



REVIEW

Protein kinase C isoenzymes: a review of their structure, regulation and role in regulating airways smooth muscle tone and mitogenesis

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Abbreviations: ACh, acetylcholine; ASM, airways smooth muscle; $[Ca^{2+}]_i$, intracellular calcium concentration; DAG, diacylglycerol; DPB, 12-deoxyphorbol 13-isobutyrate; ERK, extracellular signal-regulated kinase; H_2O_2 , hydrogen peroxide; K_{ATP} , ATP-sensitive K^+ channels; LC, light chain; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase kinase; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; OAG, 1-oleoyl-2-acetyl *sn*-glycerol; PDA, phorbol-12, 13-diacetate; PDBu, phorbol 12,13-dibutyrate; PDGF, platelet-derived growth factor; PDK, phosphatidylinositol-trisphosphate-dependent kinase; PI, phosphatidylinositol; PKC, protein kinase C; PKD, protein kinase D; PKM, protein kinase M; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; PRK, protein kinase C-related kinase; PS, phosphatidylserine; RACK, receptor for activated C-kinase; VDCC, voltage-dependent calcium channel

Introduction

Protein kinase C (PKC) is a multifunctional, cyclic nucleotide-independent protein kinase that phosphorylates serine and threonine residues in many target proteins. This enzyme was identified in bovine cerebellum by Nishizuka and co-workers (Takai *et al.*, 1977; Inoue *et al.*, 1977) as a protein kinase that phosphorylated histone and protamine. Since its discovery, much interest has been shown in PKC and its role in signal transduction. Development (Otte *et al.*, 1991), memory (Alkon, 1989), differentiation (Cutler *et al.*, 1993), proliferation (Murray *et al.*, 1993) and carcinogenesis (Ashendel, 1985) all are processes for which PKC has been implicated. Once thought to be a single protein, PKC is now known to comprise a large family of enzymes that differ in structure, cofactor requirements and function. Indeed, the PKC family is the largest serine/threonine-specific kinase family known (Parker, 1992) to which many cellular responses have been credited (Nishizuka, 1995). This enzyme multiplicity, together with variation in cellular and tissue distribution, and abundance might explain why so many signal transduction functions have been attributed to this kinase. Here we briefly describe the organization and regulation of PKC and review the current understanding of their role in the regulation of airways smooth muscle (ASM) tone and mitogenesis, which have been investigated in some detail.

Classification and organization of PKC

PKC represents a structurally homologous group of proteins of which at present 10 isoforms have been identified unequivocally. The PKC family can be broadly divided into three groups that differ in their cofactor requirements. These

are known as conventional (c)PKC isoforms (α , β_1 , β_{II} and γ), that require Ca^{2+} and diacylglycerol (DAG) to become activated; novel (n)PKC isoforms (δ , ϵ , η , θ and μ) that require only DAG; and the atypical (a)PKC isoforms, namely ζ , ι and λ (the mouse homologue of human PKC ι), that require neither Ca^{2+} nor DAG. All PKC family members possess a phosphatidylserine (PS) binding domain for membrane interaction (though the specific lipid activators of aPKC's are not yet known). The general structure of a PKC molecule consists of a catalytic and a regulatory domain found at the C- and N-terminus respectively. Both domain structures are composed of a number of conserved regions (C1–C4) interspersed with regions of lower homology, so-called variable domains, V0–V5 (Figure 1).

The most recently discovered members of the 'PKC-superfamily' are the protein kinase C-related kinases (PRKs), which display sequence homology with PKC's and, like aPKC's, are insensitive to Ca^{2+} , DAG and phorbol diesters. These enzymes were discovered independently by Palmer *et al.* (1994) and Kitagawa *et al.* (1995) (who named it PKN) utilizing PCR and low-stringency screening, and are regulated by small GTP-binding proteins such as Rho. Indeed, PRK's belong to the Rho-activated kinase family that include rhotekin and rhophilin (see Mellor & Parker, 1998).

Another novel serine/threonine protein kinase that binds phorbol diesters was identified in 1994 by Valverde and colleagues but was not classified into a particular kinase family. This enzyme shows greatest homology (41%) with myosin light chain kinase (MLCK) (Valverde *et al.*, 1994) and was named protein kinase D (PKD), although it may represent the mouse equivalent of human PKC μ (which at 916 amino acids is six residues shorter than PKD) or of an undiscovered human homologue of human PKC μ (Johannes *et al.*, 1994). However, the 54 amino acid difference between PKD and PKC μ exceeds the number seen for other isoforms when interspecies comparisons are made and considerable controversy,

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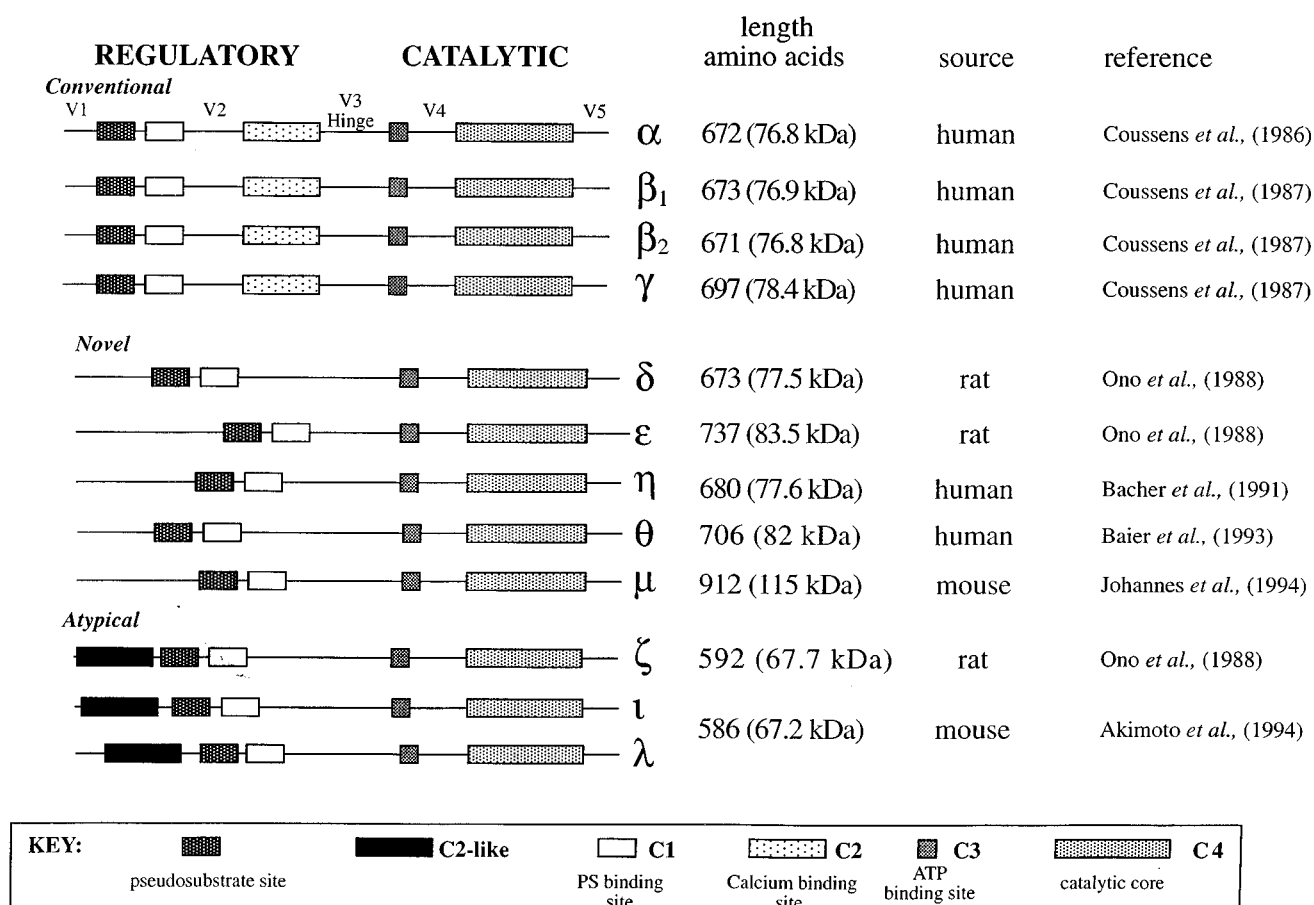


Figure 1 Diagrammatic representation of the domain structure of PKC isoenzymes. The PKC family can be divided into three sub-families consisting of cPKC's, namely α , β_1 , β_2 and γ , the nPKC's namely δ , ϵ , η , θ and μ that do not require Ca^{2+} (as they do not possess the C2 domain) and the aPKC's, namely ζ , ι (human) and λ (mouse) that require neither Ca^{2+} nor DAG for activation. The length and relative sizes of the isoforms are given to demonstrate the variability within this kinase family. V regions represent poorly conserved (variable) sequences between the isoforms and separate the highly conserved functionally-specific domains. The V3 region acts as a link between the regulatory and catalytic domains and also provides a site for proteolytic cleavage by calpains and trypsin, which results in constitutive and activator-dependent kinase activity (Mochly-Rosen *et al.*, 1990). V0 is an N-terminal domain not present in the cPKC's. The C domains are conserved sequences throughout the PKC family. C1 features the DAG and PS binding sites. At the N-terminus of the C1 domain is located a pseudosubstrate site that regulates PKC activity. These 'so-called' autoinhibitory domains contain a consensus phosphorylation sequence but no phosphorylatable residue, thus controlling kinase activity by blocking access of the substrate through occupation of the catalytic site (Soderling, 1990). C2 is the Ca^{2+} -binding domain present only in cPKC's. The C-terminus of all PKCs features the highly conserved catalytic domain (the C3 and C4 regions), separated by a short variable (V4) insert present only in PKC γ (Parker, 1992). Various sub-domains within the C3-C4 regions have also been identified (Hanks *et al.*, 1988). The C3 region, which is essential for enzyme activity, represents the conserved ATP-binding site sequence found in all isoforms in which an invariant lysine residue (lys-380) is essential for kinase activity (Freisewinkel *et al.*, 1991). The C4 domain represents the catalytic core of PKC's. For further details on the structure of PKC, interested readers should refer to Azzi *et al.* (1992), Hug & Sarre (1993), Stabel & Parker (1991), Dekker & Parker (1994) and Mellor & Parker (1998).

thus, surrounds the issue of whether PKD/PKC μ are true PKC family members (see Ron & Kazanietz, 1999).

Finally, protein kinase B (also known as RAC-PK (related to A- & C-kinase) was identified as a serine/threonine protein kinase having a catalytic domain resembling PKC and PKA (hence the nomenclature) (Coffer & Woodgett, 1991; Jones *et al.*, 1991). This kinase has been implicated in a number of signalling pathways, most recently in those controlling ASM proliferation (Walker *et al.*, 1998).

Regulation of PKC

Activation

Membrane association Activation of cPKC's, regardless of the stimulus, seemingly involves translocation from the cytosol

to specific binding domains at cell membranes and can involve four basic interactions. First, these isoforms can form catalytically inactive, membrane-associated complexes in a Ca^{2+} -dependent manner; second, they may form two membrane contacts in the C1 and C2 domains (see legend to Figure 1 for description of PKC domain sequence); third, membrane interactions can occur that facilitate conformational changes of the PKC molecule, which are distinct from an activation event; and fourth, additional changes can occur following DAG/phorbol diester binding to the membrane-associated kinase. See Zidovetzki & Lester, 1992; Lester *et al.*, 1990; Bazzi & Nelsestuan 1987 for further details.

The activity of PKC is controlled by its compartmentalization within the cell. Mochly-Rosen (1995) indicated that specific anchoring proteins (immobilized at particular intracellular sites) localize the kinases to their sites of action. These include 'receptors for activated C-kinase' (RACKS), annexins

and other cytoskeletal proteins. For a protein to be considered a RACK, certain criteria must be fulfilled: (i) it should bind PKC in the presence of activators; (ii) PKC binding to recombinant RACK1 should not be inhibited by a pseudosubstrate peptide; (iii) binding of PKC to RACK1 should be saturable and specific; and (iv) RACKS should contain a sequence homologous to a PKC binding motif.

Diacylglycerol and phorbol diester interactions Following an increase in $[Ca^{2+}]_i$, PKC interacts with the cell membrane in an inactive, but conformationally distinct, form (Bell & Burns, 1991; Huang, 1989). With respect to cPKC's and nPKC's, the availability of DAG is essential for their activation through its ability to facilitate the penetration of these isoenzymes into the cell membrane (Lester *et al.*, 1990). Using synthetic lipid bilayers, it has been shown that the region in PKC required for penetration contains cysteines and one or more primary amines. This sequence may have homology with a region in phospholipase A_2 that acts as a phospholipid-binding site for the enzyme (Lester *et al.*, 1990).

The effects of different phorbol diesters on the activation of PKC's α , β_1 , γ , δ and ϵ have been investigated (Ryves *et al.*, 1991) in an attempt to identify isoenzyme-selective activators without success.

Role of calcium Activation of cPKC's is critically dependent $[Ca^{2+}]_i$, as well as PS. Indeed, the level of Ca^{2+} under resting conditions is insufficient to activate PKC. Upon receptor stimulation, and subsequent Ca^{2+} mobilization, the $[Ca^{2+}]_i$ increases transiently and of sufficient magnitude to promote translocation of inactive PKC from the cytosol to the plasma membrane for attachment and activation (Zidovetzki & Lester, 1992). When attached to the membrane, the affinity of PKC for Ca^{2+} is increased (Nishizuka, 1988) such that activation of the enzyme is achieved.

Fatty acids Phosphatidylserine has been shown to bind electrostatically to peptides bearing sequence homology to the pseudosubstrate binding site in PKC (Mosier & McLaughlin, 1991). Since unsaturated fatty acid chain length in DAG and PS affect their potential action as PKC cofactors (Lapetina *et al.*, 1985), hydrophobic membrane-interactions must also apply in the activation process (Brumfeld & Lester, 1990).

Other membrane phospholipids may ultimately link extracellular signals to intracellular events through PKC (Nishizuka, 1992). *cis*-Unsaturated fatty acids such as arachidonic, linoleic and oleic acid can activate PKC in the absence of DAG and PS (Hansson *et al.*, 1986), suggesting an activation mechanism that differs from that effected by DAG. Phosphatidylcholine only activates PKC if a *cis*-fatty acid is present (Chen *et al.*, 1992), even in the presence of DAG. This is the case for all types of PKC. It has been suggested that fatty acids exert their effect at the regulatory domain of PKC by altering pseudosubstrate binding and unblocking the catalytic site, thereby activating the kinase (Chauhan *et al.*, 1990).

Proteolysis

It is well known that some, if not all, PKC isoforms can be proteolytically cleaved at the V3 region by the calcium-activated protease, calpain (Kishimoto *et al.*, 1983), to generate a cofactor-independent free catalytic subunit known as protein kinase M (PKM). Calpain exists as two distinct isoforms that have a low (μ -calpain) and high (m-calpain) requirement for Ca^{2+} respectively, where m and μ reflect millimolar and micromolar $[Ca^{2+}]$ respectively (Michetti *et al.*,

1995). The physiological relevance of the 'calpain-product' was not understood at the time of its discovery but it should not be regarded as an 'unregulated' enzyme since its generation is, in fact, regulated by proteolysis (Shea *et al.*, 1994). A Ca^{2+} /phospholipid-independent kinase similar to PKM has been observed in several tissues such as the neutrophil (Melloni *et al.*, 1986). In that cell, the rate of disappearance of Ca^{2+} /phospholipid-dependent PKC activity correlated with the appearance of a 65 kDa Ca^{2+} /phospholipid-independent kinase that was identical to that reported for proteolytically modified PKC. Thus, the proteolytic cleavage of the membrane-bound protein can be considered an alternative mechanism of activation.

Down-regulation

Prolonged exposure of mammalian cells and tissues to phorbol diesters and bryostatins has been shown to desensitize agonist- and phorbol-diester-induced responses by a mechanism that can involve down-regulation of conventional and novel PKC isoforms (Newton, 1995; Smith *et al.*, 1985; Huwiler *et al.*, 1994). Although calpains have been implicated in this down-regulation (Eto *et al.*, 1995a; Kishimoto *et al.*, 1989), the evidence is not convincing as it derives from studies with small molecule inhibitors of limited specificity (see Lee *et al.*, 1997). It seems likely that increased vesicle trafficking including lysosomal endocytosis may account for down-regulation of some PKC species (Goode *et al.*, 1994; 1995; Junco *et al.*, 1994). Indeed, isoform-specific PKC down-regulation has been observed in *Schizosaccharomyces pombe* in response to phorbol diesters (Goode *et al.*, 1995). Alternatively, activation of the ubiquitin/proteasome pathway may play a dominant role in the down-regulation of certain PKC isoforms including PKC α and PKC ϵ (Lee *et al.*, 1996; 1997). All PKCs except the δ isoform express, so-called, PEST sequences (hydrophilic polypeptide segments enriched in proline (P), glutamic acid (E), serine (S) and threonine (T)), that target proteins for degradation by the proteasome (Rechsteiner & Rogers, 1996). An additional level of complexity has been the finding that dephosphorylation of activated PKCs predisposes them to ubiquitination (Lee *et al.*, 1997).

Oxidation

PKC isolated from many tissues is subject to oxidation by a number of agents including peroxide, *N*-chlorosuccinimide and periodate. In all cases, oxidation is dependent on the presence of Fe^{2+} and yields a modified form of the enzyme that is constitutively active. Thus, in the case of cPKC's, Ca^{2+} and phospholipid are not required for activity (Palumbo *et al.*, 1992; Gopalakrishna & Anderson, 1987; 1989; Allen *et al.*, 1994; Webb *et al.*, 1997a). Oxidative inactivation of PKC by hydrogen peroxide (H_2O_2) is enhanced by Ca^{2+} or by phorbol diesters in the absence of Ca^{2+} . Gopalakrishna *et al.* (1989) demonstrated that ATP protects the catalytic site from oxidation indicating that the ATP-binding domain features redox-sensitive residues. Under this condition, oxidants increase cofactor-independent PKC activity by exerting an affect at the regulatory site. Using rat brain PKC, it was also demonstrated that mild oxidation of PKC increases Ca^{2+} /phospholipid-independent activity whereas further oxidation inactivates the enzyme (Allen *et al.*, 1994).

N-Chlorosuccinimide as well as H_2O_2 down-regulate phorbol diester binding to PKC (Gopalakrishna *et al.*, 1987) indicating that oxidation of the DAG-binding domain

markedly reduces the affinity of phorbol diesters and DAG for oxidized PKC. This inactivation was shown to be more rapid for membrane-associated PKC than the cytosolic form. At micromolar concentrations, periodate activates PKC through oxidation at the regulatory site, whereas at higher concentrations of the oxidant the enzyme is inactivated through an action at the catalytic domain (Gopalakrishna *et al.*, 1991). Thus, selective regulatory site oxidation constitutively activates PKC whereas oxidation at the regulatory and/or catalytic domain, inactivates the enzyme irreversibly. We have shown (Webb *et al.*, 1997a) that constitutively active cPKC isoforms exist in bovine ASM and behave as 'unregulated' kinases due to selective oxidation of the regulatory domain. In this respect, it is interesting that treatment of smooth muscle cells with phorbol diesters increases the concentration of intracellular reactive oxygen species (Mietus-Snyder *et al.*, 1997) raising the potential for exaggerated PKC-driven responses.

Mechanistically, oxidation of PKC may represent a unique method of regulation. Just as the apparently 'unregulated' activity of PKM is, in fact, controlled by proteolysis, so is the 'unregulated' activity of oxidized PKC at the regulatory site. Indeed, the constitutive activity is abolished by further oxidation through inactivation of the enzyme at the catalytic core. It is possible that activation of PKC may occur under conditions of oxidative stress, typified by inflammatory disorders such as chronic obstructive pulmonary disease, asthma and arthritis.

Nitrosylation

Protein kinase C contains certain thiol residues that may serve to regulate the enzymes' activity by acting as substrates for S-nitrosylation. Gopalakrishna *et al.* (1993; 1995) demonstrated (using a number of nitric oxide-generating agents such as sodium nitroprusside) irreversible inactivation of PKC activity and a loss of phorbol diester binding due to disulphide bridge formation following oxidation of the nitrosylated thiols. Inhibition of calpain by S-nitrosylation also may represent an indirect process through which PKC is regulated (Michetti *et al.*, 1995).

Phosphorylation and autophosphorylation

Until recently, there have been few published studies to suggest that PKC is phosphorylated by kinases other than itself. However, it is now appreciated that PKC isoforms are phosphorylated at an 'activation loop' C-terminal serine/threonine residue in the sequence TFCGTP by phosphatidylinositol-trisphosphate-dependent kinase (PDK1). This is followed by an additional phosphorylation (probably PDK-mediated) or autophosphorylation at a more C-terminal serine/threonine residue in the sequence FSF/FTY. Nineteen amino-acids N-terminal to this FSF/FTY phosphorylation motif is another autophosphorylation site (the 'so-called' TP site) (Bornancin & Parker, 1996; 1997; Stempka *et al.*, 1997; Li *et al.*, 1997). The order of these final two phosphorylation events is not elucidated, though it is clear that each event induces necessary conformational changes in the PKC molecule that result in altered thermal stability, resistance to phosphatases and catalytic activity (Stempka *et al.*, 1997; Newton & Koshland, 1990; Bornancin & Parker, 1996; 1997).

Autophosphorylation of PKC requires great flexibility of the protein structure, and the region surrounding these sites of phosphorylation contains no common recognition sequence or similarity with substrate phosphorylation sites (Flint *et al.*, 1990). A number of features of PKC autophosphorylation are evident. In particular, it requires higher concentrations of PS than are required for phosphorylation of exogenous substrates (Bazzi & Nelsestuan, 1992; Newton & Koshland, 1990) due to different PS requirements of aggregation by divalent cations.

Tissue expression of PKC isoenzymes

The expression and distribution of PKC isoforms varies markedly between cells and tissues (Nishizuka, 1988). Some isoforms (e.g. PKC α) are ubiquitously expressed whereas others seem to be restricted to certain tissues (see Table 1).

In ASM, similar patterns of expression of PKC isoforms have been found in the larger central airways of several species (Table 2). In bovine trachealis there is expression of conventional α -, β_1 -, and β_2 -PKC isoforms as well as the

Table 1 Distribution of PKC isoenzymes between selected mammalian cells and tissues

Cell/Tissue*	PKC α	PKC β_1	PKC β_2	PKC γ	PKC δ	PKC ϵ	PKC η	PKC ζ	PKC θ	PKC $\lambda/1$	PKC μ
Central Nervous System	+	+	+	+	+	+	+	+	+	+	?
Heart	+	+	+	—	+	+	+	+	+	+	?
Small Intestine	?	?	?	—	+	?	?	?	?	+	?
Kidney	+	+	+	—	+	+	?	+	?	?	+
Liver	+	+	+	—	+	+	?	+	+	+	?
Airways Smooth Muscle	+	+	+	—	+	+	+	+	+	+	+
Lung	+	+	+	—	+	+	+	+	+	+	+
Neutrophil	+	+	+	—	+	?	?	+	?	?	?
Monocyte	+	+	+	—	+	+	+	+	—	—	+
Macrophage	+	+	+	—	+	+	+	+	—	—	+
Eosinophil	+	+	+	—	+	+	—	—	—	+	+
Platelet	+	+	+	—	+	+	?	+	+	?	?
T-Lymphocyte	+	+	+	—	+	+	+	+	?	?	?
B-Lymphocyte	+	+	+	—	+	+	+	+	?	?	?
Vascular Smooth Muscle	+	+	+	—	+	+	+	?	?	+	?
Retina	+	+	+	—	+	+	?	+	?	?	?
Spleen	+	+	+	—	+	+	+	?	?	+	?
Testis	+	+	+	—	+	+	?	+	?	+	?
Ovary	+	+	+	—	+	+	?	+	?	+	?
Pancreas	+	+	+	—	?	+	?	?	?	+	?
Thymus	+	+	+	—	?	—	?	?	?	?	?

*Distribution may vary between species. + Isoform detected at the protein and/or mRNA level; — Isoform absent; ? Expression unknown.

Table 2 Protein and mRNA expression of PKC isoforms in airways smooth muscle from different mammalian species

	Human ¹ ASM protein	Human ¹ ASM mRNA	Canine ² trachealis protein	Bovine ³ trachealis protein	Bovine ³ trachealis mRNA	Porcine ⁴ ASM protein	Human ⁵ Cultured ASM
<i>PKC isoenzyme</i>							
α	+	+	—	+	ND	+	+
β_I	±	+	+	+	ND	+	+
β_{II}	+	+	+	+	ND	+	+
γ	—	ND	—	—	ND	—	ND
δ	—	+	+	+	+	+	+
ε	+	+	+	+	+	+	+
ζ	+	—	+	—	+	+	+
η	±	+	—	—	ND	ND	+
ι/λ	ND	—	ND	ND	ND	+	+
θ	—	—	+	+	+	—	+
μ	—	ND	ND	ND	ND	+	+

¹=Data taken from Webb *et al.* (1997a); ²=Data taken from Donnelly *et al.* (1995); ³=Data taken from Webb *et al.* (1997b); ⁴=Data taken from Togashi *et al.* (1997); ⁵=Data taken from Carlin *et al.* (1999); ND=isoform not detected; —=isoform absent; ±=borderline expression; +=isoform detected

novel δ -, ε and θ -PKC variants together with trace amounts of atypical PKC ζ (Webb *et al.*, 1997a). Canine trachealis shows a similar profile, except that PKC α is apparently absent (Donnelly *et al.*, 1995). With the availability of monoclonal antibodies to additional members of the aPKCs, a recent investigation also identified PKC μ and ι/λ isoforms in porcine trachealis (Togashi *et al.*, 1997). In human trachealis, there is expression of the conventional α , β_I , and β_{II} PKC isoforms as well as novel (δ , ε , η , θ) and atypical (ζ) variants (Webb *et al.*, 1997b). Where discrepancies between species have been noted in expression of particular isoforms, it is possible that this involves failure of antibodies raised against human epitopes to cross-react with those of different species, or to the presence of contaminating non-muscle cell types in the tissue preparations. PKC γ , often abundantly expressed in neuronal tissues, has not been detected in ASM from any species thus far investigated.

PKC and contraction

Our present understanding of smooth muscle contraction is based on the original studies of Huxley & Niedergerke (1954) and Huxley & Hanson (1954). Using striated muscle these two groups of investigators independently advanced the 'sliding filament' theory, which proposes that contraction of muscle is the result of a relative interdigitation between two populations of protein filaments, actin and myosin, with the reversible formation of stress-bearing cross-bridges. Many studies have been conducted to elucidate the biochemical basis and regulation of smooth muscle contraction (Allen *et al.*, 1994; Somlyo & Somlyo, 1994) and significant differences have been discovered between the processes that govern force generation and force maintenance (Dillon *et al.*, 1981; Aksoy *et al.*, 1982; Giembycz & Raeburn, 1992). An understanding of smooth muscle contraction and how enzymes, such as PKC, are involved initially requires knowledge of the structure of the contractile proteins themselves and how they interact with each other ultimately to effect force.

Force generation

Myosin is a hexameric protein that forms thick bipolar filaments in muscle cells; it is comprised of a single pair of asymmetric heavy chains and two pairs of light chains. Morphologically, myosin is composed of a head and a tail

region. The tail of many myosin molecules combine to form the body of each myosin filament whilst the head, which has a globular appearance, possesses the binding site for attachment to actin and two domains of catalytic (myosin ATPase) activity that provide the energy required for force development. Both pairs of light chains are also located at the globular head of myosin and it is those of 20 kDa (LC₂₀) that are believed to exert important regulatory control over the actin-myosin interaction (see below). Actin is the other major contractile protein. In solution, actin (known as globular- or G-actin) exists as spherical monomers that polymerises in cells into thin filaments, termed filamentous- or F-actin. In force generation, actin performs two important functions. First, each G-actin subunit of the F-actin chain features a binding site for the globular heads of myosin, which permits the formation, breaking and reformation of cross-bridges (force) between the filaments by a process called cross-bridge cycling. Second, actin activates myosin ATPase when LC₂₀ are phosphorylated by a Ca²⁺- and calmodulin-requiring enzyme known as myosin light chain kinase (MLCK) (Small & Sobieszek, 1977; Sobieszek & Small, 1977; Aksoy *et al.*, 1976). Thus, in the absence of Ca²⁺, MLCK is catalytically inactive. However, when [Ca²⁺]_i increases some 4–10 fold above the resting level, Ca²⁺ bind to calmodulin exposing one or more hydrophobic domains for which MLCK has high affinity, and a catalytically-active Ca²⁺/calmodulin/MLCK complex is formed. LC₂₀ phosphorylation then ensues enabling actin to activate myosin ATPase, which hydrolyses the ATP required for the formation of stress-bearing, actin-myosin cross-bridges (Figure 2).

Force maintenance

Although LC₂₀ phosphorylation undoubtedly accounts for force generation in ASM, evidence exists that a different mechanism(s), as well as or in addition to LC₂₀ phosphorylation, may be responsible for steady-state force. This conclusion was based on findings that force is maintained when LC₂₀ are dephosphorylated and myosin ATPase activity is low, and lead to the proposal of a modified stress-bearing, non-cycling, or very slowly cycling, cross-bridge between actin and myosin filaments, which was called the 'latch-bridge' (Dillon *et al.*, 1981; Chatterjee & Murphy, 1983). However, subsequent studies identified serious inconsistencies with the original 'latch-bridge' hypothesis (see Giembycz & Raeburn, 1992 for

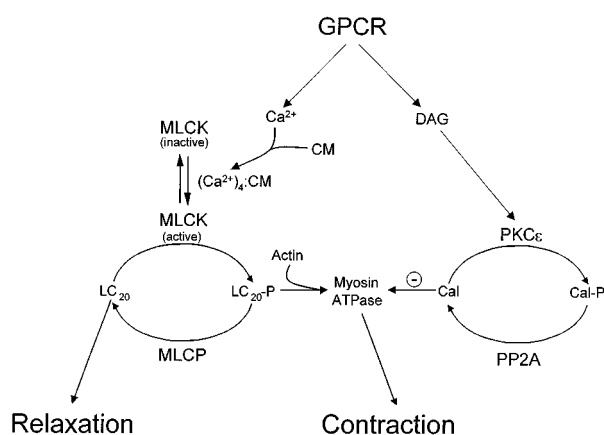


Figure 2 Putative model of force generation and maintenance in ASM involving MLCK, PKC and calponin. When a Ca^{2+} -mobilizing agonist, acting through a G-protein-coupled receptor (GPCR), is applied to an ASM cell of the contractile phenotype, there occurs a transient increase in the $[\text{Ca}^{2+}]_i$ of sufficient magnitude to occupy all four Ca^{2+} -binding sites on calmodulin (CM). In this form Ca^{2+} -bound CM is able to activate myosin light chain kinase (MLCK), which phosphorylates the 20 kDa light chains of myosin (LC_{20}) permitting actin activation of myosin ATPase, cross-bridge cycling and, in turn, force generation. When the $[\text{Ca}^{2+}]_i$ returns towards the resting levels, Ca^{2+} dissociate from CM and MLCK is dephosphorylated by myosin light chain phosphatase (MLCP). However, in many cases, force is maintained. One explanation for Ca^{2+} -independent force generation is the activation of a novel protein kinase C (PKC) isoform such as $\text{PKC}\epsilon$ by diacylglycerol (DAG), which is also generated by the contractile agonist and phosphorylates an actin-linked protein, calponin (Cal). Under resting conditions Cal inhibits the ability of actin to activate myosin ATPase and so keeps the muscle relaxed. However, in the phosphorylated state, Cal dissociates from actin thereby relieving its inhibitory effect on myosin ATPase. Ca^{2+} -independent contraction then ensues due to the phosphorylation of LC_{20} by the resting activity of MLCK. Cal is thought to be regulated by reversible phosphorylation and protein phosphatase 2A (PP2A) has been shown to dephosphorylate Cal to a form that prevents actin-activated myosin ATPase activity (see text for further details).

discussion) and led Murphy *et al.* (1990) to re-define latch as a '... state characterized by moderate phosphorylation [of LC_{20}] levels when high forces are associated with reduced contraction rates and ATP consumption.' Nevertheless, the general finding that ATP utilization and $[\text{Ca}^{2+}]_i$ in ASM are low during force maintenance and that LC_{20} phosphorylation can be clearly dissociated from myosin ATPase activity has led to the proposal that tonic force is controlled and/or regulated, at least in part, by a different mechanism.

One explanation is that cross-bridges, generated during the development of force, are in some way modified to a 'latch' state, which is responsible for force maintenance. Implicit in this hypothesis is that LC_{20} phosphorylation is both necessary and sufficient to explain this modified actin-myosin interaction and represents a, so-called, myosin-linked process. Another possibility that has received much attention and is not mutually exclusive with the first, is that latch is due to an undefined actin-linked mechanism(s) that may involve the participation of other proteins including caldesmon, calponin and/or one or more of the actin intermediate filaments. Both postulates are being rigorously investigated, including the potential role played by PKC. These are discussed below.

Myosin-linked regulation – role of LC_{20} phosphorylation
Phorbol diesters are used widely as activators of conventional and novel PKCs due to their ability to substitute for DAG, and Rembold & Murphy (1988) exploited this pharmacological

property to assess if latch is a myosin-linked process that is mediated by PKC through the phosphorylation of LC_{20} . Using porcine carotid arterial smooth muscle strips these investigators reported that the phorbol diester, phorbol 12, 13-dibutyrate (PDBu), evoked a delayed, slow increase in force that was preceded by Ca^{2+} mobilization and LC_{20} phosphorylation. Given that phorbol diesters can promote Ca^{2+} influx into vascular smooth muscle (see Beech, 1997), Rembold & Murphy (1988) reasoned that the PDBu-induced contraction was mediated by Ca^{2+} through their ability to promote the MLCK-dependent phosphorylation of LC_{20} and cross-bridge cycling. Consistent with this interpretation is the finding that although PKC catalyses the phosphorylation of LC_{20} on several residues, this is not associated with an increase in myosin ATPase activity (Ikebe *et al.*, 1987; Bengur *et al.*, 1987). Murphy and co-workers thus concluded that LC_{20} phosphorylation could account quantitatively for the mechanical effect of PDBu without the need to involve an undefined (actin-linked) PKC-dependent mechanism.

Verification of this conclusion is provided by Singer *et al.* (1989) who observed that PDBu promoted the phosphorylation of LC_{20} at S^1 , S^2 and S^{19} in rabbit aortic smooth muscle, the latter of which was phosphorylated by MLCK but not PKC. The knowledge that S^{19} phosphorylation is Ca^{2+} -dependent suggests that PDBu evokes this response through the Ca^{2+} /calmodulin-dependent activation of MLCK with much of the Ca^{2+} being derived extracellularly through voltage-dependent Ca^{2+} channels (VDCCs).

Of the five main types of VDCCs thus far defined, it is the L-, or long-lasting, type that is most abundant in the plasma membrane of smooth muscle cells including ASM (Small & Foster, 1994). The use of 1,4-dihydropyridine agonists and antagonists as probes has led to the discovery that L-type VDCCs are large oligomeric proteins with dissimilar subunits that, in non-skeletal muscle, combine to form an $\alpha_1\alpha_2-\delta\beta$ complex. In the heart, the α_1 subunit is recognized as a target for channel modulation. In particular, it is phosphorylated by PKC that, together with the β subunit (also a substrate for PKC), results in channel activation by relieving an inhibitory constraint exerted by the N-terminus of α_1 (Shistik *et al.*, 1998). Whether such regulation is conferred by PKC in smooth muscle is unclear, but some indirect evidence to support this idea is available.

Studies with phorbol diesters In ASM from the cow (Park & Rasmussen, 1986; Knox *et al.*, 1993), guinea-pig (Menkes *et al.*, 1986), rabbit (Schramm & Grunstein, 1989), rat (Chopra *et al.*, 1994) and humans (Yang & Black, 1996; Rossetti *et al.*, 1995), low concentrations of phorbol diesters elicit slow tonic contractions similar to those evoked in the vasculature (see above). Significantly, force development is antagonized by inhibitors of PKC, including Ro 31-8220 and Ro 31-7549 (Chopra *et al.*, 1994), as well as by staurosporine (Yang & Black, 1995). The rate and magnitude of tension generation is enhanced by Ca^{2+} ionophores and agonists of L-type VDCCs such as BAY K 8644 (Part & Rasmussen, 1985; Yang & Black, 1996). Conversely, chelation of extracellular Ca^{2+} or application of nifedipine, diltiazem and verapamil attenuates phorbol diester-induced contractions (Park & Rasmussen, 1985; Knox *et al.*, 1993). Taken together, these data suggest that phorbol diesters promote force generation in ASM, at least in part, by a PKC-dependent process that involves an increase in the open-state probability of L-type VDCCs (possibly by direct phosphorylation of the α_1 subunit), Ca^{2+} influx, LC_{20} phosphorylation and activation of the contractile machinery. This sequence of events is supported by the finding that

phorbol diesters amplify K^+ -induced contractions of guinea-pig, canine and bovine trachealis (Menkes *et al.*, 1986; Gunst *et al.*, 1994; Yang & Black, 1996). However, more recent data have challenged this hypothesis. For example, phorbol 12,13-diacetate (PDA) and an activating PKC fragment suppressed, rather than enhanced, inward Ca^{2+} currents through L-type VDCCs in porcine tracheal smooth muscle cells by a mechanism that was antagonized by the PKC inhibitor, calphostin C (Yamakage *et al.*, 1995). Moreover, it has been demonstrated that phorbol diesters can contract smooth muscle by inhibiting, directly or indirectly, the activity of myosin light chain phosphatase (MLCP). This phenomenon, which leads to LC_{20} phosphorylation at S^{19} and force development at a constant Ca^{2+} concentration (Masuo *et al.*, 1994; Peiper *et al.*, 1996; Iizuka *et al.*, 1999), is known as Ca^{2+} sensitization (Somlyo & Himpens, 1989; Somlyo & Somlyo, 1994; Kitazawa *et al.*, 1989; 1991a) and is well recognized as a mechanism of force generation/maintenance in ASM of several species including the cow (Takuwa *et al.*, 1986; rabbit (Yoshii *et al.*, 1999; Iizuka *et al.*, 1999) and dog (Iizuka *et al.*, 1997).

Recent studies by Bremerich *et al.* (1998) have confirmed that PDBu increases LC_{20} phosphorylation in β -escin-permeabilized canine trachealis at a constant Ca^{2+} concentration. However, these investigators provided two pieces of evidence against the idea that LC_{20} phosphorylation accounts for force development under these conditions. First, PDBu promotes the phosphorylation of LC_{20} on residues (S^1 , S^2 , T^9) that are not thought to contribute to contraction (Ikebe *et al.*, 1987; Bengur *et al.*, 1987; Kamm & Stull, 1989). In fact, phosphorylation of T^9 has been shown to suppress the increase in myosin ATPase activity associated with phosphorylation of S^{19} , a major site for MLCK (Kamm & Stull, 1989). In addition, PKC does not affect actin sliding velocity in an *in vitro* motility assay when LC_{20} are phosphorylated by MLCK at S^{19} (Umemoto *et al.*, 1989). Thus, it has been speculated (Bremerich *et al.*, 1998) that phorbol diesters promote the PKC-dependent phosphorylation of LC_{20} at residues that do not affect force. This conclusion is supported by the additional observation that under certain conditions PDBu can increase LC_{20} phosphorylation without concomitant force development (Gerthoffer, 1986; Gerthoffer *et al.*, 1989). Second, calphostin C and a pseudosubstrate peptide inhibitor of PKC block PDBu-induced LC_{20} phosphorylation but not Ca^{2+} sensitization. Collectively, therefore, it has been proposed that at a constant Ca^{2+} concentration PDBu increases force in ASM by a PKC-independent mechanism that is unrelated to its ability to phosphorylate LC_{20} (Bremerich *et al.*, 1998). In this respect PDBu potentiates the effect of endothelin-1 on ovine tracheal smooth muscle (Katoch, 1997) and carbachol on guinea-pig ileum (Sasaguri & Watson, 1989) under conditions where PKC is down-regulated implying that the phorbol diester is acting at a different intracellular locus. A number of potential targets have been identified that can act as receptors for phorbol diesters including Unc-13 (Kazanietz *et al.*, 1995) and *n*-chimaerin (Ahmed *et al.*, 1993; reviewed in Ron & Kazanietz, 1999). While the role of these proteins in regulating smooth muscle tone is unexplored, it is noteworthy that the C-terminal domain of chimaerins is thought to act as a GTPase-activating protein for members of the Rho subfamily of small GTP-binding proteins (Ahmed *et al.*, 1993). In airways and other smooth muscle, p21rhoA has been implicated in Ca^{2+} sensitization (Hirata *et al.*, 1992; Fujihara *et al.*, 1997; Gong *et al.*, 1996; 1997; Akopov *et al.*, 1998; Yoshii *et al.*, 1999; Iizuka *et al.*, 1999) and it was recently reported that PDBu-induced contractions of rabbit trachealis at constant Ca^{2+}

concentration were partially reversed by Y-27632, an inhibitor of rho-associated coiled coil-forming protein kinase (ROCK) I and ROCK II (also known as p160ROCK and ROK α rho kinase respectively) (Yoshii *et al.*, 1999). ROCKs lie downstream of p21rho in smooth muscle and can phosphorylate and, thereby, inactivate, MLCP (Amano *et al.*, 1996).

Given the aforementioned discussion, it is clear that the available evidence is insufficient to formulate a unifying hypothesis that adequately explains phorbol diester-induced contraction of ASM. While certain pharmacological experiments point to the activation of VDCCs, Ca^{2+} influx and MLCK-driven LC_{20} phosphorylation as described for vascular smooth muscle, electrophysiological investigations and studies with permeabilized smooth muscles have provided little corroborating support and, in fact, suggest that phorbol diesters may regulate tone independently of PKC. However, as described below, this later idea should be viewed with caution since most of the evidence against a role for PKC is derived from permeabilized preparations from which proteins essential to the contractile process may be lost.

Studies with agonists Phorbol diesters have the ability to activate at least nine immunologically and, presumably, functionally distinct isoenzymes that comprise the conventional and novel PKC families. As shown in Table 2, multiple cPKCs and nPKCs are co-expressed in ASM from all species thus far examined and it is important to appreciate that phorbol diesters will, theoretically, activate all of these. Therefore, data arising from such studies cannot be considered representative of the effects of receptor agonists which, almost certainly, will selectively activate specific isoforms. Furthermore, phorbol diesters do not permit an evaluation of the role of aPKCs in biological processes since this subfamily of enzymes lacks the diglyceride-binding domain.

Like phorbol diesters, agonism of some G-protein-coupled receptors on ASM also activates L-type VDCCs including the ET_A - (Grunstein *et al.*, 1991; Yang *et al.*, 1994), 5-HT $_{2A}$ (Watts *et al.*, 1994) and M_3 muscarinic receptor (Tomasic *et al.*, 1992; Kamashima *et al.*, 1992). Therefore, it is conceivable that, as in vascular smooth muscle, this effect is mediated by PKC and leads to contraction through the activation of MLCK. However, although inhibitors of VDCCs, such as nifedipine and verapamil, effectively inhibit L-type Ca^{2+} currents in ASM cells of some species (Kotlikoff, 1988) they fail, in many cases, to significantly affect tension generation (Coburn, 1979; Farley & Miles, 1978; Cheng & Townley, 1983; Gunst & Pisoni, 1986; Duncan & Douglas, 1984; Ahmed *et al.*, 1985). This discrepancy has led to the belief that most of the Ca^{2+} necessary for agonist-induced contraction of ASM is derived from the sarcoplasmic reticulum whereas Ca^{2+} influx through VDCCs is required for refilling the internal Ca^{2+} stores (Daniel *et al.*, 1992). Exceptions to this general rule include the ability of L-type VDCC antagonists to suppress 5-HT- and carbachol/methacholine-induced tension generation in guinea-pig trachea and rat bronchioles (Watts *et al.*, 1994; Kamishima *et al.*, 1992). The 5-HT results are particularly important since contraction is not associated with inositol phospholipid hydrolysis (Watts *et al.*, 1994) implying that activator Ca^{2+} are mobilised by an $Ins(1,4,5)P_3$ -independent process. Although this might involve the novel second messengers, cyclic ADP ribose or sphingosine 1-phosphate, which can both mobilise intracellular Ca^{2+} in response to agonists that act through G-protein-coupled receptors (Higashida, 1977; Meyer zu Heringdorf *et al.*, 1998), it is likely that much of the Ca^{2+} are derived extracellularly and enter the cell through VDCCs. Indeed, the additional finding that 5-HT-induced contractions

are abolished by Ro 31-8220, Ro 31-7549 and GF 109203X (Watts *et al.*, 1994; Chopra *et al.*, 1994) suggests that cPKCs and/or nPKCs are involved in the nifedipine-sensitive and insensitive components of contraction. Since 5-HT fails to hydrolyse inositol-containing phospholipids in guinea-pig trachea, it is tempting to speculate that the 5-HT_{2A} receptor subtype couples to PLD, which will indirectly provide the diglyceride necessary to activate nPKCs from phosphatidylcholine.

An interesting, and equally confounding, finding is that ACh reversibly inhibited whole cell L-type Ca²⁺ currents in guinea-pig tracheal myocytes by a mechanism blocked by the selective M₃ muscarinic antagonist 4-DAMP, but not AF-DX 116 (M₂-selective) (Yamashita & Kokubun, 1997). Moreover, neither phorbol 12-myristate 13-acetate (PMA) nor the DAG analogue, 1-oleoyl-2-acetyl *sn*-glycerol (OAG), mimicked this effect suggesting that the inhibition of ACh-induced Ca²⁺ influx is mediated by a PKC-independent mechanism. Acetylcholine similarly inhibits VDCCs in porcine tracheal smooth muscle cells but, in this tissue, PKC seemingly plays a role since calphostin C and a pseudosubstrate peptide inhibitor antagonized this effect (Yamakage *et al.*, 1995). The reason for these contrary results is unknown. Likewise, the discrepancy between the results of these studies and those of Tomasic *et al.* (1992) and Kamishima *et al.* (1992) described above, where M₃ muscarinic receptor activation enhanced L-type VDCCs was reported, equally are difficult to reconcile but differences between patch clamp methods have been proposed (Yamakage *et al.*, 1995).

Another mechanism that could account for agonist-induced force maintenance is Ca²⁺ sensitization. Indeed, there is a consensus that agonists that act through G-protein-coupled receptors sensitize the contractile machinery to Ca²⁺ primarily by inhibiting MLCP, although exactly how this occurs is controversial (Kitazawa *et al.*, 1991a,b; 1999; Li *et al.*, 1998; Shirazi *et al.*, 1994; Parker *et al.*, 1998). Agonist-induced Ca²⁺ sensitization has been described for canine (Gerthoffer, 1996; Iizuka *et al.*, 1997; Bremerich *et al.*, 1997), human (Yoshii *et al.*, 1999) and rabbit trachealis (Yoshii *et al.*, 1999) and a role for PKC in this response has been sought. Using β -escin-permeabilized canine tracheal smooth muscle cells, Bremerich *et al.* (1997) demonstrated that at a constant submaximal concentration of Ca²⁺ and in the presence of GTP, ACh-induced Ca²⁺ sensitization was neither prevented nor reversed by calphostin C, chelerythrine or a pseudosubstrate peptide inhibitor of PKC. Of particular significance was the result of an additional experiment in which the DAG analogue, OAG, similarly failed to mimic the effect of ACh (Bremerich *et al.*, 1997). In other permeabilized smooth muscle preparations stimulated with GTP γ S, PKC inhibitors are also inactive (Akopov *et al.*, 1998; Fujita *et al.*, 1995; Itoh *et al.*, 1994) or poorly active (Iizuka *et al.*, 1999) and there is a wealth of literature implying that neither inhibition nor down-regulation of PKC affect agonist-induced contractions in many intact smooth muscles (Oishi *et al.*, 1992; Sasaguri & Watson, 1989; Yoshida *et al.*, 1994; Fujita *et al.*, 1994). Taken together, these results do not implicate PKC in agonist-induced Ca²⁺ sensitization and point to other effectors such as the small GTP-binding protein p21rho (Akopov *et al.*, 1998; Yoshida *et al.*, 1994; Otto *et al.*, 1996; Itoh *et al.*, 1994; Fujita *et al.*, 1995; Fujihara *et al.*, 1997; Amano *et al.*, 1996). However, it is conceivable that permeabilization of smooth muscle may alter signal transduction by allowing essential cofactors and integral proteins to be lost through the pores. This possibility was formally investigated in a recent publication by Kitazawa *et al.* (1999) in which methods of permeabilization of aortic smooth

muscle strips were compared. The most salient observation of that study was the absence of Ca²⁺ sensitization in tissue permeabilized with Triton X-100 due to the depletion of PKC and a novel endogenous smooth muscle-specific inhibitor of MLCP, CPI-17 (Eto *et al.*, 1995b; 1997) from the tissue (Kitazawa *et al.*, 1999). Indeed, reconstitution of tissues with PKC α and CPI-17 increased LC₂₀ phosphorylation and promoted contraction at a constant Ca²⁺ concentration by a mechanism that was abolished by Go6976, a selective inhibitor of cPKCs (Kitazawa *et al.*, 1999). Significantly, CPI-17 is phosphorylated by PKC on T³⁸ and phospho-CPI-17 inhibits the catalytic subunit and holoenzyme of MLCP with equally high potency resulting in LC₂₀ phosphorylation. These data have culminated in a new, simple model of Ca²⁺ sensitization in smooth muscle that is independent of regulation at the level of the thin actin filaments (discussed below). In its simplest form, phorbol diesters and agonists evoke Ca²⁺-independent contractions through the PKC-dependent phosphorylation of CPI-17, which increases basal MLCK activity indirectly through the inhibition of MLCP.

Actin-linked regulation – role of caldesmon, calponin and the actin-intermediate filaments

Although most investigators support the general view that LC₂₀ phosphorylation adequately explains Ca²⁺-dependent smooth muscle contraction, this concept ignores the observation that under certain conditions force can develop without LC₂₀ phosphorylation (see Gerthoffer, 1991). For example, saponin-permeabilized, single smooth muscle cells isolated from ferret aorta contract in response to phenylephrine and PKM, a constitutively active fragment of PKC, in the absence of Ca²⁺ and LC₂₀ phosphorylation by a mechanism that is abolished by a pseudosubstrate peptide inhibitor of the α - and β -isoforms of PKC (Collins *et al.*, 1992). Moreover, phorbol diesters and many contractile agonists promote the phosphorylation of a number of, so-called, late phase proteins that may play auxiliary regulatory roles in smooth muscle contraction (Park & Rasmussen, 1986; Rasmussen *et al.*, 1987; Walsh *et al.*, 1996; Pohl *et al.*, 1997). Indeed, exposure of bovine tracheal smooth muscle strips, labelled with inorganic phosphate, to PDBu results in a slow, well sustained phosphorylation of several contractile/cytoskeletal proteins including caldesmon, synemin, filamin and α - and β -desmin that parallels closely the development of force (Park & Rasmussen, 1986; Rasmussen *et al.*, 1987). Significantly, these proteins are also phosphorylated by carbachol with similar kinetics. Another actin-associated protein, calponin, is also phosphorylated in ASM by contractile stimuli (Pohl *et al.*, 1997), and has led to the view that the PKC-dependent phosphorylation of one or more of these proteins may promote, or facilitate, the actin-myosin cross-linking that maintains tonic force.

Caldesmon Caldesmon is an acidic, actin-binding protein associated with the thin actin filaments that also interacts with calmodulin and myosin (see Walsh, 1990) and is ubiquitously expressed in smooth muscle including the trachealis (Park & Rasmussen, 1986; Gerthoffer & Pohl, 1994; Gerthoffer *et al.*, 1997). The postulate that caldesmon might regulate the actin-myosin interaction stemmed from the *in vitro* observation that it could reversibly bind to either calmodulin or actin depending on the Ca²⁺ concentration (Sobue *et al.*, 1981). Although it is now known that caldesmon remains attached to actin during the contraction-relaxation cycle in intact smooth muscle, persuasive evidence is available that it may play a prominent

role in regulating smooth muscle contraction. Thus, in a variety of smooth muscles caldesmon inhibits actin-activated myosin ATPase without affecting LC₂₀ phosphorylation (Ngai & Walsh, 1984; Clark *et al.*, 1986). Myosin ATPase activity is also attenuated by caldesmon in the absence of actin (Hemric & Chalovich, 1988; Ikebe & Reardon, 1988). These observations suggest that there must be some intrinsic mechanism by which the inhibition of myosin ATPase activity can be relieved. One possibility is that caldesmon is regulated by reversible phosphorylation. Indeed, Sobue & Sellers (1991) reported that caldesmon is a substrate for several protein kinases, including PKC, and there is evidence that caldesmon is phosphorylated in intact trachealis during agonist-induced contraction (Gerthoffer & Pohl, 1994; Gerthoffer *et al.*, 1997). In vascular smooth muscle, the residues phosphorylated are proline-directed serines (i.e. S⁷⁸⁹PTKV and S⁷⁵⁹PAPK), which is characteristic of a mitogen-activated protein (MAP) kinase consensus sequence (Adam *et al.*, 1992; 1993). Thus, in intact smooth muscle extracellular-regulated kinase (ERK)-1 and/or ERK-2, rather than PKC, may be the endogenous caldesmon kinase. In this respect, purified caldesmon is phosphorylated by murine ERK-2 in a cell-free system and addition of ERK-2 to Triton X-100-permeabilized canine ASM fibres potentiates Ca²⁺-induced tension development (Gerthoffer *et al.*, 1997). Conversely, unphosphorylated caldesmon produces a concentration-dependent inhibition of actin sliding velocity in an *in vitro* motility assay (Shirinsky *et al.*, 1992), presumably through its ability to inhibit myosin ATPase and prevent actin-myosin interactions. Further support for a MAP kinase/caldesmon cascade in regulating smooth muscle contractility derives from pharmacological studies where phenylephrine-induced contraction of ferret aortic strips in the absence of extracellular Ca²⁺ was significantly suppressed by PD 098059 (Dessy *et al.*, 1998), a selective inhibitor of MAP kinase kinase-1 (MEK-1).

If ERK-1 and/or ERK-2 are direct upstream regulators of caldesmon phosphorylation where does this leave PKC? Khalil & Morgan (1992) have reported that Ca²⁺-independent, phenylephrine-induced contraction of ferret aortic smooth muscle cells is associated with a sustained translocation of PKC ϵ to the plasma membrane. This effect is accompanied by a transient association of MAP kinase with the membrane followed by a second re-localization towards the vicinity of the contractile filaments prior to active force development (Khalil & Morgan, 1992). The finding that inhibitors of PKC abolished the intracellular redistribution of ERK-1/ERK-2 suggests that members of this MAP kinase family may transmit signals from membrane-bound PKC ϵ to the contractile machinery. Indeed, ERK-1/ERK-2 is a substrate for PKC and is capable of phosphorylating caldesmon on residues that are phosphorylated *in vivo* during smooth muscle contraction (Adam *et al.*, 1992).

Calponin Shortly after the discovery of caldesmon, Takahashi *et al.* (1986; 1987) identified a basic, heat-stable protein in chicken gizzard and bovine aortic smooth muscle that displayed properties consistent with the regulation of the actin-myosin interaction. This protein was named calponin and has been identified in many tissues including ASM (Gerthoffer & Pohl, 1994; Pohl *et al.*, 1997). *In vitro*, calponin interacts with actin and calmodulin in a way similar to caldesmon (see above). In addition, calponin inhibits actin-activated myosin ATPase activity without affecting LC₂₀ phosphorylation, an effect that (in a reconstituted cell-free system) is regulated by reversible protein phosphorylation. Indeed, under these conditions calponin is a substrate for PKC

(Winder & Walsh, 1990), in particular PKC ϵ (Horowitz *et al.*, 1996b), and displays a dramatic reduction in affinity for actin and is unable to inhibit myosin ATPase activity in the phosphorylated state. An activity capable of dephosphorylating calponin has been found in smooth muscle and identified as a type 2A protein phosphatase (Winder *et al.*, 1992). Moreover, dephosphorylation of calponin by this phosphatase restored actin binding and the inhibition of myosin ATPase (Winder *et al.*, 1992). Functionally, phosphorylation of calponin by PKC results in contraction of porcine coronary artery (Mino *et al.*, 1995) whereas unphosphorylated calponin relaxes single permeabilized vascular smooth muscle cells precontracted with phenylephrine and PKC ϵ (Horowitz *et al.*, 1996a,b). Similarly, calponin causes a profound inhibition of actin sliding velocity in a cell-free motility assay whereas phosphocalponin is inactive (Pohl *et al.*, 1997). In intact canine tracheal smooth muscle strips, metabolically labelled with inorganic phosphate, carbachol promoted the phosphorylation of calponin and the appearance of an additional, more acidic, isoform over the four variants that were expressed in unstimulated cells (Gerthoffer & Pohl, 1994; Pohl *et al.*, 1997). The subcellular localization of calponin is also affected by PKC-dependent phosphorylation. In unstimulated smooth muscle cells isolated from the ferret portal vein, calponin is primarily cytosolic where it is associated with filamentous structures (presumably F-actin). However, following exposure to phenylephrine, which activates PKC in these cells, calponin redistributes to a region of the cell just underneath the plasma membrane (Parker *et al.*, 1994). The identity of the PKC isoform(s) involved in calponin phosphorylation in ASM has not been explored, although cPKCs or nPKCs are possible candidates given that GF 109203X inhibits the tonic phase of histamine-induced tension generation in human bronchus (Yang & Black, 1995). In the vasculature, a strong case has been made for the novel isoform, PKC ϵ , since it is Ca²⁺-independent, translocates to the plasma membrane upon stimulation, produces contractile responses in permeabilized muscle strips clamped at low free [Ca²⁺] and is activated by phorbol diesters (see Walsh *et al.*, 1996). Based upon these data, it has been postulated that agonist-induced, Ca²⁺-independent contraction of smooth muscle is mediated by PKC through its ability to phosphorylate calponin. This, in turn, facilitates its release from actin thereby relieving the inhibition of myosin ATPase. Slow, sustained contraction then ensues due to the phosphorylation of LC₂₀ by the basal activity of MLCK (Walsh *et al.*, 1996; Figure 2).

More recent studies have shown that calponin binds to both PKC ϵ and ERK-1/ERK-2 in ferret aorta and that it translocates to the cell's cortex with kinetics identical to the association of PKC ϵ and ERK-1/ERK-2 to the plasma membrane (Menice *et al.*, 1997). Of potential significance is that the interaction of ERK with calponin does not result in phosphorylation. Moreover, the amount of calponin in ERK immunoprecipitates decreases at late time-points after agonist (phenylephrine) exposure whereas increased amounts are found at early time-points in PKC ϵ immunoprecipitates. Collectively, these results have led Morgan and co-workers to question the original idea that calponin may directly prevent the actin-myosin interaction (see above). Instead, they propose that calponin is associated with ERK under resting conditions and with PKC ϵ in cells exposed phenylephrine where it acts as an adapter protein to facilitate the migration of ERK and PKC ϵ to the plasma membrane (Menice *et al.*, 1997). Indirect support for this hypothesis is that calponin dissociates from the thin actin filaments after agonist exposure before measurable force is generated (Parker *et al.*, 1994; Menice *et*

al., 1997), which is difficult to reconcile with a direct modulation of the actin-myosin interaction.

Desmin, synemin and filamin In addition to caldesmon and calponin, four other proteins (filamin, synemin, α -desmin, β -desmin) have been found in airways and other smooth muscles (Park & Rasmussen, 1986) that, together with actin, form the actin-intermediate filaments (Small *et al.*, 1986). It has been proposed that these filaments may constitute a mechanism of force development distinct from the classical actin-myosin system described above (Granger & Lazarides, 1980; Small *et al.*, 1986). Indeed, even though few investigations have considered the functional significance of the actin-intermediate proteins, ultrastructural studies indicate that they may be specifically involved in the genesis of tonic force. This could occur by the formation of actin-actin cross-bridges promoted by proteins such as filamin that, itself, requires the phosphorylation of other proteins including synemin and desmin (Small *et al.*, 1986). Some support for this hypothesis is provided from studies conducted with bovine tracheal smooth muscle in which desmin was found to be the most abundant of the actin-intermediate filaments phosphorylated in response to carbachol and 12-deoxyphorbol 13-isobutyrate (DPB; Park & Rasmussen, 1986). Since the slow contracture evoked by the phorbol diester mimics the maintained phase of the carbachol-induced contraction, a case for phosphorylated desmin catalyzed by PKC has been made (Park & Rasmussen, 1986). Indeed, desmin can be phosphorylated on four residues by PKC (Kitamura *et al.*, 1989).

Subcellular re-distribution of PKC during contraction In canine and bovine tracheal smooth muscle, methacholine and carbachol promote a rapid translocation of PKC from the cytosol to the membrane implying a functional change from the inactive to the active form (Yamakage, 1992; Langlands & Diamond, 1992). Generally, this effect is transient and less well sustained than phorbol diester-induced responses (Haller *et al.*, 1990). Similar data have been obtained with intestinal smooth muscle, where PKC β_{II} is implicated in methacholine-induced contraction (Xu *et al.*, 1991), and the vasculature where endothelin, histamine and phenylephrine elicit rapid translocation of PKC from the cytosol to the membrane (Haller *et al.*, 1990; Khalil & Morgan, 1992).

The identity of the PKC isoforms involved in agonist-induced contraction of ASM is unknown. However, in vascular smooth muscles, data are emerging that vessel type, even within the same species and in response to the same agonist, dictates the complement of isoforms activated. This phenomenon is elegantly illustrated in studies performed by Morgan and colleagues with permeabilized vascular smooth muscle from the ferret. Thus, at a constant Ca^{2+} concentration, Lee *et al.* (1999) found that a pseudosubstrate inhibitor of cPKCs inhibited phenylephrine-induced contraction of the portal vein but not aorta. Conversely, a peptide targeted against the N-terminus (translocation domain) of PKC ϵ blocked contractions of the aorta elicited by phenylephrine but not those of the portal vein under identical experimental conditions. These functional data were supported by Western blotting where PKC α and PKC ϵ were strongly expressed in portal vein and aorta respectively (Lee *et al.*, 1999). Moreover, digital imaging of the distribution of PKCs in these two muscles before and after exposure to phenylephrine found that PKC α translocated from the cytosol to membrane in cells of the portal vein but not aorta (Khalil *et al.*, 1994; Lee *et al.*, 1999). In contrast, PKC ϵ migrated to the periphery of aortic smooth muscle cells in response to phenylephrine whereas no

such redistribution was detected in the portal vein (Khalil *et al.*, 1992; Lee *et al.*, 1999). This selective effect on specific PKC isoenzymes may reflect discrete activation in a subcellular compartment or differences in the expression of, so-called, scaffold or anchoring proteins such as RACKs to which these enzymes interact.

PLD and PKC-mediated, Ca^{2+} -independent contraction

Several agonists that act through G-protein-coupled receptors including ACh and bradykinin activate PLD in [3H]-palmitic acid-labelled porcine and guinea-pig tracheal smooth muscle respectively in the presence of a primary alcohol as indicated by a significant increase in the formation of [3H]-phosphatidylalcohol (Pyne & Pyne, 1993a,b; Mamoon *et al.*, 1999). Such an effect leads to the formation of phosphatidic acid and, ultimately, DAG by the action of phosphatidic acid phosphohydrolase. Since agonist-induced PLD activation is not associated with Ca^{2+} mobilization, this signal transduction pathway could account for Ca^{2+} -independent contraction of smooth muscles elicited by phorbol diesters and receptor-mediated agonists through the selective activation of nPKC isoforms such as PKC ϵ (see above). However, ACh and bradykinin also stimulate PLC in ASM (Takuwa *et al.*, 1986; Pyne & Pyne, 1993b; see Farmer, 1995) liberating DAG for the activation of cPKC and nPKC isoforms. Although the functional significance of this effect is obscure, it is clear that multiple PKC isoforms could be activated in response to a single stimulus that may act in a complex fashion to fine-tune various functional responses including contraction and proliferation.

PKC and relaxation

Na^+/K^+ -dependent ATPase, Na^+-H^+ exchanger and $Na^+/K^+/Cl^-$ -co-transporter as targets for PKC

High concentrations of phorbol diesters including DPB cause relaxation of methacholine-contracted ASM (Schramm & Grunstein, 1989; Menkes *et al.*, 1986), and evidence is available that this may be due to the activation of the Na^+/K^+ -ATPase. Mechanistically, an increase in extracellular Na^+ would lower the [Ca^{2+}]_i through enhanced Na^+/Ca^{2+} exchange and also hyperpolarize the membrane. Indirect evidence for such a mechanism is the greater relaxation produced in DPB-treated rabbit tracheal smooth muscle when the ATPase is activated by loading the muscle with Na^+ than that produced in the absence of DPB. In addition, an inhibitory effect of DPB on agonist-induced contractions is prevented by inhibiting the ATPase with ouabain or by incubating the muscle in K^+ -free media. This effect is independent of the activation of the Na^+-H^+ exchanger and of Ca^{2+} -influx through VDCCs (Schramm & Grunstein, 1989). Human ASM also relaxes in response to a high concentration of PDBu but this is then followed by contraction (Yang & Black, 1996). Consistent with the results obtained in rabbit trachea, PDBu-induced relaxation is inhibited by ouabain whereas the contractile component is augmented (Yang & Black, 1996).

In guinea-pig trachea, phorbol diesters promote either relaxation (Morrison & Vanhoutte, 1991) or contraction followed by a relaxation (Souhrada & Souhrada, 1989a,b). Moreover, in a study where electrical and mechanical measurements were made simultaneously, phorbol diesters produce a triphasic electrical response: an initial rapid depolarization of the membrane and associated muscle

contraction, hyperpolarization of the membrane and coincident relaxation followed by a second slow depolarization and a gradually developing contracture (Souhrada & Souhrada, 1989a). The biochemical basis for this triphasic effect is complex. All phases of the response were suppressed, to some extent, by verapamil indicating that the phorbol diester promotes Ca^{2+} influx through L-type VDCCs. In addition, the first and second phase were inhibited by amiloride (*c.f.* rabbit trachea) implicating an alteration in Na^+/H^+ exchange that ultimately modifies the $[\text{Ca}^{2+}]_i$. The second (relaxation) component was also inhibited by ouabain suggesting that it is due, in part, to the activation of the Na^+/K^+ ATPase which would lower $[\text{Ca}^{2+}]_i$ through enhanced $\text{Na}^+/\text{Ca}^{2+}$ exchange. The final slow contraction was attenuated by the diuretic frusemide, although whether this is through the inhibition of the $\text{Na}^+/\text{K}^+/\text{Cl}^-$ -co-transporter is unknown. In bovine trachealis, 5-N,N-hexamethylene amiloride and frusemide, inhibitors of the Na^+/H^+ exchanger and $\text{Na}^+/\text{K}^+/\text{Cl}^-$ -co-transporter respectively, have no effect on PDBu-induced contractions which might point to differences in regulation between species.

ATP-sensitive K^+ channels as a target for PKC

Path clamp studies using porcine tracheal smooth muscle cells have established that ACh and PMA inhibit the activation of ATP-sensitive K^+ channels (K_{ATP}) in response to levromakalim (Nuttall & Farley, 1997) implying that a cPKC or nPKC is responsible. Staurosporine antagonized the effect of both ACh and PMA from which the authors suggest that activation of the muscarinic receptor on porcine trachealis inhibits K_{ATP} by a PKC-dependent mechanism. By reducing the open state probability of K_{ATP} , PMA and ACh would promote hyperpolarization, prevent further Ca^{2+} entry through VDCCs and so facilitate relaxation (Nuttall & Farley, 1997). However, given the pharmacology of staurosporine, this interpretation must be viewed cautiously.

Subcellular re-distribution of PKC during relaxation

In methacholine-contracted bovine trachealis, verapamil evokes relaxation and dissociation of PKC from the membrane presumably through an effect on L-type VDCCs (Langlands & Diamond, 1994). Sodium nitroprusside and the β -adrenoceptor agonist, isoprenaline, which are believed to act *via* cyclic GMP and cyclic AMP respectively, also release PKC from the membrane fraction and relax the tissue (Langlands & Diamond, 1992; 1994).

PKC and mitogenesis

Mitogenesis is believed to play an important role not only in normal homeostasis but also in the development of increased smooth muscle content in the airway wall in diseases such as chronic severe asthma. The mitogens involved in this process *in vivo* are undefined, but cell culture-based studies conducted over the last 10 years have identified several ASM mitogens, which are also contractile agonists. These include α -thrombin (Panettieri *et al.*, 1995), histamine (Panettieri *et al.*, 1990), leukotriene D_4 (Cohen *et al.*, 1995), thromboxane (Noveral & Grunstein, 1992), phenylephrine (Noveral & Grunstein, 1994), endothelin-1 (Noveral *et al.*, 1992; Glassberg *et al.*, 1994; Panettieri *et al.*, 1996; Whelchel *et al.*, 1997) and 5-HT (Herschenson *et al.*, 1995). The receptors for these ligands in ASM and other cell types are G protein-coupled to PLC and

the activation of PKC (Yang *et al.*, 1994; Panettieri *et al.*, 1995; Noveral & Grunstein, 1994). This has led investigators to hypothesize that, in addition to a possible role in the regulation of contraction, activation of PKC may also be an important signalling event for the mitogenic response of ASM. In many other cell types, activation of PKC, either directly by phorbol diesters or indirectly by mitogens, has been examined in greater detail and found to be linked to several early events associated with the onset of mitogenesis. These include activation of the Na^+/H^+ exchanger, which promotes intracellular alkalinization, increased cellular protein synthesis and the induction of nuclear proto-oncogenes such as *c-fos* and *c-myc*.

Studies with PKC activators

Panettieri *et al.* (1991a) first demonstrated a possible role for PKC in human ASM mitogenesis by showing that PMA induced DNA synthesis and the expression of *c-fos*. Few subsequent studies have characterized the effect of direct stimulation of PKC using phorbol diesters or synthetic DAG analogues. Instead, many investigators have examined more physiological, receptor-mediated stimuli in ASM cells in which endogenous DAG levels have been manipulated or PKC depletion strategies based on prolonged pretreatment with phorbol esters have been used (Lew *et al.*, 1997; Hirst *et al.*, 1995a; Abe *et al.*, 1994; 1998). In vascular smooth muscle, PKC depletion markedly attenuates phorbol diester-induced phosphorylation of a 76 kDa (p76) protein (presumably autophosphorylated PKC) and abolishes both the proliferative response and expression of *c-fos* (Kariya *et al.*, 1987; 1989; Taubman *et al.*, 1989; Rao & Berk, 1992). In bovine tracheal smooth muscle, stimulation of proliferation by β -hexosaminidases (mannosyl-rich lysosomal hydrolases) acting at mannose-recognizing receptors, was enhanced by increasing endogenous DAG levels using the DAG kinase inhibitor R59022; while depletion of PKC by prolonged treatment with phorbol esters reduced proliferation (Lew *et al.*, 1997). In our own studies, we have shown that prolonged pre-treatment of ASM cells with 4 β -PMA, but not the inactive 4 α -isomer, markedly attenuated the subsequent proliferative response induced by foetal bovine serum (Hirst *et al.*, 1995a) or platelet-derived growth factor (PDGF)-BB (Hirst *et al.*, 1994).

Studies with PKC inhibitors

In search of evidence that activation of PKC is a necessary event for proliferation of ASM, investigators have also examined the effect of inhibitors of PKC. Lew *et al.* (1997) found that pre-treatment of bovine tracheal smooth muscle cells with calphostin C prevented proliferation following activation of mannose receptors by β -hexosaminidase. Proliferation of rabbit tracheal smooth muscle cells induced by foetal calf serum was also inhibited by the bisindolylmaleimide, Ro31-8220 and the less potent analogue, Ro31-7549 (Hirst *et al.*, 1995a). In the same study, these inhibitors also abolished Ca^{2+} -dependent and -independent PKC activity purified from ASM cells. We have found that PKC inhibitors are effective in preventing proliferation of human (Hirst *et al.*, 1995b) and rabbit (Hirst *et al.*, 1994) ASM induced by PDGF isoforms acting through distinct PDGF receptors. Our findings contrast with those of others, using bovine tracheal smooth muscle cells, where calphostin C (Lew *et al.*, 1997) and other selective PKC inhibitors such as GF109203X, Ro31-8220 and chelerythrine (Walker *et al.*, 1998) failed to inhibit PDGF-stimulated DNA synthesis, but are in agreement with other findings

reported in human ASM in which proliferation, induced by PDGF-BB, was dependent not only on tyrosine kinase-dependent mechanisms, but also on PLC-dependent processes in which PKC activation is believed to be a downstream event (De *et al.*, 1996).

Functional role of PKC in proliferation

Little information is currently available on the PKC isoforms that control cell growth or the state of differentiation of ASM. Assender *et al.* (1994) reported that non-proliferating human and rat vascular smooth muscle cells showed a similar qualitative pattern of PKC isoform expression (α , δ and ζ PKC) to proliferating smooth muscle cells in culture. Analysis of the mRNA, however, revealed that unlike their proliferating counterparts, contractile smooth muscle cells did not express mRNA for PKC ϵ . Thus, while PKC α was found in smooth muscle cells in all states of differentiation, expression of PKC ϵ was increased as smooth muscle cells became more proliferative. Expression of PKC β was not detected in either freshly isolated or proliferating smooth muscle cells. In another study, PKC α was down-regulated when rat vascular smooth muscle cells assumed the proliferative-secretory phenotype (Haller *et al.*, 1995). When the cells reassumed their contractile state, PKC α expression reverted to normal levels.

Similar evidence for phenotype-dependent expression of PKC isoforms is also emerging in ASM. For example, in canine tracheal smooth muscle, Halayko *et al.* (1996), using an antibody which recognizes the α and β PKC isoforms (note that PKC α was reported not to be expressed in canine trachealis (Donnelly *et al.*, 1995)), reported that expression of PKC β was increased 8–10 fold in proliferating compared to contractile cells. This is consistent with our own unpublished observations in human ASM in which we have found increased expression of PKC β_1 , as well as PKC ϵ in proliferating cells, compared to their serum-deprived, non-proliferating counterparts (Hirst, Webb and Giembycz, unpublished observations).

The functions of the various PKC isoforms expressed in ASM have not been investigated in any detail and we must look to other tissues for possible clues. In non-muscle cells, the PKC β_{II} and ζ isoenzymes are reported to be essential for differentiation and proliferation (Murray *et al.*, 1993; Berra *et al.*, 1993). In human arterial smooth muscle cells, PKC α activation is necessary for progression from G₁ to the S phase of the cell cycle and subsequent proliferation (Marrilley *et al.*, 1996) and selective depletion of PKC α by antisense strategies prevents this response (Leszczynski *et al.*, 1996). In contrast, PKC δ , also expressed in ASM, has previously been shown to retard cell cycle progression in G₁ through an effect on cell cyclins (Fukumoto *et al.*, 1997), suggesting that activation of different PKC isoforms in smooth muscle may lead either to growth acceleration or inhibition. Assender *et al.* (1994) reported that PKC ζ was present in both proliferating and contractile vascular smooth muscle cells of the rat. A study examining PKC ζ activity in chicken gizzard smooth muscle suggests that this isoform differs very markedly from other PKC isoforms in its susceptibility to down-regulation by phorbol diesters or inhibition by PKC inhibitors such as sphingosine, chelethyrine, staurosporine or calphostin C (Clemont-Chomienne & Walsh 1995). Given that ASM proliferation in response to a range of stimuli can be prevented by prolonged exposure to phorbol diesters or the presence of inhibitors of PKC, it might seem appropriate to conclude that at least in ASM, PKC ζ is not involved directly in the proliferative process. However, a recent preliminary report documented that transfection of human ASM cells with a

dominant negative PKC ζ mutant inhibited PDGF-stimulated DNA synthesis by up to 80% (Black *et al.*, 1998). In a follow-up study, the same investigators demonstrated that proliferation was associated with up-regulation of PKC ζ without any change in the expression of other PKC isoforms (Carlin *et al.*, 1999; Table 2).

While there seems little agreement over the patterns of expression of particular PKC isoforms in either contractile or proliferative vascular ASM, the ability of Ro31-8220 and Ro31-7549 to abolish Ca²⁺-dependent and -independent PKC activity purified from ASM, and to inhibit proliferation (Hirst *et al.*, 1995a), is consistent with a role of at least one or more of these isoenzymes in the proliferative process. Furthermore, both Ro31-8220 and Ro31-7549 potently and selectively inhibit α , β_I , β_{II} and ϵ PKC isoenzymes from other cell systems (Wilkinson *et al.*, 1993), which are also expressed in ASM obtained from several species including humans (Table 2).

Interaction of PKC with other signalling molecules

Although most investigators now agree that activation of PKC is important in the cascade of biochemical events that lead to proliferation, not all agonists that activate PKC cause proliferation in smooth muscle cells. In rat cultured aortic smooth muscle cells, rendered quiescent in a defined serum-free medium, both angiotensin II (Geisterfer *et al.*, 1989) and arginine-vasopressin (Geisterfer & Owens 1989) fail to induce proliferation, but cause marked increases in cellular protein synthesis with subsequent hypertrophy. Despite failure to induce proliferation, both angiotensin II and arginine-vasopressin stimulate early signal transduction events in cultured vascular smooth muscle cells such as activation of PLC, mobilization of intracellular Ca²⁺, activation of PKC and induction of proto-oncogenes (Berk *et al.*, 1988; Griendling *et al.*, 1987; Berk *et al.*, 1989; Taubmann *et al.*, 1989). Likewise, in human ASM cells, histamine and bradykinin promote Ca²⁺ mobilization (Yang *et al.*, 1994; Truett & van Breemen, 1988; 1989), phosphatidylinositol-4,5-bisphosphate hydrolysis (Yang *et al.*, 1994; Daykin *et al.*, 1993) and *c-fos* expression, but only histamine increases DNA synthesis (Panettieri *et al.*, 1991b). This suggests that while these events appear critical for force development in ASM, additional or more sustained regulatory signals are required to promote cell proliferation (Figure 3).

In support of a requirement for additional mechanisms for full mitogenesis, Whelchel *et al.* (1997) showed that activation of ERK-2 was necessary for endothelin-1-stimulated rat tracheal smooth muscle proliferation. In the same study, these authors also demonstrated that activation of ERK-2 was prevented following PKC depletion (by phorbol diester pre-treatment), emphasizing a requirement for one or more of these enzymes. Similar results have also been reported using the PKC inhibitor, Ro31-8220, which partially reduced endothelin-1-stimulated activation of MAP kinase in bovine tracheal smooth muscle (Malarkey *et al.*, 1995). Abe *et al.* (1994) had previously demonstrated a similar requirement for PKC in ERK-2 activation in ASM following stimulation with H₂O₂ but this is not a universal finding as other agonists of G protein-coupled receptors, such as thrombin, are able to activate ERK-2 in PKC-depleted cells. However, in other smooth muscles, thrombin is known to up-regulate the expression of both PDGF-A chain ligands and their corresponding α -receptors (Stouffer & Runge, 1998). These receptors have intrinsic tyrosine kinase activity and retain the ability to activate ERK-2 following PKC depletion (Whelchel *et al.*, 1997). Concomitant up-regulation of PDGF ligands and

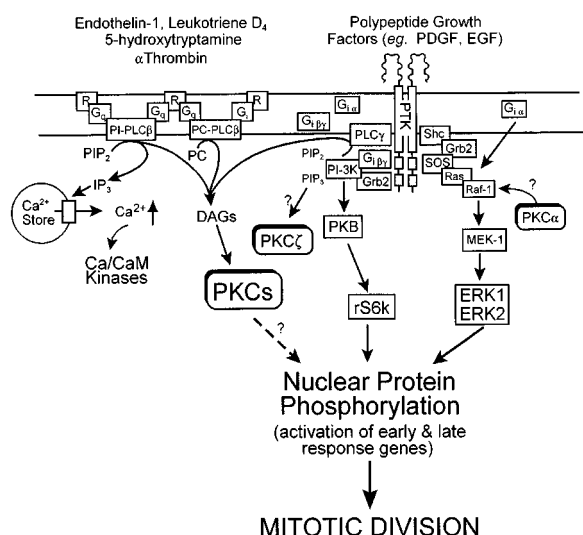


Figure 3 Possible mechanisms that regulate ASM mitogenesis induced by agonists that activate G-protein (G)-coupled receptors (R) and polypeptide growth factor receptor tyrosine kinases (PTK). G protein agonist-induced extracellular kinase (ERK) activation by G_{12} -coupled, but not PTK-linked receptors, occurs by a pertussis toxin-sensitive, Raf-1-dependent pathway, in which G_{12} subunits associate with Ras. Alternatively, interaction of polypeptide growth factors with their receptors results in recruitment of the guanine nucleotide releasing binding protein, Grb2 at the membrane. Grb2 localizes the guanine nucleotide exchange factor, Sos, to Ras. Activation of Ras results in recruitment to the membrane of Raf-1 and phosphorylation and activation of MEK-1 (MAP kinase/ERK-kinase activating kinase). MEK-1 directly phosphorylates and activates ERK1/ERK2 which translocate to the cell nucleus. Activation of PKC is implicated in ASM mitogenesis, though the importance of phosphatidylinositol (PI)- or phosphatidylcholine (PC)-dependent-PLC-derived diacylglycerols (DAGs) is disputed. Currently, no clear consensus for the site of integration of PKC activation in activation of ERK in ASM has been established, although in other systems PKC α may phosphorylate and activate Raf-1. The dotted arrow illustrates possible direct effects of PKC on nuclear transcription elements. Recent evidence suggests proliferation induced by certain Gi-coupled receptor agonists (α -thrombin) may be dependent on activation of PI3kinase by G_{12} subunits. Localized generation of PI 3,4,5-trisphosphate (PIP₃) by PI-3kinase recruits protein kinase B (PKB) to the plasma membrane where it can be fully activated prior to activation of the S6 ribosomal kinase (p70^{S6k}). PIP₃ may also activate PKC ζ and this may offer another integration site for PKC in ASM mitogenesis.

receptors by thrombin may also help explain the potent and efficacious mitogenic activity of this proteinase on ASM.

Further evidence that PKC- and ERK-dependent processes are implicated in agonist-induced ASM mitogenesis is the observation that the MEK-1 inhibitor, PD 098059, prevents proliferation of rat tracheal myocytes elicited by endothelin-1 and PDGF (Whelchel *et al.*, 1997). Thus, it would seem that for a full mitogenic stimulus to be propagated in ASM cells, PKC and ERK signalling cascades act co-operatively and converge at a locus upstream of MEK-1. Tolan *et al.* (1999) have also reported that PKC plays an intermediary step in the activation of the MEK-1/ERK cascade by PDGF in ASM. One possible mechanism that could explain the integration of these two signalling pathways is the activation of Raf-1 (a serine/threonine kinase that lies upstream of MEK-1) by PKC (Figure 3). Indeed, Kolch *et al.* (1993) have reported that Raf-1 is a substrate for PKC α and that phosphorylation results in an increase in catalytic activity. Evidence that such a mechanism operates in ASM derives from studies where phorbol diesters (Vichi *et al.*, 1999) as well as agonists that act through G-protein-coupled receptors that utilize PKC such as endothelin-

1 (Shapiro *et al.*, 1996) and 5-HT (Hershenson *et al.*, 1995), activate Raf-1. Furthermore, H₂O₂ is reported to promote ERK activation in ASM by successive activation of PKC, Raf-1 and MEK-1 (Abe *et al.*, 1998). Finally, Lew *et al.* (1997) have demonstrated that induction of proliferation in bovine tracheal smooth muscle by β -hexosaminidases and activation of the ERK pathway was prevented by the PKC inhibitor calphostin C, an effect which appeared to be mediated at the Ras/Raf-1 locus.

In summary, the available data suggest that activation of PKC is necessary, but not sufficient, for the induction of G protein-coupled receptor-dependent ASM cell proliferation. Additional pathways clearly contribute to the proliferative response. In cultured ASM cells, the critical signalling event appears to be prolonged activation of ERKs. A component of this process is PKC-dependent, as indicated by PKC inhibitor and depletion studies. It is proposed that activation of ERKs by PKC could be mediated by direct phosphorylation of Raf-1, possibly by PKC α . As we learn more, other mechanisms will almost certainly be implicated in the proliferative response of ASM. In bovine trachealis, activation of phosphatidylinositol-3-kinase (PI3kinase) has also been proposed as a key event in mediating PDGF- and α -thrombin-stimulated proliferation (Scott *et al.*, 1996; Walker *et al.*, 1998). Novel (ϵ and η) and atypical (ζ and λ) isoforms of PKC have been shown to be regulated by PI3kinase. Thus, activation of PI3kinase may explain the marked reduction in PDGF-stimulated DNA synthesis reported in human ASM cells transfected with a dominant negative PKC ζ (Black *et al.*, 1998; Carlin *et al.*, 1999). Initial data, however, show little involvement of PKC in the response, but a requirement for activation of MAP kinase-dependent mechanisms for a full mitogenesis was identified (Walker *et al.*, 1998). The apparent PKC-independent mitogenic response may reflect the choice of agonist used to induce proliferation, possible secondary mediator interactions (Stouffer & Runge, 1998), or the species investigated. With the recognition of the diversity of agonists which induce proliferation of ASM *in vitro* and the involvement of multiple intracellular signalling pathways in this process, future studies are needed to characterize the relative importance of each of these intracellular mechanisms in mediating the mitogenic signal. Identification of the critical pathways that modulate ASM growth and division are essential before we can evaluate the role of increased smooth muscle content in the pathogenesis of diseases such as chronic severe asthma or develop novel therapeutic approaches to control these events.

Discussion

Since the discovery of PKC in 1977, much has been learned of the biochemistry and molecular biology of this expanding family of closely related enzymes. Fundamental advances have been made in understanding the regulation of these proteins and how they are positioned in various signalling pathways in a variety of organisms as diverse as budding yeast and mammals. However, the functional role of individual PKC isoenzymes, many of which are co-expressed in the same cell, are poorly studied and present a particular challenge for future research. The availability of small molecule inhibitors that have a degree of selectivity for the three main PKC sub-families have provided clues to their role in processes including smooth muscle contraction and mitogenesis (reviewed here). The use of antisense technology also has shed some light of the functions these enzymes subserve in various cells and tissues. Nevertheless, newer

agents with high degrees of selectivity for individual PKC isoforms are urgently required. Until then, we can only

speculate on the functions of the most extensive serine/threonine protein kinase family thus far identified.

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