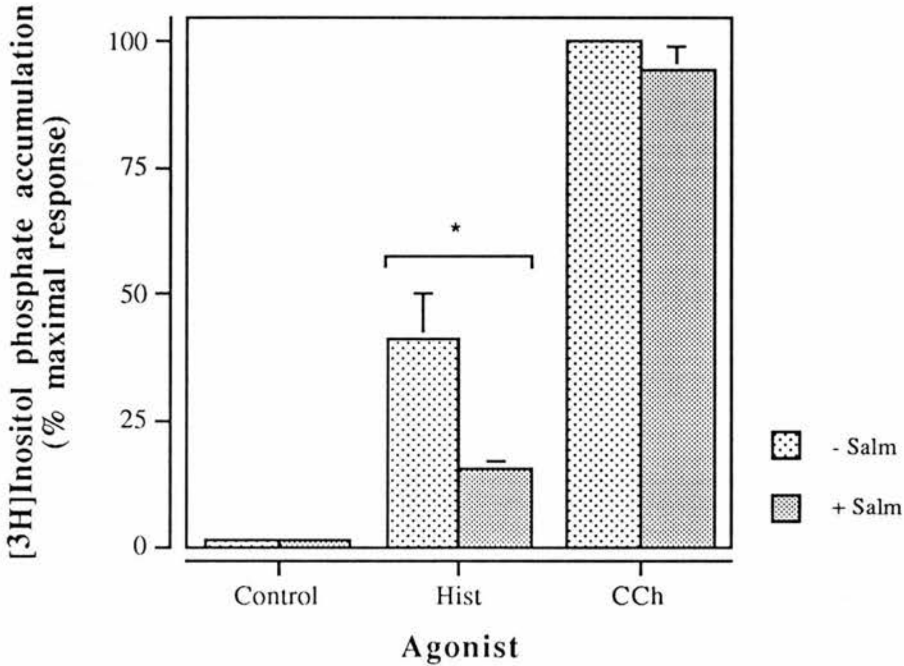

Figure 5.4.1.1 Effect of salmeterol on prolonged (30 minute) carbachol- and histamine-stimulated [^{3}H]inositol phosphate accumulation
[^{3}H]Inositol labelled BTSM slices (2.3.2) were pre-incubated in 24 well tissue culture plates for 30 min in the presence or absence of 100 nM salmeterol. The slices were subsequently incubated (30 min) with 100 μM CCh, 1 mM Hist or buffer in the presence of 10 mM LiCl. Total [^{3}H]InsPs were separated from neutralised TCA extracts (2.4.2) by Dowex AG 1-X8 anion-exchange chromatography (2.6.1) and quantified by liquid scintillation counting. Results are exressed as the percentage of the CCh-stimulated [^{3}H]InsP response in the absence of salmeterol (449,424 ± 90,837 DPM/50 μl slices), and represent the mean ± SEM of five or six separate experiments, each performed in duplicate. * Denotes $p < 0.05$ for comparisons between salmeterol-treated and -unteated slices (two-tailed, unpaired student's t-test).

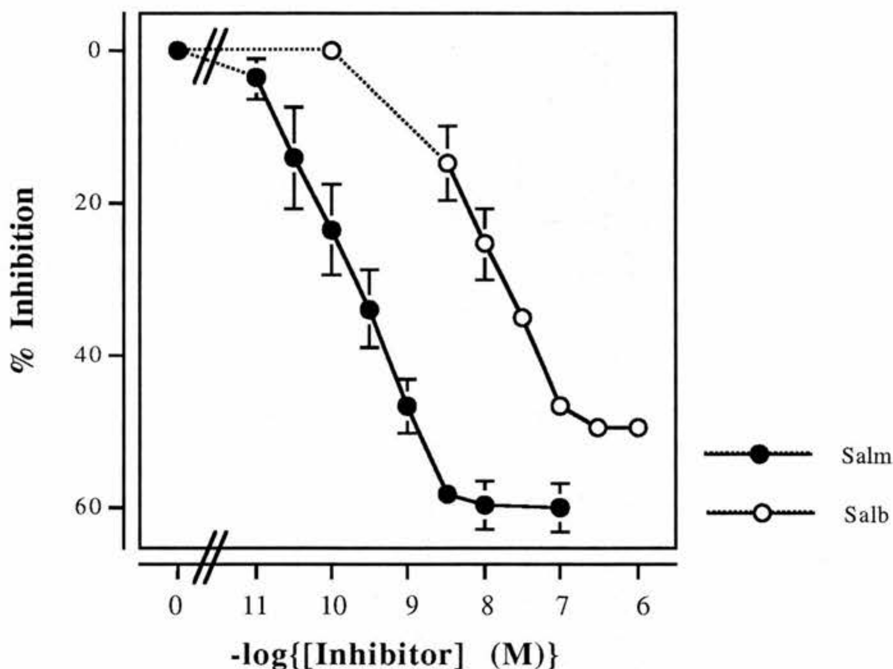

Figure 5.4.1.2 Salmeterol and salbutamol concentration-response curves for inhibition of histamine-stimulated [^{3}H]inositol phosphate accumulation

[^{3}H]Inositol labelled BTSM slices (2.3.2) in 24 well tissue culture plates were pre-incubated for 30 min in the presence of the indicated concentrations of either salmeterol or salbutamol. The slices were then further incubated (30 min) with 1 mM Hist in the presence of 10 mM LiCl. Total [^{3}H]InsPs were separated from neutralised TCA extracts (2.4.2) by Dowex AG 1-X8 anion-exchange chromatography (2.6.2) and quantified by liquid scintillation counting. Results are expressed as the percentage inhibition of the 1 mM Hist-stimulated [^{3}H]InsP response in the absence of added inhibitor (133,569 ± 26,928 DPM/50 µl slices) and represent the mean ± SEM of three separate experiments, each performed in duplicate.

lower potency of Salb for this effect (IC_{50} = 290 nM) than described here. This discrepancy may reflect differences in the experimental conditions since Hist and Salb were added simultaneously in the study by Hall and Hill.

5.4.2 Time-course for salbutamol- and salmeterol-mediated inhibition of histamine-stimulated [^{3}H]inositol phosphate accumulation

While β_2-adrenoceptor agonists are capable of relaxing ASM pre-contracted with a variety of agents, it would appear that the relative extent of relaxation is dependent on both the contractile agonist (e.g. Russell, 1984; Torphy *et al.*, 1985) and the initial contractile state of the tissue (Torphy, 1984). For example, muscarinic cholinoceptor-mediated contraction is relatively resistant to β_2-adrenoceptor-induced relaxation (Hall *et al.*, 1990; Madison and Brown, 1988; Offer *et al.*, 1991). Salm and Salb are unable to inhibit the [^{3}H]InsP response to the muscarinic agonist CCh but significantly attenuate the response to Hist in BTSM (see above). In addition, these two β-adrenoceptor agonists exhibit a differential inhibitory effect on both the [^{3}H]InsP response (Figure 5.4.1.2) and the contractile response (Ellis *et al.*, 1995) stimulated by Hist. It is therefore possible that the effects of Salm and Salb on [^{3}H]InsP accumulation may explain, at least in part, their differential relaxant activity.

A close temporal correlation between the Salm- and Salb-mediated relaxation of methacholine-induced tone and intracellular cAMP accumulation has been observed in BTSM (Ellis *et al.*, 1995). In this model Salm resulted in a slowly developing reversal of induced tone accompanied by a progressive accumulation of cAMP, with both responses maximal after 30-40 min. By contrast, Salb resulted in rapid

effects on both induced tone and cAMP which were maximal within 5 min and declined thereafter. Hence the relaxant effects of β_2-adrenoceptor agonists in this model would seem to be secondary to elevation of cAMP.

The time-course of Salm- and Salb-mediated inhibition of Hist-stimulated [^{3}H]InsP accumulation was investigated in order to address whether or not the differential inhibition of phosphoinositide hydrolysis could account for the long-term relaxant effects of Salm and the shorter-term relaxant effects of Salb. [^{3}H]Inositol pre-labelled BTSM slices were incubated with the β_2-adrenoceptor agonists or buffer for 0-24 h prior to a 30 min stimulation with Hist, and the resulting accumulation of [^{3}H]InsPs quantified. Figure 5.4.2 shows that while the [^{3}H]InsP response was highly sensitive to short-term treatment with both β_2-adrenoceptor agonists, the [^{3}H]InsP response gradually recovered at later time-points and no inhibition of Hist-stimulated [^{3}H]InsP accumulation was observed 12 h after Salm or Salb addition. At the earliest time-point analysed (1 h) the inhibition of [^{3}H]InsP accumulation was significantly greater in Salm-treated BTSM slices than in those treated with Salb. At later time-points (3, 6, 12 and 24 h) however, no such differential inhibition was evident. Control values for Hist-stimulated [^{3}H]InsP accumulation remained remarkably constant over the duration of the time-course (data not shown).

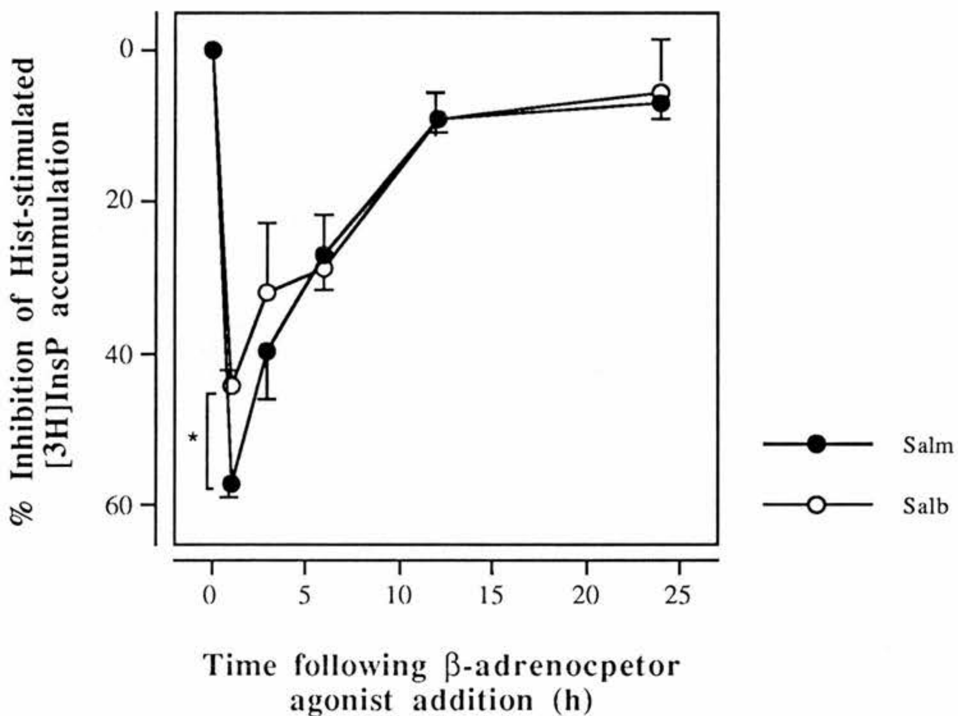

Figure 5.4.2 Time-course of salmeterol and salbutamol inhibition of histamine-stimulated [^{3}H]inositol polyphosphate accumulation

[^{3}H]Inositol labelled BTSM slices (2.3.2) were pre-incubated for the times indicated with concentrations of Salm or Salb (100 nM and 5 μM respectively) that gave equivalent (maximal) inhibition of Hist-stimulated [^{3}H]InsP accumulation after 30 min pre-incubation (see figure 5.4.1.2). The slices were then stimulated with 1 mM Hist for 30 min in the presence of 10 mM LiCl. Total [^{3}H]InsPs were separated from neutralised TCA extracts (2.4.2) by Dowex AG 1-X8 anion-exchange chromatography (2.6.1) and quantified by liquid scintillation counting. Results are expessed as the percentage of the Hist-stimulated [^{3}H]InsP response in the absence of inhibitor and represent the mean ± SEM of three separate experiments, each performed in triplicate. * Denotes $p < 0.05$ for comparisons between salmeterol and salbutamol treated slices (two-tailed, paired student's t-test).

5.4.3 Effect of salmeterol on carbachol- and histamine-stimulated inositol 1,4,5-trisphosphate mass

BTSM slices were incubated with a maximally effective concentration of Salm for 30 min prior to stimulation with agonist and subsequent measurement of Ins(1,4,5)P_3 mass. In agreement with Chilvers and co-workers (1989b), CCh (100 μM) resulted in a transient increase in Ins(1,4,5)P_3 that was maximal at 5 sec and decreased to sub-basal values by 30 sec (data not shown). No difference was observed between Ins(1,4,5)P_3 accumulation in control BTSM slices and those pre-treated with 100 nM Salm (0-300 sec). Further experiments were conducted to establish the effect of Salm pre-treatment on the peak (5 sec) Ins(1,4,5)P_3 accumulation in response Hist- and CCh. Data from freshly prepared BTSM slices and tissue incubated overnight in RPMI medium were not significantly different and were pooled for statistical analysis. Figure 5.4.3 shows that Hist stimulation results in only a very modest increase in Ins(1,4,5)P_3 accumulation at 5 sec, which is significantly inhibited by Salm pre-treatment. The basal and CCh-stimulated accumulation of Ins(1,4,5)P_3 however is unchanged by such treatment.

5.5 EFFECT OF DEXAMETHASONE ON CARBACHOL- AND HISTAMINE-STIMULATED PHOSPHOINOSITIDE HYDROLYSIS IN BOVINE TRACHEAL SMOOTH MUSCLE

In addition to inhibition of inflammatory mediator release (Bersenstein *et al.*, 1987; Fitzke and Dieter, 1991), glucocorticoids have also been shown to modulate ASM responses to contractile agonists (e.g. Powell *et al.*, 1993). Furthermore, activation of glucocorticoid receptors with dexamethasone (Dex) in RBL-2H3 cells (Berenstein *et al.*, 1987) and

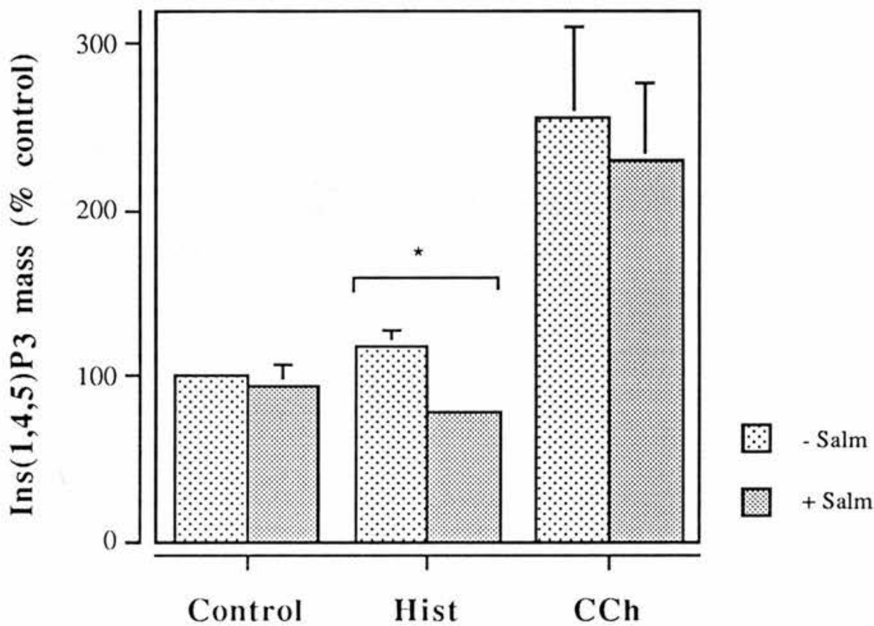

Figure 5.4.3 Effect of salmeterol on peak (5 second) histamine- and carbachol-stimulated Ins(1,4,5)P_3 accumulation

BTSM slices (2.2) were incubated in the presence or absence of salmeterol for 30 min at 37 °C in either oxygenated KHB or RPMI medium (2.7.1). The slices were then further incubated for 5 sec with Hist (1 mM), CCh (100 μM) or buffer. Reactions were terminated with 1 M TCA and the Ins(1,4,5)P_3 present in neutralised TCA extracts (2.4.2) quantified using a radioreceptor assay (2.7.3). Results are expressed as the percentage Ins(1,4,5)P_3 mass in control slices in the absence of salmeterol (17.0 ± 3.6 pmol/mg protein), and represent the mean ± SEM of 12 separate experiments, each performed in duplicate or triplicate. * Denotes $p < 0.05$ for comparisons between salmeterol-treated and -untreated BTSM slices (unpaired, two -tailed student's t-test).

macrophages (Fitzke and Dieter, 1991) results in inhibition of agonist-stimulated phosphoinositide hydrolysis. The latter study showed this effect to be specific for glucocorticoids since cortisone exhibited a similar action, but other steroids such as progesterone were without effect. These authors concluded that the effect of dexamethasone on agonist-stimulated phosphoinositide hydrolysis was due to an inhibition of the incorporation of [^{3}H]inositol into the membrane phospholipids rather than an inhibition of PLC. Fitzke and Dieter (1991) demonstrated that prolonged (> 6 h) exposure to dexamethasone was necessary for this steroid to influence zymosan-stimulated phosphoinositide hydrolysis in macrophages and also noted a similar time-dependent inhibition of zymosan-stimulated InsP formation in response to protein- and RNA-synthesis inhibitors. These results strongly suggest an indirect action of glucocorticosteroids on phosphoinositides involving *de novo* synthesis of proteins.

In order to address whether or not such a corticosteroid-mediated inhibition of agonist-stimulated phosphoinositide hydrolysis could occur in BTSM, the ability of Dex to influence CCh- and Hist-stimulated accumulation of [^{3}H]InsPs and incorporation of [^{3}H]inositol into the inositol phospholipids was assessed.

5.5.1 Effect of dexamethasone on carbachol- and histamine-stimulated [^{3}H]inositol phosphate accumulation

[^{3}H]Inositol-labelled BTSM slices were pre-incubated for 24 h with 1 μM Dex prior to further incubation (30 min) with varying concentrations of either CCh or Hist. The resulting accumulation of [^{3}H]InsPs was quantified and is illustrated in figure 5.5.1. Dex (1 μM) had no effect on [^{3}H]InsP formation in response to either Hist (10 nM - 1 mM) or CCh (10

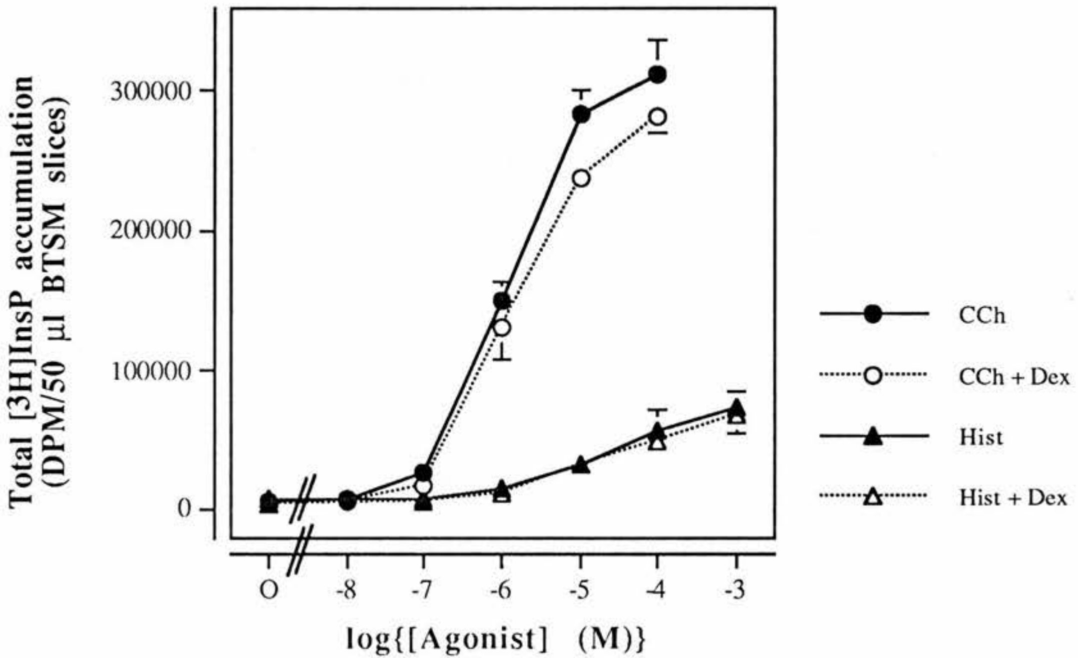

Figure 5.5.1 Effect of dexamethasone on carbachol- and histamine-stimulated [^{3}H]inositol phosphate accumulation in bovine tracheal smooth muscle

[^{3}H]Ins labelled BTSM slices (2.3.2) were pre-incubated for 24 h with 1 µM Dex prior to agonist stimulation (30 min) with 100 µM CCh or 1 mM Hist. [^{3}H]InsPs were separated from neutralised acidified chloroform/methanol extracts (2.5.1) by Dowex AG 1-X8 anion-exchange chromatography (2.6.1) and quantified by liquid scintillation counting. Results represent the mean ± SEM of two-three separate experiments.

nM - 100 μM). Addition of Dex 6 or 18 h prior to agonist stimulation was also without effect on agonist-stimulated [^{3}H]InsP formation (data not shown).

5.5.2 Effect of dexamethasone on carbachol- and histamine-stimulated [^{3}H]inositol incorporation into inositol phospholipids in bovine tracheal smooth muscle slices

BTSM slices were labelled by pre-incubation (24 h) with [^{3}H]inositol (2.3.2) prior to treatment (24 h) with Dex (1 μM) and further incubation (30 min) with either CCh (10 nM - 100 μM) or Hist (10 nM - 1 mM). The incorporation of [^{3}H]inositol into the inositol phospholipids was quantified (2.5.1) and is illustrated in figure 5.5.2. Despite the [^{3}H]inositol label being present for 48 h, labelling of the CCh-accessible [^{3}H]phosphoinositide pool to isotopic equilibrium was not reached. Hence a definite increase in incorporation of [^{3}H]inositol is observed with 1 μM CCh. 1 μM Dex had no significant effect on the degree of [^{3}H]inositol incorporation observed under basal or agonist-stimulated conditions. Addition of Dex 6 or 18 h prior to agonist stimulation was also without effect on agonist-stimulated [^{3}H]inositol incorporation.

5.6 DISCUSSION

The experiments described in this chapter were designed principally to modulate the agonist-stimulated InsPP response in order to shed light on the factors regulating InsPP metabolism in BTSM. While stimulation with Hist and CCh results in broadly similar patterns of InsPP generation, i.e. a transient accumulation of Ins(1,4,5)P_3 and dominant metabolism of Ins(1,4,5)P_3 via the 5-phosphatase pathway, differences in the regulation

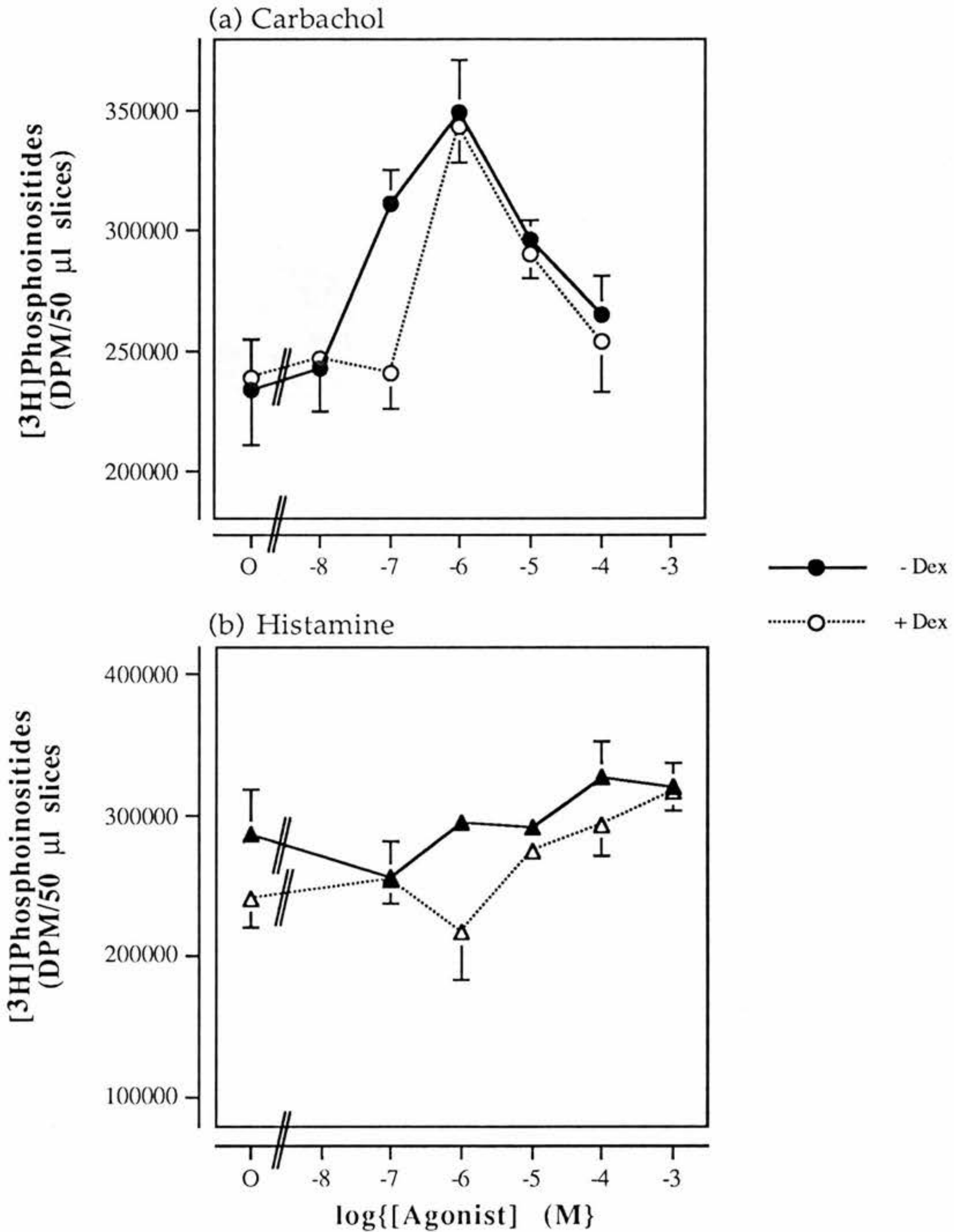

Figure 5.5.2 Effect of dexamethasone on CCh- and Hist-stimulated [^{3}H]inositol incorporation into membrane phospholipids

[^{3}H]Ins labelled BTSM slices (2.3.2) were incubated for 24 h in the presence or absence of 1 µM Dex prior to agonist stimulation (30 min) with 100 µM CCh or 1 mM Hist. [^{3}H]Phosphoinositide incorporation was quantified by measuring the radioactivity in the lower phase of partitioned acidified chloroform/methanol extracts (2.5.1). Results represent the mean ± SEM of two-three separate experiments.

of these responses clearly do exist. Stimulation of BTSM slices with a maximally effective concentration of Hist produces an [^{3}H]InsP response only 35 % of that seen in response to a maximally effective CCh concentration. This reduction in [^{3}H]InsP accumulation is accompanied by a 54 % reduction in the peak Ins(1,4,5)P_3 mass concentration and a small but significant increase in the proportion of Ins(1,4,5)P_3 metabolised by the 3-kinase pathway. Hence the decrease in Ins(1,4,5)P_3 available for metabolism results in a relative increase in metabolism through the 3-kinase pathway. Due to the much higher affinity of the 3-kinase than the 5-phosphatase for Ins(1,4,5)P_3 it is likely that this 'shift' reflects a relative reduction in the amount of Ins(1,4,5)P_3 metabolised via the 5-phosphatase rather than an enhancement of 3-kinase activity. It cannot be ruled out however, that the metabolism of Ins(1,4,5)P_3 may be subject to direct receptor-mediated regulation. This possibility is supported by the fact that the 5-phosphatase is located predominantly on the inner leaflet of the plasma membrane where it may come into contact with the active receptor/G-protein complex.

The PKC-stimulating phorbol ester PDBu had a major but differential effect on the Hist- and CCh-stimulated [^{3}H]InsP responses. While [^{3}H]InsP accumulation following CCh- and Hist addition were clearly decreased in response to a 30 min pre-incubation with a high concentration (10 μM) of this phorbol ester, PDBu had both a greater potency and greater efficacy for inhibition of the Hist-stimulated [^{3}H]InsP response than that stimulated by CCh. A similar heterogeneity of PKC-mediated feedback inhibition of agonist-stimulated InsP responses has also been observed in a number of cell-types. For example, in rat hepatocytes the Ins(1,4,5)P_3 response to angiotensin II stimulation is attenuated, whereas the response to epidermal growth factor is

completely blocked following activation of PKC (Johnson and Garrison, 1987). In addition phorbol ester treatment of bovine adrenal chromaffin cells inhibits the peak Hist- and bradykinin-stimulated Ins(1,4,5)P_3 responses by 70 % and 30 % respectively (Boarder and Challiss, 1992). A more dramatic difference can be observed in Rat-1-fibroblasts (Plevin *et al.*, 1991) where application of PMA results in an inhibition of the lysophosphatidic acid stimulated inositol phosphate formation whereas the response to endothelin-1 is completely unaffected. Furthermore a differential regulatory activity of PKC can be observed between different cell-types. For example Ward and Cantrell (1990) have demonstrated that antigen-stimulated phosphoinositide metabolism is inhibited following PKC activation in Jurkat cells but not in T lymphoblasts.

The mechanisms underlying the PKC-mediated inhibition of agonist-stimulated InsP responses and the apparent agonist and/or tissue specificity of this regulation are currently unclear. Several studies have demonstrated that the receptor number and affinity for its agonist are unchanged following addition of phorbol ester (e.g. Brock *et al.*, 1985; Orellana *et al.*, 1985, 1987; Vincentini *et al.*, 1985). A notable exception however, is the α_1-adrenoceptor from liver: following perfusion of liver membranes with PMA, a 30-40 % reduction in receptor number was observed (Lynch *et al.*, 1985). Nonetheless, the large number of different agonist responses which can be modulated by PKC activation suggest a common component of the signal transduction mechanism, and not the receptor, is the site of PKC action. In support of this hypothesis guanosine nucleotide- (Murray *et al.*, 1989; Orellana *et al.*, 1987) or fluoroaluminate- (Yang *et al.*, 1994) stimulated inositol phosphate formation is also inhibited by PKC activation. These results suggest a post-receptor site of action for PKC, possibly by phosphorylation of the G-

protein or PIC enzyme. To date there does not appear to be any evidence for PKC-mediated phosphorylation of G_q, however both $G_{s\alpha}$ (Pyne *et al.*, 1992a) and $G_{i\alpha}$ (Bushfield *et al.*, 1991) may be subject to such modulation. In addition PKC may phosphorylate and thereby inhibit PIC-β_1 (Ryu *et al.*, 1990). The differential inhibition of agonist-stimulated InsP responses by PKC may therefore arise as a result of the expression or activation of distinct G-protein and PIC isozymes. This differential regulation could also be mediated *in vivo* by the activation of alternative PKC isoforms and therefore it is interesting to note that while rat RBL-2H3 cells contain several PKC isoforms, only PKC_α and PKC_ε can inhibit antigen-induced phosphoinositide hydrolysis (Ozawa *et al.*, 1993).

It is also possible that some of the effects observed following PKC activation could be secondary to adenylyl cyclase activation and cAMP accumulation since treatment of cultured guinea-pig tracheal smooth muscle cells with either PMA or bradykinin results in enhanced adenylyl cyclase activity (Stevens *et al.*, 1994). The bradykinin-stimulated activation of adenylyl cyclase is mediated through PLD rather than PLC signallling (Stevens *et al.*, 1994) and does not involve the activation of PKC_α (Pyne *et al.*, 1994).

Whether a PKC-mediated feedback inhibition of the PIC response occurs *in vivo* is difficult to judge since the phorbol ester used in this study is relatively stable and induces highly unphysiological and global activation of multiple PKC isoforms. Its cellular counterpart - $PtdInsP_2$-derived DAG - is likely to be subject to rapid metabolism. Since the CCh-stimulated [^{3}H]InsP response does not readily desensitise in this tissue, with a linear accumulation of [^{3}H]InsP evident for at least 90 min (Chilvers *et al.*, 1989a), it would appear most unlikely that PKC activation has a major curtailing influence on M_3-mediated $PtdInsP_2$ hydrolysis.

The Hist response however does desensitise with time (Hall and Hill, 1988) and exhibits a greater sensitivity to inhibition by PDBu. Therefore the possibility of a physiological PKC-mediated feedback loop regulating Hist receptor signalling is far more likely. Clearly, with the advent of more selective PKC isozyme inhibitors and more detailed knowledge of the precise PKC isoforms targetted by $PtdInsP_2$-derived DAG it may, in the future, be possible to dissect these pathways further. It should also be noted that PLC-mediated phospholipid hydrolysis may not be the only route of DAG formation in ASM since bradykinin clearly induces phosphatidylcholine hydrolysis in cultured guinea-pig tracheal smooth muscle cells (Pyne and Pyne, 1993). There is therefore a strong possibility of cross-talk between the different signal transduction pathways in this tissue.

In addition to regulating PLC activity, PKC activation may also modulate the enzymes responsible for $Ins(1,4,5)P_3$ metabolism (see chapter three). However any effect of PKC on the these enzymes must be 'balanced' since there was no significant difference in the routing of Hist- or CCh-stimulated $Ins(1,4,5)P_3$ metabolism in the presence of PDBu.

A further differential activity on CCh- and Hist-stimulated [^{3}H]InsP formation is exhibited by the β_2-adrenoceptor agonists Salm and Salb. Salm did not affect the accumulation of [^{3}H]InsP in response to a maximally effective concentration of CCh, or the peak CCh-stimulated increase in $Ins(1,4,5)P_3$ mass. The Hist-stimulated [^{3}H]InsP response however was significantly attenuated by both Salm and Salb. Salm was the more potent in its inhibition of the [^{3}H]InsP response to Hist, and this agent also significantly reduced the Hist-stimulated $Ins(1,4,5)P_3$ mass response. Maximally effective concentrations of Salm and Salb elicited a similar degree of inhibition of the Hist-stimulated [^{3}H]InsP response (50-

60 %) which was also comparable to that observed for isoprenaline (68 %, Hall and Hill, 1988).

The mechanisms underlying the inhibition of Hist-stimulated [^{3}H]InsP accumulation by β_2-adrenoceptor agonists remain to be elucidated but could be mediated at several levels of the inositol phosphate signal transduction pathway. A reduction in the available substrate (PtdIns(4,5)P_2) is unlikely since [^{3}H]inositol incorporation into membrane phospholipids is unaffected by Salb in Hist-stimulated BTSM slices (Hall and Hill, 1988). Alternatively, β_2-adrenoceptor agonists may affect the H_1 receptor, the receptor-G-protein complex, or even directly inhibit PIC. Since agents which increase intracellular levels of cAMP can inhibit fluoroaluminate-stimulated [^{3}H]InsP formation, a post-receptor mediated mechanism has been implicated in the β_2-adrenoceptor mediated inhibition of phosphoinositide hydrolysis (Hall *et al.*, 1990a). Hence alterations in the G-protein-PIC coupling mechanism may underlie the effect of β_2-adrenoceptor agonists on receptor-stimulated [^{3}H]InsP accumulation. Indeed the possibility for G-protein regulation by β-adrenoceptor agonists is exemplified by the phosphorylation *in vitro* of $G_{s\alpha}$ by PKA (Pyne *et al.*, 1992b). It should also be noted that $\beta\gamma$ G-protein subunits liberated following β-adrenoceptor-mediated stimulation of G_s can activate β-adrenergic and muscarinic acetylcholine receptor kinases (Kameyama *et al.*, 1993; Koch *et al.*, 1993). These kinases may phosphorylate the receptors or G-proteins involved in phosphoinositide hydrolysis thereby serving to uncouple the receptor from its G-protein. Hence, as discussed above for PKC activation, the recruitment of distinct G-protein and PIC isoenzymes may explain the differential susceptibility of various agonists to β_2-adrenoceptor activation.

It has been suggested that the relative resistance of muscarinic receptor-mediated contraction to relaxation with β_2-adrenoceptor agonists may be explained, at least in part, by the ability of muscarinic receptor stimulation to inhibit cAMP accumulation in ASM tissue and cells (Jones *et al.*, 1987; Sankary *et al.*, 1988; Yang *et al.*, 1991). ASM contains two subtypes of muscarinic cholinoceptors, M_2 and M_3 (e.g. Yang *et al.*, 1991; Mahesh *et al.*, 1992). The latter are coupled to phosphoinositide hydrolysis while the former (the predominant receptor subtype in most species thus far studied) appears to be linked via G_i to the inhibition of adenylyl cyclase. Activation of muscarinic cholinoceptors may also serve to inhibit adenylyl cyclase via a functional inactivation of G_s (Pyne *et al.*, 1992c). The ability of $\beta\gamma$ G-protein subunits to bind to- and inactivate $Gs\alpha$ could possibly underlie this latter effect (Hildebrandt and Kohnken, 1990). The relative importance of M_2 and M_3 muscarinic receptor subtypes in determining the extent of inhibition of adenylyl cyclase is highly controversial (see Eglen *et al.*, 1994b and c; Roffel *et al.*, 1994a). Watson and Eglen (1994) suggested that M_2 receptors are primarily responsible for this inhibition since the selective M_2 antagonist methoctramine augmented the relaxant potency of isoprenaline in guinea-pig trachea at a concentration associated with minimal M_3 receptor occupancy. This data is supported by the observation in guinea-pig ileum that following alkylation of M_3 receptors, M_2 receptor activation inhibits the relaxant responses to β-adrenoceptor agonists (Reddy *et al.*, 1994). However, a similar study in guinea-pig trachea failed to reveal such an effect (Eglen *et al.*, 1994a). An alternative proposal is that the extent of β-adrenoceptor-induced relaxation is controlled instead by the level of M_3 receptor-mediated tone (Roffel *et al.*, 1994b). Indeed small alterations in the contractile tone are associated with large shifts in the potency of

isoprenaline for relaxation (van Amsterdam *et al.*, 1989). To date the study of M_2/M_3 receptor-mediated regulation of ASM contraction has been severely hampered by the absence of a highly selective M_2 receptor antagonist. The future development of more subtype-specific muscarinic receptor agonists, antagonists and antisera may lead to greater understanding of muscarinic receptor functions.

When the Hist-stimulated [^{3}H]InsP response was monitored at different times following Salm/Salb pre-incubation, a difference between the effects of the two β_2-adrenoceptor agonists was only observed at the earliest time-point studied (1 h), with no difference in the apparent duration of inhibition between the two agents. Hence it is unlikely that the inhibition of Hist-stimulated InsP formation by Salm explains the prolonged relaxant effects of this agonist observed both *in vitro* and *in vivo* (see Jack, 1991). The spasmolytic effects (relaxation of pre-contracted tissue) of these agents probably reflect the increased cAMP formation (Ellis *et al.*, 1995), while the inhibition of Hist-mediated phosphoinositide hydrolysis may be important in the maintenance of the relaxed state (anti-spasmogenic effect).

β_2-adrenoceptor agonists have proved to be useful tools in the treatment of asthma and hence the role of these agents in ASM relaxation has been a topic of much interest in recent years. β_2-adrenoceptor agonists may exert their relaxant effect by regulating the intracellular Ca^{2+} concentration. β-adrenoceptor stimulation of ASM is often accompanied by membrane hyperpolarisation (Allen *et al.*, 1985; Fujiwara *et al.*, 1988), which can be induced by both cAMP-dependent (Kume *et al.*, 1989) and -independent (Kume *et al.*, 1992, 1994) activation of high conductance Ca^{2+}-activated potassium channels (BK_{Ca}). Membrane hyperpolarisation

would prevent influx of Ca^{2+} through voltage-dependent Ca^{2+}-channels and could thereby influence the sustained (tonic) phase of contraction.

When considering the effects of β-adrenoceptor agonists, it should be appreciated that cAMP may activate PKG in addition to PKA: cAMP activates PKG with an EC_{50} (80 nM) only slightly greater than that for PKA (30 nM) in canine trachealis (Torphy *et al.*, 1982). Moreover, the relaxant potencies of cyclic nucleotide analogues against K^+-induced contractions of guinea-pig trachea strongly correlate with their EC_{50} values for PKG but not PKA (Francis *et al.*, 1988). Further to the effects of increased levels of cAMP, β-adrenoceptor agonists may also stimulate direct receptor-mediated effects to induce relaxation of ASM as evidenced by the cAMP-independent action of isoprenaline on BK_{Ca}.

In addition to modulating Ca^{2+} signalling by agonist-stimulated InsP formation, agents which activate both PKC and PKA may also be able to regulate intracellular Ca^{2+} release downstream of Ins(1,4,5)P_3 formation through the phosphorylation of the Ins(1,4,5)P_3 receptor. Consensus sequences for phosphorylation by PKA have been identified on the InsP_3R-1 (Furuichi *et al.*, 1989; Mignery *et al.*, 1990) and InsP_3R-2 (Sudhof *et al.*, 1991) receptors. Although these sequences are often conserved between tissues, phosphorylation of Ins(1,4,5)P_3 receptors by PKA appears to have different effects in different cell types. For example the sensitivity of the receptor from cerebellar microsomes is decreased by PKA-mediated phosphorylation (Supattapone *et al.*, 1988) whereas Ca^{2+} release from permeabilised hepatocytes is potentiated by pre-treatment of the cells with the catalytic subunit of PKA (Burgess *et al.*, 1991). The Ins(1,4,5)P_3 receptor is also known to be a target for PKC (Ferris *et al.*, 1991) and phosphorylation by this kinase is associated with accelerated Ca^{2+} release (Matter *et al.*, 1993).

A final attempt to manipulate InsPP formation and metabolism in BTSM utilised the glucocorticoid dexamethasone. While glucocorticoids have been shown to inhibit agonist-stimulated InsP formation in a number of tissues, the data obtained in BTSM show that dexamethasone is without effect on either InsP formation, or incorporation of inositol into the membrane lipids. These results are in agreement with Michoud and co-workers (1994) who demonstrated that treatment of cultured rat tracheal smooth muscle cells with 100 nM dexamethasone had no effect on the subsequent 5-hydroxytyptamine-stimulated PLC activity.

The experiments detailed in this chapter provide further insight into the factors regulating InsPP metabolism in ASM. It would appear from the experiments performed in the presence of PDBu that receptor-PIC coupling can be modulated in an agonist-specific way by PKC-mediated feedback. The Hist experiments indicate that a reduction in the concentration of Ins(1,4,5)P_3 (compared to CCh-stimulated concentrations) may facilitate a relative increase in its metabolism through the 3-kinase pathway. Whether this reflects a direct effect of PKC in regulating the activity of the 3-kinase and 5-phosphatase enzymes themselves, or is simply due to the higher affinity of the 3-kinase enzyme for Ins(1,4,5)P_3 is uncertain. The reduced formation of InsPs in response to both Hist and CCh in the presence of PDBu highlight the possibility for cross-talk between different signal transduction pathways (e.g. inhibition of phosphoinositide hydrolysis by the PLD-mediated production of DAG). The susceptibility of both Hist- and CCh-stimulated InsP responses to PDBu indicate similar components may be involved in the hydrolysis of phosphoinositides in response to these two agonists. The differential sensitivities of the two responses to PDBu suggest however, that the Hist-

and CCh- receptors themselves (and the G-proteins/PIC isozymes/phosphoinositide pools they recruit) may be subject to differential regulation. A similar differential inhibitory effect is exerted by β_2-adrenoceptor agonists, i.e. the Hist-stimulated InsP response can be inhibited by both Salm and Salb, while the CCh-stimulated response is unaffected by these agents. The effect of Salm and Salb on Hist-stimulated InsP accumulation further substantiates a role for cross-talk between signal transducing pathways in the regulation of InsP formation. However, the very similar time-course for the inhibition of Hist-stimulated [^{3}H]InsP accumulation by these agents, despite differing cAMP accumulation and relaxant effects, suggests that inhibition of phosphoinositide hydrolysis may not be the primary mechanism underlying β-adrenoceptor-mediated relaxation of ASM. In contrast to many other tissues studied, InsP accumulation does not appear to be regulated by the glucocorticoid dexamethasone in BTSM.

CHAPTER SIX
AGONIST-STIMULATED ACCUMULATION OF INOSITOL 4,5-BISPHOSPHATE IN BOVINE TRACHEAL SMOOTH MUSCLE

6.1 INTRODUCTION

Muscarinic receptor stimulation of BTSM slices results in a Li^+-sensitive accumulation of a novel inositol bisphosphate isomer, namely [^{3}H]Ins(4,5)P_2 (see section 3.4). This isomer has previously been detected in only a limited number of mammalian cell types: thyrotropin-releasing hormone-stimulated GH_3 cells (Dean and Moyer, 1987; Hughes and Drummond, 1987), unstimulated WRK1 rat mammary tumour cells (Wong *et al.*, 1992) and CCh-stimulated rat cerebral cortex slices (Batty *et al.*, 1989; Jenkinson *et al.*, 1992). Jenkinson and co-workers (1992) have carried out the only detailed study of the accumulation of [^{3}H]Ins(4,5)P_2 to date and, in agreement with the data described here and with Hughes and Drummond (1987), showed that the accumulation of [^{3}H]Ins(4,5)P_2 is Li^+-sensitive.

Since the accumulation of Ins(4,5)P_2 in BTSM, GH_3 cells and in rat cerebral cortex slices may be agonist-stimulated, it is possible that this novel InsP_2 isomer may play some role in signal transduction. Ins(4,5)P_2 has been shown to be as effective as Ins(1,4,5)P_3 in mobilising Ca^{2+} from non-mitochondrial intracellular stores in permeabilised guinea-pig hepatocytes (Burgess *et al.*, 1984) and Swiss mouse 3T3 cells (Irvine *et al.*, 1984) albeit with a much lower potency. Ins(4,5)P_2 does not appear to accumulate to very high concentrations even under agonist-stimulated conditions as it represents only 2.9 ± 0.1 % and 1.71 ± 0.21 % of the total

$[^3H]InsP_2$ pool following 30 min CCh stimulation in rat cerebral cortex slices (Batty *et al.*, 1989) and BTSM slices (see section 3.4.1) respectively. It is possible however, that this isomer may play a significant calcium-signalling role at later time-points in the agonist-stimulated response, when the $Ins(1,4,5)P_3$ concentration has returned to basal values, and may be of particular importance in patients receiving Li^+ therapy for manic depression or other affective disorders. $Ins(4,5)P_2$ has also been shown to be an effective inhibitor of the Ca^{2+}-ATPase of human erythrocytes *in vitro* (Davis *et al.*, 1991). In addition, $Ins(4,5)P_2$ may function as an extracellular signalling molecule since superfused $Ins(4,5)P_2$ causes a concentration-dependent increase in luteinising hormone release from non-permeabilised rat pituitary cells (Przylipiak *et al.*, 1990).

The origins and metabolic fate of $Ins(4,5)P_2$ in the cell is unknown. There are several possible routes for both the formation and metabolism of this compound which are summarised in figure 6.1. $Ins(4,5)P_2$ may be formed by the action of a 1-phosphatase on $Ins(1,4,5)P_3$, or possibly $Ins(1,3,4,5)P_4$ followed by subsequent hydrolysis by a 3-phosphatase. A novel $Ins(1,4,5)P_3$ 1-phosphatase has been described in *Dictyostelium discoideum* (Van Lookeren Campagne *et al.*, 1988) but no mammalian counterpart has been identified to date. The well-characterised $Ins(1,4)P_2/Ins(1,3,4)P_3$ 1-phosphatase has no activity against either $Ins(1,4,5)P_3$ or $Ins(1,3,4,5)P_4$ (Inhorn and Majerus, 1987). Recent interest in the role of $InsP_5$ and $InsP_6$ in cell signalling was sparked by the observation of an agonist-stimulated conversion of $Ins(1,3,4,5,6)P_5$ to $Ins(3,4,5,6)P_4$ in rat pancreatoma cells (Menniti *et al.*, 1990). Hence mammalian cells appear to contain an alternative 1-phosphatase enzyme, the substrate specificity of which is currently unknown. The accumulation of $[^3H]Ins(3,4,5)P_3$ in avian erythrocytes has been

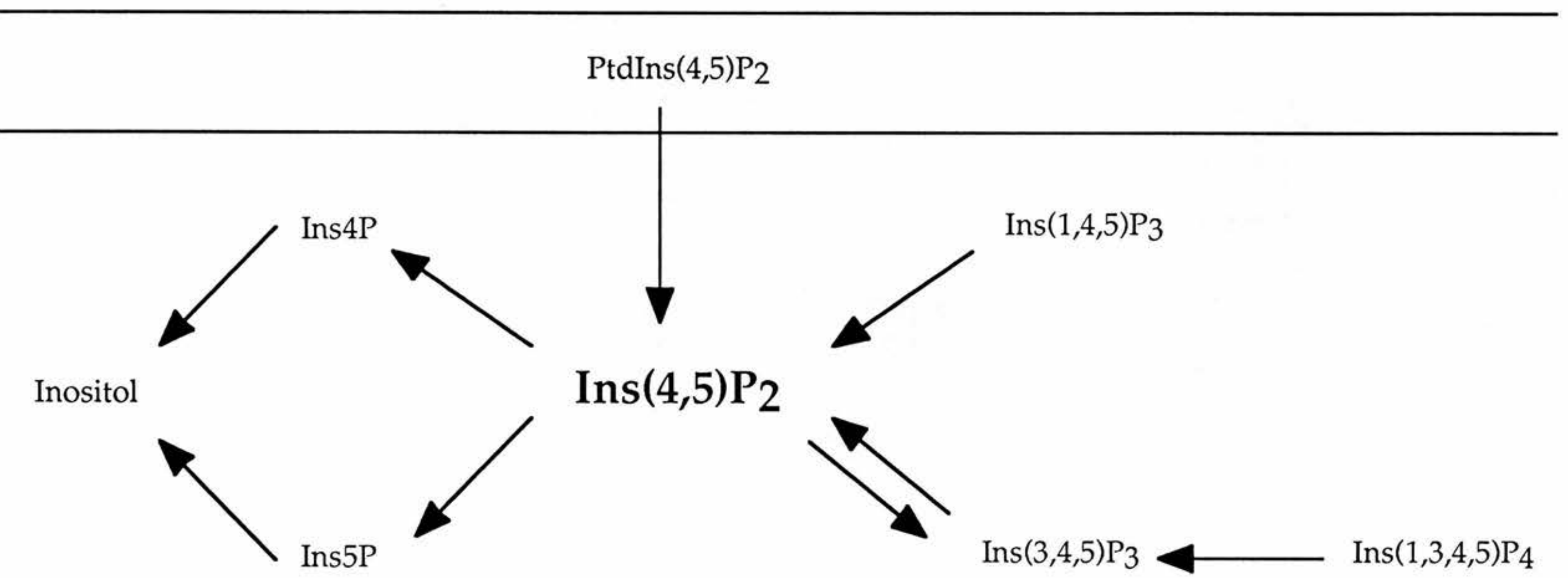

Figure 6.1. Possible pathways for the formation and metabolism of inositol 4,5-bisphosphate

documented (Stephens *et al.*, 1989); however this study indicated that $Ins(1,3,4)P_3$ was formed by a 6-phosphatase action on $Ins(1,3,4,6)P_4$.

An alternative route of $Ins(4,5)P_2$ formation could be a PLD-mediated hydrolysis of $PtdIns(4,5)P_2$. While a role for PLD in transmembrane signalling in ASM, secondary to PKC stimulation, has been suggested (Pyne and Pyne, 1993), there is little, if any, evidence to date implicating PLD in the hydrolysis of the phosphoinositides in ASM.

The metabolism of $Ins(4,5)P_2$ is likely to proceed by a dephosphorylation pathway to form either Ins4P or Ins5P. $Ins(4,5)P_2$ has been shown to be a substrate (albeit a poor one) for the $Ins(1,4,5)P_3/Ins(1,3,4,5)P_4$ 5-phosphatase (Mitchell *et al.*, 1989), whereas the significant accumulation of Ins5P observed in muscarinic receptor-stimulated rat cerebral cortex might suggest the involvement of a 4-phosphatase (Ackermann *et al.*, 1987). In addition, it is possible that $Ins(4,5)P_2$ could be phosphorylated to $Ins(3,4,5)P_4$ since it can be metabolised slowly by an $Ins(1,4,5)P_3$ 3-kinase purified from rat brain (Morris *et al.*, 1988).

The purpose of this chapter is to assimilate the data obtained in experiments described earlier with a focus on the accumulation of $Ins(4,5)P_2$. To expand on these studies further experimental data on the effect of Li^+ and phorbol ester pre-treatment on agonist-stimulated $[^3H]Ins(4,5)P_2$ accumulation are presented which give some insight into the possible routes of formation and metabolism of this novel inositol phosphate isomer in BTSM.

6.2 CARBACHOL- AND HISTAMINE-STIMULATED INOSITOL 4,5-BISPHOSPHATE ACCUMULATION

Experiments conducted to establish the flux of Ins(1,4,5)P_3 through the 3-kinase and 5-phosphatase pathways (detailed in section 2.4) utilised H.P.L.C. to separate and quantify individual [^{3}H]InsPP isomers. An unexpected observation of these experiments was the presence in BTSM slices of a late running [^{3}H]InsP_2 peak which was easily separated from the common [^{3}H]Ins(1,3)P_2, [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins(3,4)P_2 isomers (see figure 6.2.1). This novel [^{3}H]InsP_2 isomer co-eluted with [^{3}H]Ins(4,5)P_2 generated by alkaline hydrolysis of the [^{3}H]phosphoinositides isolated from BTSM (see section 2.6.3). Alkaline hydrolysis cleaves phosphoinositides into its glycerol and inositol phosphate moieties, and hence the major [^{3}H]InsP_2 isolated by this process is [^{3}H]Ins(4,5)P_2. Since no labelled alditols would be formed following periodate oxidation of [2-^{3}H]Ins(4,5)P_2, this technique could not be used to positively identify the isomeric composition of the late running [^{3}H]InsP_2. It may be possible to give a clearer identification of this isomer by examining the products formed following its dephosphorylation by a purified Ins(1,4,5)P_3/Ins(1,3,4,5)P_4 5-phosphatase.

Under basal conditions [^{3}H]Ins(4,5)P_2 was hardly detectable, however its accumulation increased following muscarinic cholinoceptor stimulation with CCh and was dramatically enhanced in the presence of 5 mM LiCl (see figure 6.2.2). Following prolonged (30 min) CCh (100 μM) stimulation in the presence of 5 mM LiCl, [^{3}H]Ins(4,5)P_2 represented 1.27 $\pm$ 0.06 % of the total retrieved [^{3}H]InsPPs and 8.61 $\pm$ 1.18 % of the total [^{3}H]InsP_2 pool. Time-course experiments indicated that the accumulation of [^{3}H]InsP_2 following receptor stimulation was delayed, only accumulating to levels significantly above basal after 5 min (Figure 6.2.3).

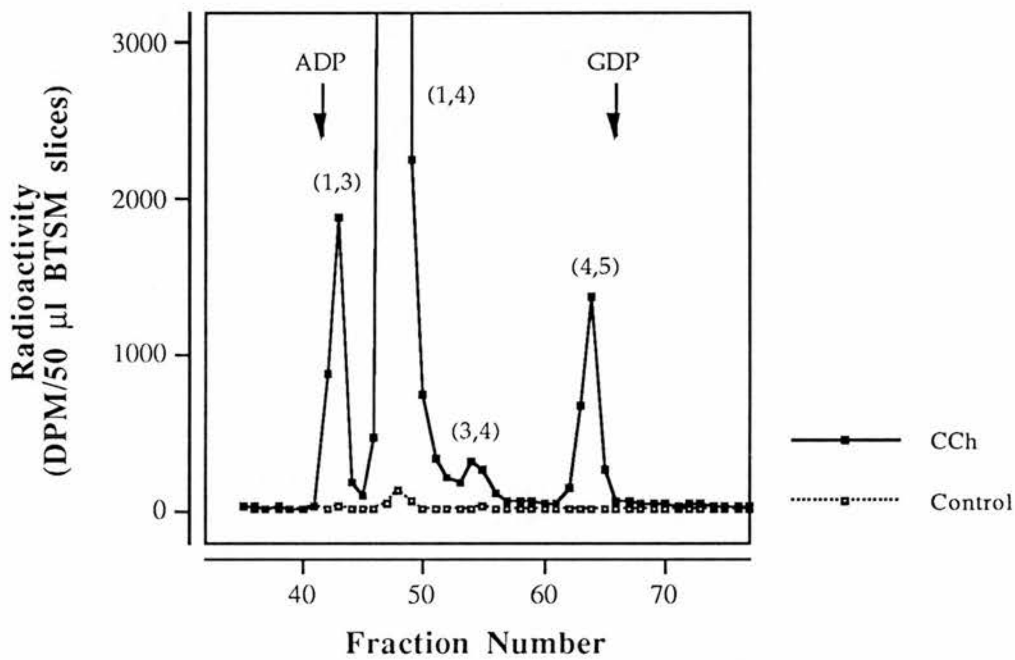

Figure 6.2.1 Typical H.P.L.C. elution profile obtained following carbachol stimulation of bovine tracheal smooth muscle slices

[^{3}H]Inositol pre-labelled BTSM slices (2.3.1) were incubated for 30 min at 37 °C in the presence of 5 mM LiCl and 100 µM CCh. [^{3}H]InsP$_2$s were separated from pooled triplicate TCA extracts (2.4.2) using a Partisphere 5 SAX H.P.L.C. column (2.6.3) and quantified by liquid scintillation counting.

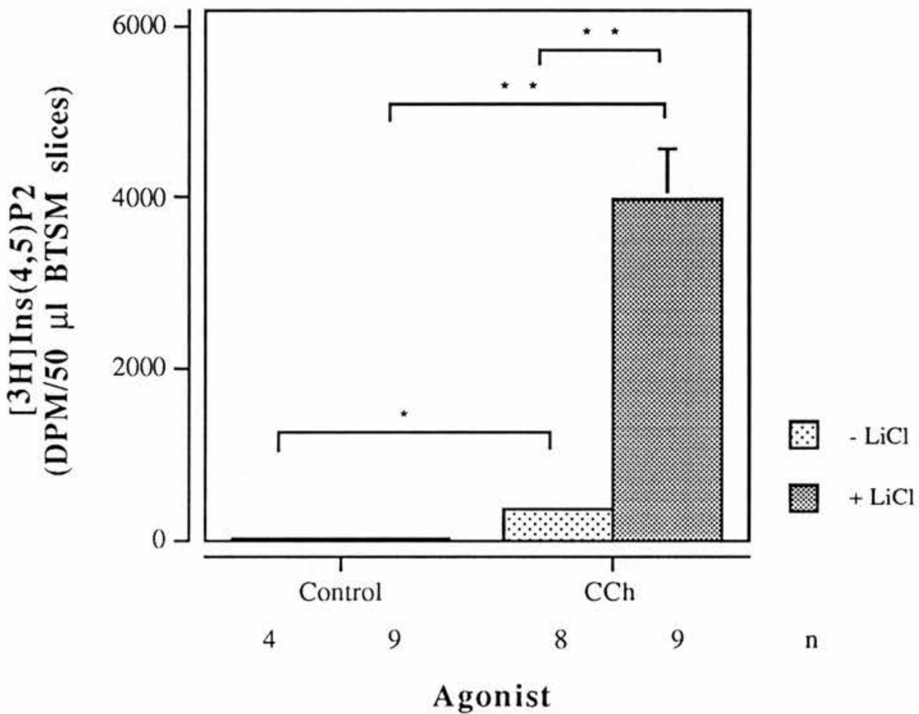

Figure 6.2.2 Effect of lithium ions on prolonged carbachol-stimulated inositol 4,5-bisphosphate accumulation

$[^3H]$Inositol pre-labelled BTSM slices (2.3.1) were incubated for 30 min at 37 °C in the presence or absence of 5 mM LiCl and 100 µM CCh. $[^3H]Ins(4,5)P_2$ was separated from pooled triplicate TCA extracts (2.4.2) using a Partisphere 5 SAX H.P.L.C. column (2.6.3) and quantified by liquid scintillation counting. Results represent the mean ± SEM of n separate experiments. * Represents $p < 0.05$; * * represents $p < 0.01$ (two-tailed, unpaired student's t-test).

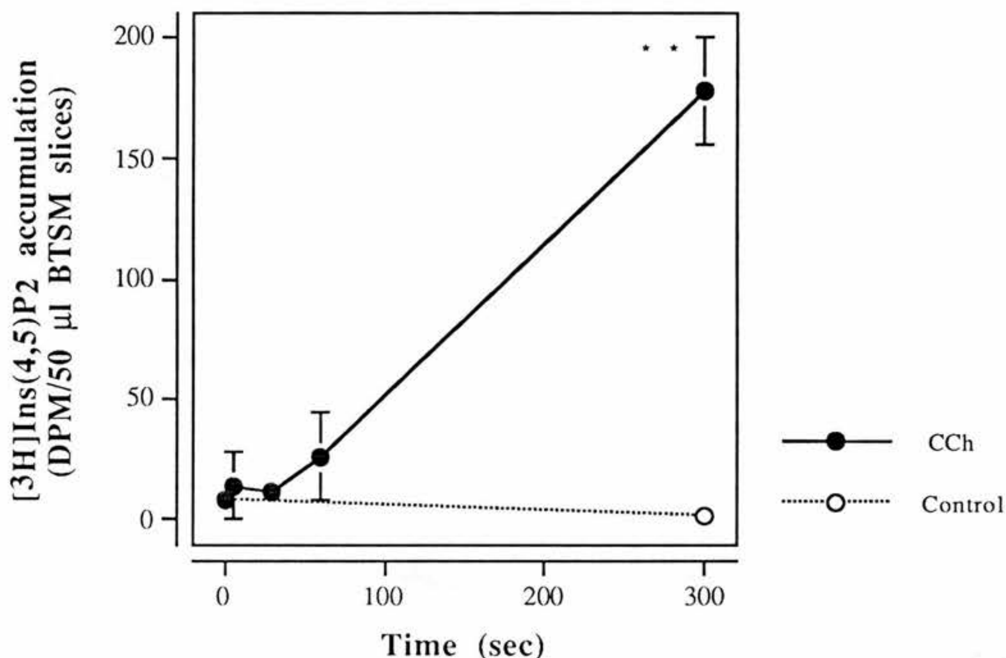

Figure 6.2.3 Time-course of carbachol-stimulated inositol 4,5-bisphosphate accumulation

[^{3}H]Inositol pre-labelled BTSM slices (2.3.1) were incubated with 100 µM CCh or vehicle in the presence of 5 mM LiCl in a final volume of 300 µl. Reactions were terminated at the times indicated by the addition of 300 µl 1 M TCA. [^{3}H]Ins(4,5)P_2 was separated from pooled triplicate neutralised extracts (2.4.2) by H.P.L.C. (2.6.3) and the radioactivity associated with each isomer quantified by liquid scintillation counting. Results represent the mean ± SEM of three separate experiments. * Represent $p < 0.05$; ** represents $p < 0.01$ for comparisons with basal values (two-tailed, paired student's t-test).

This delayed accumulation of $[^3H]Ins(4,5)P_2$ contrasts the very rapid generation and accumulation of $[^3H]Ins(1,4,5)P_3$ and $[^3H]Ins(1,4)P_2$ (see section 3.4.2) and would suggest an indirect route for the formation of $[^3H]Ins(4,5)P_2$ following stimulation with CCh.

In order to gain some insight into the pathways responsible for the formation of $[^3H]Ins(4,5)P_2$ the effects of different agonists on $[^3H]Ins(4,5)P_2$ accumulation were examined. Experiments were performed comparing the effects of CCh (100 μM) and Hist (1 mM) stimulation on $[^3H]$InsPP accumulation in BTSM. Stimulation with agonists for 30 min in the presence of Li^+ resulted in a 1,751-fold increase in the accumulation of $[^3H]Ins(4,5)P_2$ in response to 100 μM CCh and a 106-fold increase in response to 1 mM Hist stimulation. These 'fold' stimulations may be misleading however, due to the negligible accumulation of $[^3H]Ins(4,5)P_2$ under basal conditions (2 ± 1 DPM/50 μl BTSM slices). CCh stimulation produces relatively more $[^3H]Ins(4,5)P_2$ (as a percentage of the total $[^3H]$InsPPs) than Hist stimulation (see fig 6.2.4). Hence it would seem that muscarinic-cholinoceptor stimulation of BTSM slices is significantly more effective in producing $[^3H]Ins(4,5)P_2$ than stimulation with histaminergic agonists.

6.3 EFFECT OF PHORBOL DIBUTYRATE ON CARBACHOL- AND HISTAMINE-STIMULATED [3H]INOSITOL 4,5-BISPHOSPHATE ACCUMULATION

It is possible that $[^3H]Ins(4,5)P_2$ may be formed secondary to PKC stimulation and a subsequent activation of PLD-mediated $[^3H]PtdIns(4,5)P_2$ hydrolysis. Hence it is interesting to consider the effects of phorbol esters on $[^3H]Ins(4,5)P_2$ accumulation to determine if PKC

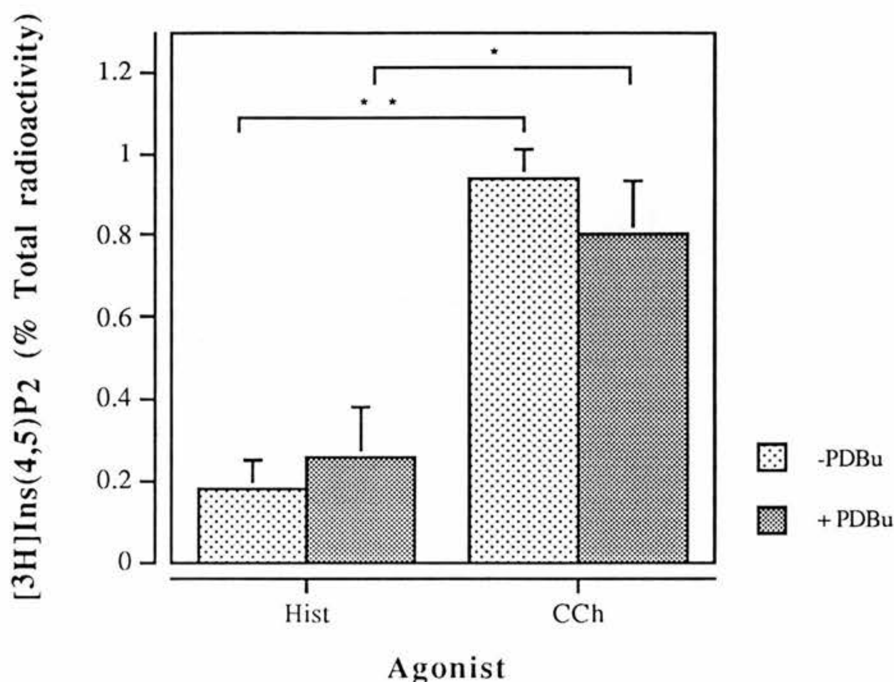

Figure 6.2.4 Effect of phorbol dibutyrate on histamine- and carbachol-stimulated [^{3}H]inositol 4,5-bisphosphate accumulation

[^{3}H]Inositol pre-labelled BTSM slices (2.3.1) were pre-incubated for 30 min at 37 °C in the presence or absence of 100 nM PDBu prior to Hist (1 mM)- or CCh (100 μM)-stimulation (30 min) in the presence of 5 mM LiCl. [^{3}H]Ins(4,5)P_2 was separated from pooled triplicate TCA extracts (2.4.2) using a Partisphere 5 SAX H.P.L.C. column (2.6.3) and quantified by liquid scintillation counting. Results are expressed as the percentage of total retrieved radioactivity associated with [^{3}H]Ins(4,5)P_2 and represent the mean ± SEM of three or five experiments for Hist and CCh incubations respectively. * Represents $p < 0.05$; * * represents $p < 0.01$ (two-tailed, unpaired student's t-test).

stimulation may alter the pattern of [^{3}H]Ins(4,5)P_2 formation. Pre-treatment of BTSM slices with 100 nM PDBu resulted in a decrease in the accumulation of [^{3}H]Ins(4,5)P_2 in both CCh- and Hist-stimulated BTSM slices (22.0 ± 15.5 % and 82.9 ± 12.4 %, section 5.3.1). However, these decreases reflected the observed decreases in total [^{3}H]InsPPs (12.5 ± 2.1 and 71.1 ± 3.8 for CCh- and Hist-stimulated slices respectively) and therefore the percentage of [^{3}H]InsPPs present as [^{3}H]Ins(4,5)P_2 remained unaltered (two-tailed, unpaired student's t-test) by PDBu treatment (see figure 6.2.4).

6.4 EFFECT OF LITHIUM IONS ON [^{3}H]INOSITOL 4,5-BISPHOSPHATE ACCUMULATION

As described above, the CCh-stimulated accumulation of [^{3}H]Ins(4,5)P_2 is highly sensitive to Li^+. Jenkinson *et al.* (1992) demonstrated a bell-shaped Li^+-concentration curve for the CCh-stimulated accumulation of [^{3}H]Ins(4,5)P_2 in rat cerebral cortex slices. If a similar response to Li^+ occurs in BTSM it is possible that an even greater accumulation of [^{3}H]Ins(4,5)P_2 may occur at lower, therapeutically relevant concentrations of Li^+. Hence the accumulation of [^{3}H]InsPP isomers in response to prolonged CCh stimulation over a range of LiCl (0 - 10 mM) concentrations was studied. The results are shown in table 6.4. At low concentrations of LiCl (≤ 10 mM) [^{3}H]Ins(4,5)P_2 accumulates in a manner similar to the Li^+-sensitive [^{3}H]InsP_1s with a progressive increase in [^{3}H]Ins(4,5)P_2 levels, suggesting a Li^+-sensitive metabolism of this isomer. The EC_{50} for the effect of Li^+ on [^{3}H]Ins(4,5)P_2 accumulation is 1.06 mM compared to 1.12 mM and 0.87 mM for [^{3}H]Ins1/3P and [^{3}H]Ins4P accumulation respectively. Extending the Li^+ concentration range

[^{3}H]Ins(x)PP	Radioactivity associated with [^{3}H]Ins(x)PP (% Control) [LiCl] (mM)							
	0 (n = 4)	0.01 (n = 4)	0.1 (n = 4)	0.3 (n = 4)	1 (n = 4)	3 (n = 4)	10 (n = 4)	30 (n = 1)
1/3	100 ± 17	111 ± 13	129 ± 8	232 ± 26	490 ± 38	925 ± 162	1208 ± 470	1004
4	100 ± 20	116 ± 15	136 ± 11	279 ± 39	774 ± 59	1497 ± 259	1894 ± 963	725
1,3	100 ± 17	91 ± 7	104 ± 17	117 ± 10	127 ± 18	131 ± 23	131 ± 25	140
1,4	100 ± 17	108 ± 13	104 ± 10	139 ± 10	153 ± 5	213 ± 41	480 ± 167	634
3,4	100 ± 14	92 ± 3	114 ± 19	112 ± 9	119 ± 19	111 ± 15	159 ± 34	264
4,5	100 ± 19	105 ± 11	140 ± 16	237 ± 31	525 ± 28	1191 ± 231	1415 ± 644	642
1,3,4	100 ± 18	89 ± 4	105 ± 114	115 ± 6	125 ± 8	127 ± 20	131 ± 33	153
1,4,5	100 ± 15	99 ± 15	101 ± 10	105 ± 6	113 ± 6	89 ± 15	101 ± 27	90
2,4,5	100 ± 31	356 ± 256	242 ± 113	158 ± 74	70 ± 27	180 ± 108	258 ± 121	71
$InsP_4$	100 ± 14	102 ± 13	259 ± 139	123 ± 3	121 ± 8	123 ± 21	124 ± 35	114
Total [^{3}H]InsPs	100 ± 16	107 ± 11	118 ± 10	190 ± 16	377 ± 23	706 ± 129	910 ± 361	644

Table 6.4 Effect of lithium ions on the carbachol-stimulated accumulation of [^{3}H]inositol polyphosphate isomers

[^{3}H]Inositol pre-labelled BTSM slices (2.3.1) were stimulated with 100 μM CCh in the presence of the indicated concentration of LiCl for 30 min. Individual [^{3}H]InsPP isomers were separated from pooled triplicate TCA extracts (2.4.2) using a Partisphere 5 SAX H.P.L.C. column (2.6.3) and quantified by liquid scintillation counting. Results are expressed as the radioactivity associated with each [^{3}H]InsPP isomer as a percentage of the value obtained in the absence of lithium ions, and represent the mean ± SEM of n separate experiments.

however, demonstrated a marked attenuation of $[^3H]Ins(4,5)P_2$ accumulation in the presence of 30 mM LiCl (see table 6.4, figure 6.4.1). Hence, in accordance with the results of Jenkinson *et al.* (1992) a bell-shaped Li^+ concentration-response curve for the accumulation of $[^3H]Ins(4,5)P_2$ is observed if LiCl is increased to high (> 10 mM) concentrations. It is important to note that 30 mM LiCl also caused a marked decrease in $[^3H]Ins4P$ accumulation (table 6.4, figure 6.4.2) and a parallel increase in $[^3H]Ins(1,4)P_2$ accumulation (table 6.4) implying that this concentration of Li^+ can inhibit the 1-phosphatase responsible for the conversion of $Ins(1,4)P_2$ to Ins4P. The near identical concentration-dependence of both $[^3H]Ins4P$ and $[^3H]Ins(4,5)P_2$ on Li^+ ions suggest that similar enzymes might be involved in the formation and metabolism of these two $[^3H]InsPs$.

6.5 DISCUSSION

The demonstration of an agonist-stimulated accumulation of $[^3H]Ins(4,5)P_2$ in BTSM is of particular interest since the accumulation of this isomer cannot be accounted for by any conventional routes of mammalian InsPP metabolism. The agonist-stimulated nature of its accumulation from very low basal levels suggests that $Ins(4,5)P_2$, or one of its precursors or metabolites, may play an important role in signalling in this tissue. Furthermore, the sensitivity of its accumulation in the presence of Li^+ ions suggests that the flux of the inositol headgroup through $Ins(4,5)P_2$ may be substantial *in vivo*.

Two alternative routes of formation of $Ins(4,5)P_2$ are PLD action on $PtdIns(4,5)P_2$ or metabolism of $Ins(1,4,5)P_3$. Muscarinic cholinergic stimulation of BTSM slices produced a greater proportion of $[^3H]Ins(4,5)P_2$

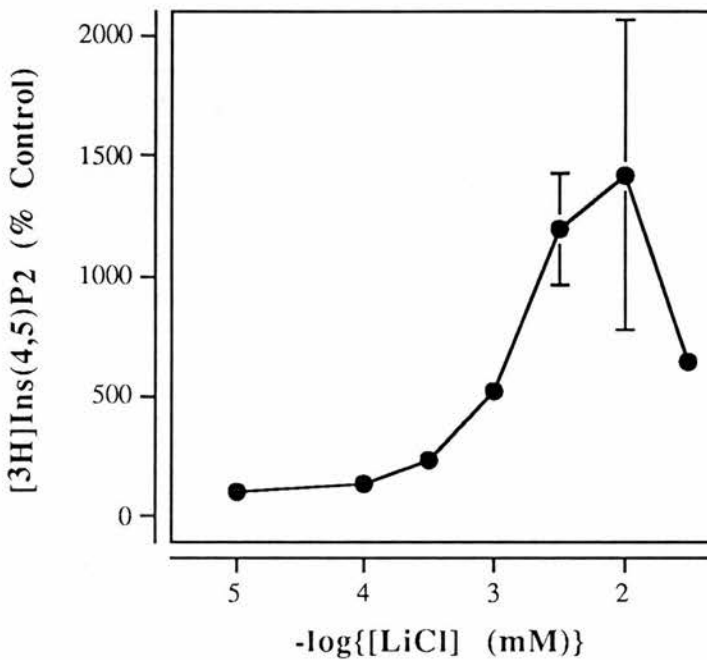

Figure 6.4.1 Effect of lithium ion concentration on carbachol-stimulated [^{3}H]inositol 4,5-bisphosphate accumulation

[^{3}H]Inositol pre-labelled BTSM slices (2.3.1) were stimulated with 100 μM CCh in the presence of the indicated concentration of LiCl for 30 min. [^{3}H]Ins(4,5)P_2 was separated from pooled triplicate TCA extracts (2.4.2) using a Partisphere 5 SAX H.P.L.C. column (2.6.3) and quantified by liquid scintillation counting. Results are expressed as the radioactivity associated with [^{3}H]Ins(4,5)P_2 as a percentage of the value obtained in the absence of LiCl, and represent the mean ± SEM of four separate experiments.

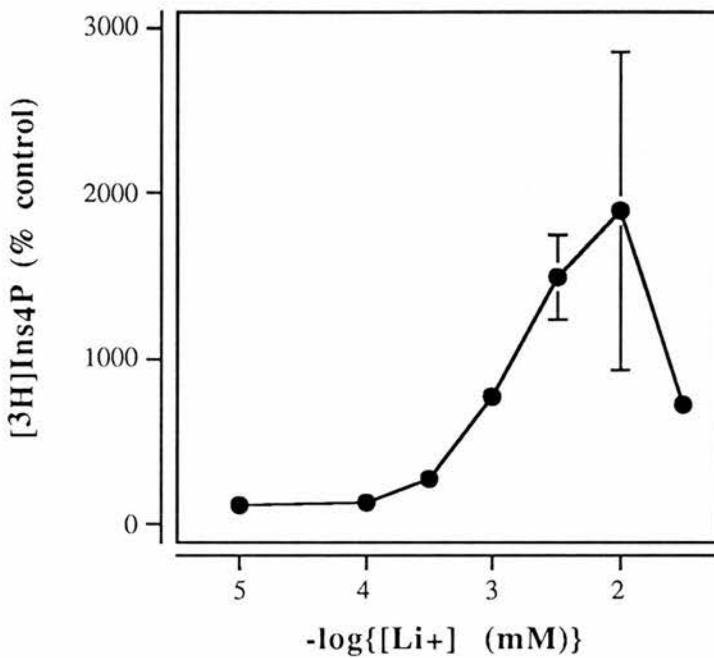

Figure 6.4.2 Effect of lithium ion concentration on carbachol-stimulated [^{3}H]inositol 4-monophosphate accumulation

[^{3}H]Inositol pre-labelled BTSM slices (2.3.1) were stimulated with 100 μM CCh in the presence of the indicated concentration of LiCl for 30 min. [^{3}H]Ins4P was separated from pooled triplicate TCA extracts (2.4.2) using a Partisphere 5 SAX H.P.L.C. column (2.6.3) and quantified by liquid scintillation counting. Results are expressed as the radioactivity associated with [^{3}H]Ins4P as a percentage of the value obtained in the absence of LiCl, and represent the mean ± SEM of four separate experiments.

(in comparison to total [^3H]InsPPs) than histaminergic stimulation. It is possible therefore that CCh stimulation may effect Ins(4,5)P_2 accumulation by routes other than Ins(1,4,5)P_3 metabolism. However, as detailed in the previous chapter, Ins(1,4,5)P_3 can be channelled differentially through the 3-kinase and 5-phosphatase pathways in response to CCh and Hist; equally a putative 1-phosphatase activity could be subject to such differential regulation.

Studies in a variety of cell types have indicated that receptor-mediated activation of PLD may occur through several mechanisms including protein tyrosine kinase activation (e.g. Cook and Wakelam, 1992), increases in intra- and extracellular Ca^{2+} (e.g. Lin and Gilfillan, 1992; Wu *et al.*, 1992) and activation of G-proteins (MacNulty *et al.*, 1992). However PLD activation is most widely recognised to occur secondary to PKC activation since in most cells phorbol esters cause persistent activation of both PKC and PLD (see Shukla and Halenda, 1991). It would seem unlikely that a PKC-stimulated PtdIns(4,5)P_2-specific PLD activity was responsible for the [^3H]Ins(4,5)P_2 accumulation in the present study for the following reasons: (1) CCh-stimulated activation of PLD in BTSM and bradykinin-stimulated PLD activation in guinea-pig ASM cells is rapid and transient and therefore could not (alone) account for the delayed and progressive accumulation of [^3H]Ins(4,5)P_2 observed in BTSM (Challiss *et al.*, manuscript in preparation; Pyne and Pyne, 1993); and (2) treatment of BTSM slices with PDBu fails to induce [^3H]Ins(4,5)P_2 accumulation or alter the proportion of [^3H]Ins(4,5)P_2 accumulating in response to either Hist or CCh. This latter observation is in agreement with the study by Jenkinson *et al.* (1992) in rat cerebral cortex in which neither activation nor inhibition of PKC had any effect on the basal or CCh-stimulated accumulation of [^3H]Ins(4,5)P_2.

$Ins(4,5)P_2$ is more likely produced by the action of a novel 1-phosphatase on $Ins(1,4,5)P_3$ similar to that described in the slime mould *Dictyostelium discoideum* (Van Lookeren Campagne *et al.*, 1988). These authors demonstrate that the major route of dephosphorylation of $Ins(1,4,5)P_3$ is via a 1-phosphatase and that the resulting $Ins(4,5)P_2$ is further degraded to Ins4P. Hence, as suggested by Bominaar *et al.* (1991), two novel enzymes catalysing the specific dephosphorylation of $Ins(1,4,5)P_3$ at the 1-position and of $Ins(4,5)P_2$ at the 5-position may exist in this organism, in addition to the more familiar $Ins(1,4,5)P_3/Ins(1,3,4,5)P_4$ 5-phosphatase and the $Ins(1,4)P_2/Ins(1,3,4)P_3$ 1-phosphatase. Jenkinson *et al* (1992) were unable to demonstrate a dephosphorylation of $[^3H]Ins(1,4,5)P_3$ to $[^3H]Ins(4,5)P_2$ with crude brain homogenates. However, Hughes and Drummond (1987) state that in the absence of added ATP, sonicated GH_3-cell preparations can, to small extent, metabolise $[^3H]Ins(1,4,5)P_2$ to a substance with identical chromatographic properties to $[^3H]Ins(4,5)P_2$. It is possible therefore that compartmentalisation or concentration of $Ins(1,4,5)P_3$ with the putative $Ins(1,4,5)P_3$ 1-phosphatase *in vivo* may allow such a reaction to proceed.

A dramatic enhancement of $[^3H]Ins(4,5)P_2$ accumulation was observed in the presence of low concentrations of Li^+. An EC_{50} of 1.06 mM was calculated for this effect, approximately one order of magnitude greater than that determined in rat cerebral cortex (94 µM, Jenkinson *et al.*, 1992). These data suggest that metabolism of either $Ins(4,5)P_2$ or possibly its primary metabolite is inhibited by Li^+. In the latter case the metabolism of $Ins(4,5)P_2$ would be subject to product inhibition. Support for this hypothesis comes from the study by Jenkinson *et al.* (1992) in rat cerebral cortex. After muscarinic receptor blockade with atropine any accumulated $[^3H]Ins(4,5)P_2$ quickly decays in the absence of Li^+, implying

that metabolism of this isomer occurs rapidly in the intact cell. In the presence of Li^+ however, the metabolism of $[^3H]Ins(4,5)P_2$ was suppressed. $Ins(4,5)P_2$ is likely to be metabolised to either Ins4P or Ins5P. As detailed earlier, $Ins(4,5)P_2$ has been shown to be a weak substrate for the $Ins(1,4,5)P_3/Ins(1,3,4,5)P_4$ 5-phosphatase (Mitchell *et al.*, 1989), however this enzyme is not Li^+-sensitive (Connolly *et al.*, 1985; Seyfred *et al.*, 1984) nor is it likely to be inhibited by Ins4P since this enzyme is largely unaffected by monophosphorylated compounds (Kennedy *et al.*, 1990). The possibility remains however, that $Ins(4,5)P_2$ may be metabolised by a novel $Ins(4,5)P_2$ 5-phosphatase such as that described in *Dictyostelium discoideum* (Bominaar *et al.*, 1991). Conversely involvement of a 4-phosphatase in the metabolism of $Ins(4,5)P_2$ is suggested by the presence of Ins5P in rat cerebral cortex (Ackermann *et al.*, 1987), a tissue in which $Ins(4,5)P_2$ has been detected.

The bell-shaped Li^+ concentration-response curve observed for $[^3H]$Ins4P accumulation can be explained by the competitive inhibitory action of Li^+ on the inositol monophosphatase enzyme, and their less potent inhibitory effect on the $Ins(1,4)P_2/Ins(1,3,4)P_3$ 1-phosphatase. At low concentrations of Li^+ inositol monophosphatase is strongly inhibited ($K_i \leq 1$ mM, Gee *et al.*, 1988; Hallcher and Sherman, 1980) resulting in a rapid accumulation of $[^3H]$Ins4P. At higher concentrations of LiCl however , the 1-phosphatase is effectively inhibited ($K_i \sim 6$ mM, Inhorn and Majerus 1987), thus decreasing the supply of $[^3H]$Ins4P and therefore its accumulation. The formation of $Ins(4,5)P_2$ from $Ins(1,4,5)P_3$ by a Li^+-sensitive $Ins(1,4,5)P_3$ 1-phosphatase akin to that found in *Dictyostelium discoideum* (Van Lookeren Campagne *et al.*, 1988) could similarly account for the bell-shaped Li^+ concentration-response curve for $[^3H]Ins(4,5)P_2$ accumulation.

$[^3H]Ins(4,5)P_2$ accumulation is stimulated in BTSM by muscarinic- and histaminergic receptor stimulation. The former receptor appears to couple more effectively to $Ins(4,5)P_2$ generation. The accumulation of this novel bisphosphate isomer is highly sensitive to Li^+, indicating that *in vivo*, in the absence of Li^+, there may be a substantial flux of the inositol headgroup through $Ins(4,5)P_2$. The lack of effect of pre-treatment with PDBu on the pattern of basal, Hist- or CCh-stimulated $Ins(4,5)P_2$ accumulation renders it unlikely that PKC-stimulated, PLD-mediated hydrolysis of $PtdIns(4,5)P_2$ is the major source of $Ins(4,5)P_2$ in this tissue. However, a role for PLD hydrolysis, activated by an alternative mechanism such as that described in human polymorphic nuclear leukocytes (Perianin *et al.*, 1993), in $Ins(4,5)P_2$ production cannot be ruled out. The most likely source of $Ins(4,5)P_2$ in BTSM appears to be $Ins(1,4,5)P_3$. A Li^+-sensitive $Ins(1,4,5)P_3$ 1-phosphatase could account for the bell-shaped Li^+ concentration-response curve observed for $[^3H]Ins(4,5)P_2$ accumulation. This therefore may represent a novel additional route for $Ins(1,4,5)P_3$ metabolism especially at later time-points of agonist stimulation when the 5-phosphatase activity decreases. $Ins(4,5)P_2$ is probably further metabolised to either Ins4P or Ins5P. While the primary metabolite of $Ins(4,5)P_2$ remains uncertain, it is clear that the enzyme responsible for the dephosphorylation of this $InsP_2$ is strongly inhibited by Li^+ ions.

SUMMARY

Excessive bronchoconstriction is an important factor governing airways calibre in conditions such as asthma and the pharmacological manipulation of the contractile response of ASM plays a central role in the treatment of this and many other respiratory diseases. Contraction is initiated following stimulation of surface receptors by spasmogens and the generation of Ins(1,4,5)P_3. This second messenger plays a pivotal role in pharmacomechanical coupling in ASM and is subject to rapid agonist-stimulated metabolism via a complex cascade of reactions which to date have been poorly studied in this tissue. A knowledge of the factors which determine the intracellular concentration of Ins(1,4,5)P_3 is of considerable importance since it may facilitate the generation of therapeutic modulators of ASM contraction and provide an understanding of the mechanisms underlying the currently used bronchodilators. The aims of this thesis were to study, in detail, the formation and metabolism of Ins(1,4,5)P_3 in an ASM model and to determine the factors which could modulate this response.

A model system was established in which BTSM slices were labelled to steady-state with *myo*-[^{3}H]inositol and subsequently stimulated with agonist for various time-periods in the presence of Li^+ ions to prevent the breakdown of InsP_1 isomers. This protocol allowed accurate quantification of InsPP accumulation following agonist-stimulation of BTSM. An important finding of the initial studies was the lack of demonstrable phosphoinositide hydrolysis following either membrane depolarisation or physiological increases in $[Ca^{2+}]_i$. In addition, no accumulation of InsPs was observed following activation of PKC with

PDBu, indicating that PKC-stimulated PtdIns-specific PLD activity is unlikely to contribute significantly to the agonist-stimulated InsP accumulation. The model therefore provided an appropriate system for monitoring the metabolism of agonist-stimulated, PtdIns(4,5)P_2-derived Ins(1,4,5)P_3. The muscarinic cholinoceptor agonist carbachol (CCh) induced a rapid and transient accumulation of [^{3}H]InsP_3 accompanied by secondary increases in [^{3}H]InsP_2 and delayed increases in [^{3}H]InsP_1. Experiments utilising cell-free preparations of BTSM indicated that, as in other tissues, Ins(1,4,5)P_3 could be metabolised by two major pathways - the 3-kinase and 5-phosphatase pathways - to yield a series of mutually exclusive InsPP products. Further experiments were therefore undertaken to determine the relative contributions of these two pathways to agonist-stimulated Ins(1,4,5)P_3 metabolism in BTSM. Following prolonged (30 min) stimulation with a maximally effective concentration of CCh the 5-phosphatase pathway accounted for 75 % of the total metabolism of [^{3}H]Ins(1,4,5)P_3. Detailed time-course analysis however revealed that the flux of [^{3}H]Ins(1,4,5)P_3 through the two enzyme pathways varied during the agonist-stimulated response. The 5-phosphatase pathway was highly dominant especially at early time-points, accounting for more than 85 % of the total metabolism over the first 5 sec following agonist-addition. The 3-kinase pathway became increasingly important at later time-points.

Since the 3-kinase pathway provides many additional putative second messenger and functional molecules the factors governing the regulation of metabolism through the two pathways may be extremely important to cell signalling and the contractile response. To gain a greater understanding of the enzymes involved, kinetic studies of the Ins(1,4,5)P_3 3-kinase and Ins(1,4,5)P_3 5-phosphatase were undertaken. The 5-

phosphatase activity was found to be predominantly particulate. The similar affinity of the soluble and particulate 5-phosphatase activities indicate that these enzymes may be identical and are likely to be Type Ia isoforms. The particulate 5-phosphatase had a higher capacity and lower affinity for $Ins(1,4,5)P_3$ metabolism (V_{max} = 45-50 nmol min^{-1} mg $protein^{-1}$; K_m = 15-20 μM) than the soluble 3-kinase (V_{max} = 323-360 pmol min^{-1} mg $protein^{-1}$; K_m = 2-2.5 μM). The affinity of the 3-kinase however was relatively low compared to studies obtained in most other tissues. A finding of particular interest in the current study was that neither the 3-kinase nor 5-phosphatase activities were significantly affected by physiologically relevant increases in $[Ca^{2+}]_{free}$. The 3-kinase is generally thought to be activated by Ca^{2+} via an increase in its V_{max}. This conclusion however is based on studies conducted in the absence of calpain inhibitors. The 3-kinase has been shown to be a substrate for calpain and sequencing studies have demonstrated that it contains several putative calpain cleavage sites. The inclusion of calpain inhibitors in the current study indicates that the intact enzyme, at least in this model system, is insensitive to changes in $[Ca^{2+}]_{free}$, and highlights the possibility for activation of this enzyme following limited calpain-mediated proteolysis. An important area of further research with clinical applications may therefore be the study of regulators of calpain activity and their effect on the ASM contractile response.

Stimulation of BTSM with a maximally effective concentration of histamine (Hist) results in a decreased (~ 30 %) $[^3H]$InsP and $Ins(1,4,5)P_3$ response compared to stimulation with a maximally effective concentration of CCh. This decrease in the initial accumulation of $Ins(1,4,5)P_3$ is associated with a relatively greater metabolism of $Ins(1,4,5)P_3$ through the 3-kinase pathway. It is quite possible that the

'stimulation' of the 3-kinase simply reflects the higher affinity of this enzyme for $Ins(1,4,5)P_3$ rather than any direct modulation of the 3-kinase or 5-phosphatase enzyme kinetics.

The Hist- and CCh-stimulated $[^3H]$InsP responses displayed differential sensitivity to both activation of PKC with PDBu, and β_2-adrenoceptor stimulation with either salmeterol (Salm) or salbutamol (Salb). Pre-treatment of BTSM slices with PDBu resulted in 99 % and 76 % maximal inhibition of the (30 min) Hist- and CCh-stimulated $[^3H]$InsP responses respectively. The mechanisms underlying the PKC-mediated selective feedback inhibition of H_1 receptor-PIC coupling are unknown but may involve phosphorylation of the G-protein/PIC signal transducing machinery. These data therefore support the suggestion that the H_1 histaminergic and M_3 muscarinic receptors are coupled to alternative G-protein and/or PIC isozymes, and that a component of the desensitisation of the InsP response to Hist is mediated via a PKC inhibitory pathway.

The Hist-stimulated $[^3H]$InsP response, unlike the CCh-stimulated $[^3H]$InsP response, was also inhibited by pre-treatment with β_2-adrenoceptor agonists. Salm, a novel, long-acting β_2-adrenoceptor agonist was a considerably more potent inhibitor of the Hist-stimulated $[^3H]$InsP response (IC_{50} = 0.24 nM) than the short-acting β_2-adrenoceptor agonist Salb (IC_{50} = 10.8 nM). In order to determine whether the differential effects of Salm and Salb on agonist-stimulated $[^3H]$InsP production underlie their differential relaxant responses the time-courses of Salm- and Salb-mediated inhibition of Hist-stimulated $[^3H]$InsP accumulation were examined. Both agents resulted in a substantial but short-term inhibition of the Hist-stimulated $[^3H]$InsP response which gradually recovered between 1 and 24 h following addition of the β_2-adrenoceptor agonist. A significant difference in the Salm- and Salb-

mediated inhibition was only observed at the earliest time-point measured (1 h). Since Salm has been shown, in an identical model, to produce relaxation over a far longer time-period than Salb, this data would suggest that the inhibition of agonist-stimulated phosphoinositide hydrolysis may not be the major mechanism underlying β_2-adrenoceptor-mediated relaxation of ASM.

An important and unexpected finding encountered during the course of these studies was the agonist-stimulated and Li^+-sensitive accumulation of a novel [^{3}H]InsP_2 isomer tentatively identified as [^{3}H]Ins(4,5)P_2. Since the origins and metabolic fate of this isomer are currently unknown further detailed experiments were conducted to try and determine its possible routes of formation and metabolism. The CCh-stimulated accumulation of this isomer only reached significant values at later time-points (> 5 min) following agonist addition. The accumulation of [^{3}H]Ins(4,5)P_2 indicates the possible existence of a PtdIns-specific PLD activity in BTSM or of a novel Ins(1,4,5)P_3 1-phosphatase. Although the first suggestion cannot be ruled out entirely, such an activity does not appear to be stimulated following PKC activation (the dominant mechanism underlying PLD activation in ASM) since treatment of BTSM with PDBu was without effect on either basal [^{3}H]Ins(4,5)P_2 accumulation or the relative amount of [^{3}H]Ins(4,5)P_2 accumulation following Hist- or CCh stimulation. [^{3}H]Ins(4,5)P_2 and [^{3}H]Ins4P accumulation exhibited bell-shaped Li^+ concentration-response curves suggesting that similar enzymes may be responsible for the formation and metabolism of these two InsPs. The accumulation of [^{3}H]Ins4P at low concentrations of Li^+ is due to the uncompetitive inhibition of the inositol monophosphatase enzyme by Li^+. At higher concentrations of Li^+ the InsPP 1-phosphatase is also inhibited thereby decreasing the supply of [^{3}H]Ins4P from

$[^3H]Ins(1,4)P_2$. It is likely therefore that $Ins(4,5)P_2$ is both formed and metabolised by Li^+-sensitive enzymes. Hence it is proposed that a novel Li^+-sensitive $Ins(1,4,5)P_3$ 1-phosphatase similar to that identified in *Dictyostelium discoideum*, and a Li^+-sensitive $Ins(4,5)P_2$ 4- or 5-phosphatase exist in BTSM and account for observed concentration effects of Li^+ on the accumulation of $[^3H]Ins(4,5)P_2$. This data indicates the presence therefore of a novel, albeit relatively minor, route of $Ins(1,4,5)P_3$ metabolism in BTSM.

The studies documented in this thesis represent the first detailed analysis of the InsPP isomers present in BTSM and have highlighted the potential for the regulation of the pattern of InsPP accumulation by both PKA and PKC. The complexity of the phosphoinositide signal transducing pathways and their multiple regulatory mechanisms testify to the importance of phosphoinositide signalling in ASM. Hist and CCh appear to generate distinct patterns of InsPP accumulation in this tissue and these responses display differential sensitivity to regulation by β_2-adrenoceptor agonists and PKC activation. These data imply that the two agonists may utilise different G-protein or PIC isozymes, and hence a future generation of highly specific G-protein and PIC inhibitors may prove to be clinically relevant. In addition, a novel $InsP_2$ isomer - $Ins(4,5)P_2$ - has been identified whose potential functional role is currently unknown and which warrants further study.

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