Protein kinase C isoenzymes in airway smooth muscle

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The protein kinase C (PKC) isoenzymes expressed by bovine tracheal smooth muscle (BTSM) were identified at the protein and mRNA levels. Western immunoblot analyses reliably identified PKC α , PKC β_1 and PKC β_{II} . In some experiments immunoreactive bands corresponding to PKC δ , PKC ϵ and PKC θ were also labelled, whereas the γ , η and ζ isoforms of PKC were never detected. Reverse transcriptase PCR of RNA extracted from BTSM using oligonucleotide primer pairs designed to recognize unique sequences in the PKC genes for which protein was absent or not reproducibly identified by immunoblotting, amplified cDNA fragments that corresponded to the predicted sizes of PKC δ , PKC ϵ and PKC ζ , which was confirmed by Southern blotting. Anion-exchange chromatography of the soluble fraction of BTSM following homogenization in Ca²⁺-free buffer resolved two major peaks of activity. Using ϵ -peptide as the substrate, the

first peak of activity was dependent upon Ca²⁺ and 4 β -PDBu (PDBu = phorbol 12,13-dibutyrate), and represented a mixture of PKCs α , β_1 and β_{II} . In contrast, the second peak of activity, which eluted at much higher ionic strength, also appeared to comprise a combination of conventional PKCs that were arbitrarily denoted PKC α' , PKC β_1' and PKC β_{II}' . However, these novel enzymes were cofactor-independent and did not bind [³H]PDBu, but were equally sensitive to the PKC inhibitor GF 109203X compared with *bona fide* conventional PKCs, and migrated on SDS/polyacrylamide gels as 81 kDa polypeptides. Taken together, these data suggest that PKCs α' , β_1' and β_{II}' represent modified, but not proteolysed, forms of their respective native enzymes that retain antibody immunoreactivity and sensitivity to PKC inhibitors, but have lost their sensitivity to Ca²⁺ and PDBu when ϵ -peptide is used as the substrate.

INTRODUCTION

The biochemical mechanisms that culminate in the generation of force by smooth muscle contractile proteins are well understood [1–3]. In airway smooth muscle, the interaction of a contractile agonist with its cognate receptor, generally evokes a transient 4–10-fold increase in the cytosolic free Ca²⁺ concentration, which is of sufficient magnitude to occupy the four Ca²⁺-binding domains on calmodulin (see [4]). In this form, the Ca²⁺/calmodulin complex activates myosin light-chain kinase (MLCK), which phosphorylates the 20 kDa light chains (LC₂₀) of the myosin molecule and permits actin to activate myosin ATPase, cross-bridge cycling and, in turn, force generation (reviewed in [4]). Although LC₂₀ phosphorylation is central to this process, evidence accumulated over the last decade implicates a different mechanism(s) in the maintenance of steady-state force. This conclusion derives from findings that force is maintained at varying degrees of LC₂₀ phosphorylation under conditions where cytosolic free Ca²⁺ concentration and actin-activated myosin ATPase activity are at basal or near basal levels [5–9]. These observations prompted Murphy and colleagues [5,10] to propose the existence of a novel stress-bearing non-cycling, or very slowly cycling, interaction between actin and myosin termed the ‘latch-bridge’, which they claimed is responsible for force maintenance. Although the specific details of this concept are still debatable, the existence of a mechanistically distinct, actin–myosin interaction that maintains tonic force is not disputed and raises the question of the nature and regulation of the ‘latch’ state.

Rasmussen et al. [11] have proposed that protein kinase C (PKC) is a potential mediator of tonic force in airway smooth muscle based upon two pieces of evidence. First, application of the phorbol diester, 4 β -PDBu (PDBu = phorbol 12,13-dibutyrate), to bovine trachealis elicited a slow, tonic contracture

during which the muscle behaved in a ‘latch-like’ way. Secondly, exposure of airway smooth muscle strips to 4 β -PDBu and carbachol resulted in the phosphorylation of five contractile/cytoskeletal proteins (caldesmon, filamin, synemin and α - and β -desmin) that paralleled closely the development of force [11,12]. Many of these observations have since been reproduced and extended in a number of independent investigations across various species and smooth muscles (e.g. [13–15]). Moreover, the contracture elicited by phorbol diesters and some Ca²⁺-mobilizing agonists is generally prevented by bisindolylmaleimide derivatives including staurosporine, Ro 31-8220, Ro 31-7549 and GF 109203X, providing further evidence that PKC participates in the genesis of this response [13–15]. Indeed, a recent report documented the ability of purportedly selective PKC inhibitors to preferentially antagonize tonic force in human airway smooth muscle evoked by Ca²⁺-mobilizing agonists [13].

Despite the apparent role of PKC in force maintenance, the evidence in support of this contention is equivocal. Thus there are several publications in which PDBu-induced contraction of airway smooth muscle is attributable to the opening of sarcolemmal voltage-dependent Ca²⁺ channels, with the resultant activation of MLCK, phosphorylation of LC₂₀ and cross-bridge cycling [16,17]. There are other reports that PKC can relax airway smooth muscle under certain conditions [18,19]. Although the reasons for these discrepancies are unclear, one possibility is that the inhibitors employed to implicate PKC in functional responses are not sufficiently selective for this enzyme family. Another highly likely explanation derives from the knowledge that PKC is a generic term that describes a heterogeneous family of enzymes that are differentially distributed between cells and tissues, exhibit differences in sensitivity to allosteric modulators and inhibitors [20,21] and which subserve (presumably) different functions in cells. Indeed, since the discovery of PKC [21,22], 12

Abbreviations used: PKC, protein kinase C; cPKC, conventional protein kinase C; BTSM, bovine tracheal smooth muscle; RT, reverse transcriptase; MLCK, myosin light-chain kinase; LC₂₀, 20 kDa light chains; HRP, horseradish peroxidase; PDBu, phorbol 12,13-dibutyrate.

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distinct isoenzymes have been identified and divided broadly into three main groups, as outlined by Nishizuka [23]: Group A or conventional PKCs (cPKC) are dependent upon calcium, diglyceride and phospholipid for their activity, and include PKC α , PKC β_1 , PKC β_{II} and PKC γ ; Group B or novel PKCs are calcium-independent, of which the δ , ϵ , η and θ isoforms are members; and Group C or atypical PKCs require only phospholipid for activation and are exemplified by PKCs ζ and λ (human equivalent of murine PKC ι [24]). In addition, recent evidence might warrant the designation of a fourth group of PKCs, which is based upon the finding that the catalytic domain of PKC μ , which was originally classified as a member of the novel PKC family, is more closely related to Ca²⁺/calmodulin-dependent protein kinases, such as MLCK, and contains signal and transmembrane moieties at its N-terminus that are absent in other PKC family members [25,26].

To date, the PKC isoenzymes expressed in airway smooth muscle and the function(s) individual variants subserve are unknown. This paper describes experiments designed to identify, at the protein and mRNA level, using Western blot analyses and reverse transcriptase (RT) PCR/Southern blotting respectively, the complement of PKC isoforms expressed in bovine trachealis.

EXPERIMENTAL

Drugs and analytical reagents

The following drugs and analytical reagents were used: PKC α , β_1 , β_{II} , γ , δ , ϵ , θ , ζ and η , and competing peptides were from Santa Cruz Biotechnology/Autogen Bioclear Ltd. (Devizes, Wiltshire, U.K.). High-molecular-mass rainbow markers, [³²P]ATP (30 Ci/mmol), [³H]4 β -PDBu (20 Ci/mmol), enhanced chemiluminescence Western blotting reagents, Hybond paper, donkey anti-rabbit, and sheep anti-mouse, horseradish peroxidase (HRP)-linked IgG were purchased from Amersham International (Little Chalfont, Buckinghamshire, U.K.). Q-Sepharose anion-exchange resin, P-81 phosphocellulose paper, X-Omat film, Tris/HCl/SDS (10%, v/v) mini-gels and hydroxyapatite were from Pharmacia (Uppsala, Sweden), Whatman (Maidstone, Kent, U.K.), Kodak (Hemel Hempstead, Herts., U.K.) and Bio-Rad (Hemel Hempstead, Herts., U.K.) respectively. GF 109203X was kindly given by Laboratoires Glaxo (Les Ulis, France). ϵ -Peptide (ERM β PKRQGSVRRRV) was custom-synthesized by the Department of Chemistry, School of Pharmacy, University of London, U.K. Histone H1S, phosphatidylserine, cyclic AMP-dependent protein kinase inhibitor peptide (IP₂₀) and all other reagents were purchased from the Sigma Chemical Company (Poole, Dorset, U.K.).

Stock solutions of 4 β -PDBu and GF 109203X were made up in DMSO and diluted in the appropriate assay buffer. All other reagents were dissolved in aqueous media.

Partial purification of PKC

Q-Sepharose anion-exchange chromatography

Bovine tracheae were obtained from a local abattoir and transported to the laboratory on ice. The trachealis from each trachea was isolated and denuded of epithelium and all connective tissue was carefully removed. Bovine tracheal smooth muscle (4 g; BTSM) was placed in buffer A [10 mM Mops (pH 7.2)/2 mM EDTA/5 mM EGTA/10 mM dithiothreitol/10% (v/v) glycerol] supplemented with the proteinase inhibitors benzamidine (2 mM), soybean trypsin inhibitor (10 μ g/ml), bacitracin (100 μ g/ml) and leupeptin (100 μ M), chopped with scissors and homogenized at full power for 10 s using a Polytron. The homogenate was centrifuged at 45000 g for 30 min at 4 °C and the supernatant

was passed through glass wool followed by a 45 μ m filter (Sartorius, Göttingen, Germany). The resulting filtrate was then applied to a Q-Sepharose anion-exchange column (Bio-Rad; 1 cm $\times$ 4 cm) pre-equilibrated with buffer A at a rate of 500 μ l/min and eluted with a linearly increasing salt gradient running from 0 to either 550 or 750 mM KCl as indicated in the text. Fractions (1 ml) were collected and assayed for PKC activity as described below.

Hydroxyapatite chromatography

In some experiments active fractions from the Q-Sepharose step were pooled, concentrated by centrifugation (Centriprep-30 columns; molecular-mass cut-off 30 kDa; Pharmacia, Uppsala, Sweden), diluted in buffer B [20 mM Mops (pH 7.2)/0.5 mM EDTA/0.5 mM EGTA/10 mM dithiothreitol/10% glycerol], to lower the conductivity to 2 mS, and loaded on to a column of hydroxyapatite (Bio-Rad; 1 cm $\times$ 4 cm) pre-equilibrated with buffer B at a rate of 500 μ l/min. The column was washed thoroughly and 1 ml fractions were eluted with a linearly increasing salt gradient running from 0 to 160 mM or 60 to 250 mM potassium phosphate as indicated in the text. PKC activity in each fraction was determined as detailed below.

Measurement of PKC activity

PKC activity was estimated by measuring the phosphorylation of histone H1S or ϵ -peptide using a modification of the method of Witt and Roskoski [27]. Assays were performed in duplicate at 30 °C and initiated by the addition of 25 μ l of each column fraction to 75 μ l of a reaction cocktail containing 20 mM Mops, 1 μ M IP₂₀, 15 mM magnesium acetate, 10 μ M ATP (supplemented with approx. 100 c.p.m./pmol [³²P]ATP), 2 mg/ml BSA, 1 mg/ml histone H1S or 150 μ M ϵ -peptide (as indicated in the text) in the presence of either EGTA (2 mM), or CaCl₂ (1.5 mM), phosphatidylserine (100 μ g/ml) and 4 β -PDBu (500 nM). In some experiments the PKC inhibitor GF 109203X was also included in the assay cocktail. Reactions were terminated after 30 min by spotting 50 μ l aliquots of the reaction mixture on to 2 cm $\times$ 2 cm P81 phosphocellulose paper squares that were left for 30 s and then immersed in orthophosphoric acid (150 mM). The paper squares were then extensively washed (4 $\times$ 5 min) with fresh orthophosphoric acid to displace any non-specifically bound ATP and inorganic phosphate, immersed in industrial methylated spirit (5 min) and diethyl ether (5 min) and allowed to dry. Bound radioactivity (representing phosphorylated substrate) was subsequently quantified by liquid-scintillation counting in 4 ml of ACS II scintillant (Amersham International) at an efficiency of approx. 60%.

Binding of [³H] 4 β -PDBu to PKC

Each column fraction (100 μ l) was added to 400 μ l of buffer C [20 mM Mops (pH 7.2)/80 mM KCl/1.2 mM CaCl₂/50 μ M EGTA/phosphatidylserine (80 μ g/ml)/0.5% (v/v) DMSO] containing 30 nM [³H]4 β -PDBu and incubated for 20 min at 30 °C. Reactions were terminated by the addition of 2.5 ml of ice-cold buffer C containing 0.3% (v/v) polyethyleneimine followed by rapid vacuum filtration over Whatman GF/B filters that had been presoaked in the same buffer for at least 60 min. Filters were washed twice with 5 ml of 0.5% DMSO and the radioactivity retained by each filter subsequently measured by liquid-scintillation counting in 4 ml of Filtron X (National Diagnostics, Hemel Hempstead, Herts., U.K.). Non-specific binding was defined in the presence of 10 μ M unlabelled 4 β -PDBu.

Table 1 Primers and conditions used in RT PCR and Southern blot analyses

Gene	Accession number	Primer		Human gene co-ordinates (5' to 3')	Product size (bp)	Annealing temperature (°C)/time (s)
		Direction	Deoxyoligonucleotide sequence			
PKC δ	M19338 X72972	Forward	5'-AAA GGC AGC TTC GGG AAG GT- 3'	1127-1146	260	58/30
		Reverse	5'-TGG ATG TGG TAC ATC AGG TC-3'	1376-1358		
		Detection	5'-GAC GAC GAC GTG GAC TGC ACC-3'	1220-1240		
PKC ϵ	X72974	Forward	5'-AGC TTG AAG CCC ACA GCC TG-3'	59-78	464	58/30
		Reverse	5'-CTT GTG GCC GTT GAC CTG ATG-3'	523-503		
		Detection	5'-GCT GCT GCA GAA CGG GAG CC-3'	322-341		
PKC ζ	X72973	Forward	5'-GTC CTC CCA GAT GGA GCT GGA AG-3'	187-209	359	54/30
		Reverse	5'-GAA GGC ATG ACA GAA TCC AT-3'	546-527		
		Detection	5'-GAG GAA GCT GTA CCG TGC CA-3'	343-362		
PKC λ	L18964 D28579	Forward	5'-TAT AAT CCT TCA AGT CAT G-3'	663-679	547	56/30
		Reverse	5'-TTA CAC ATG CCG TAG TCA GT-3'	1208-1189		
		Detection	5'-GAA GCA TGT GTT TGA GCA GG-3'	912-931		
PKC θ	L07032	Forward	5'-CTA TCA ATA GCC GAG AAA CCA TG-3'	659-681	418	54/30
		Reverse	5'-CTC ATC CAA CGG AGA CTC CC-3'	1074-1058		
		Detection	5'-ATG ATT GAG AGC ACT CAA CA-3'	901-920		
PKC η	M55284	Forward	5'-ACG GTG AGC GTG GAC CAG GT-3'	302-321	642	58/30
		Reverse	5'-GAT CGC AGA ATG TTG GCA C-3'	944-926		
		Detection	5'-CAG TTC CAG GAC CTC GTC GG-3'	473-492		
GAPDH		Forward	5'-TCT AGA CGG CAG GTC AGG TCC ACC-3'		598	58/30
		Reverse	5'-CCA CCC ATG GCA AAT TCC ATG GCA-3'			

Western immunoblot analysis of PKC isoenzymes

Approx. 2 g of BTSM, rat quadriceps femoris (control for PKC θ) and rat brain (control for all other isoforms except PKC η) were suspended in 2 ml of buffer A supplemented with the proteinase inhibitors benzamidine (2 mM), soybean trypsin inhibitor (10 μ g/ml), bacitracin (100 μ g/ml) and leupeptin (100 μ M), finely chopped with scissors and homogenized at full power for 10 s with a Polytron. The resulting homogenate was centrifuged at 100000 *g* for 60 min and the supernatant filtered through glass wool. The remaining particulate material was sonicated (3 $\times$ 5 s) in buffer A in the presence of proteinase inhibitors, frozen in liquid N₂, pulverized in a mortar and pestle and re-centrifuged as described above. Aliquots from the supernatant and solubilized particulate material were diluted (50/50) in buffer D [62.5 mM Tris/HCl (pH 6.8)/20% glycerol/2% SDS/5% (v/v) β -mercaptoethanol/0.5% (w/v) Bromophenol Blue] and denatured by boiling for 5 min. For the immunodetection of PKC isoforms, 20–100 μ g of denatured protein was loaded on to a 10% SDS ready gel and run at 200 mA for 40 min at 25 °C. Proteins were transferred on to Hybond nitrocellulose paper in buffer E [193 mM glycine/50 mM Tris/base/0.03% SDS/20% (v/v) methanol] at 200 mA and 25 °C for 3 h and the nitrocellulose membranes were subsequently blocked overnight at 4 °C in buffer F [10 mM Tris/base/150 mM NaCl/5% (w/v) non-fat milk] and then incubated for 1 h at 25 °C with the appropriate rabbit polyclonal anti-(PKC isoform)-specific IgG (diluted 1/500 in buffer F). Membranes were then washed (5 $\times$ 5 min) at 25 °C in buffer F (excluding non-fat milk) and then incubated for a further 60 min with a donkey anti-(rabbit HRP)-linked IgG [diluted 1/6000 in buffer F supplemented with 0.05% (v/v) Tween 20]. Filters were washed thoroughly (5 $\times$ 5 min washes in buffer F excluding non-fat milk) and antibody-labelled proteins were detected by enhanced chemiluminescence, photographed and then developed.

For antibody-blocked experiments, PKC antisera were pre-adsorbed with the peptide used as the immunogen, incubated overnight at 4 °C with gentle agitation and diluted to the desired

working dilution. The nitrocellulose membranes were stripped and then re-probed with the blocked antibody as described above.

Detection of PKC isoform mRNA by RT PCR and Southern blotting

Total RNA was extracted from approx. 250 mg of BTSM according to the single-step procedure of Chomczynski and Sacchi [28]. RNA (1 μ g) was reverse-transcribed using avian-myeloblastosis-virus Reverse Transcriptase according to the manufacturer's instructions, and RT-generated cDNAs encoding the PKC genes were amplified by PCR using specific primers (Table 1) designed from the reported human sequences deposited with the GenBank database [29–40]. Since gene sequences were not available for any bovine PKC isoenzyme, forward, reverse and detection primers were designed by cross-matching human cDNA sequences for each isoform with unique regions of identity present in the cDNA sequences for the same isoform from the mouse and rabbit (see Table 1 for accession numbers).

To confirm the integrity of each RNA sample, RT PCR analysis of the GAPDH gene was routinely performed using primers synthesized from sequences described by Maier et al. [41]. PCR amplification was conducted in a reaction volume of 25 μ l using a Hybaid OmniGene thermal cycler (Hybaid, Teddington, Middlesex, U.K.) and 0.5 unit of *Taq* polymerase set for 35 cycles at a denaturing temperature of 94 °C for 30 s, at a specific annealing temperature (Table 1) and at an extension temperature of 72 °C for 30 s. PCR products were subsequently size-fractionated on 1.5% (w/v) agarose gels, stained with ethidium bromide and visualized under UV light.

For Southern blotting, the 1.5% agarose gels were denatured, neutralized and transferred to nylon membranes by the 'pocket blotting' method [42], and the DNA was cross-linked to the membrane by UV light. Membranes were incubated for 4 h at 65 °C in buffer G [6 $\times$ SSC (0.15 M NaCl/0.015 M sodium citrate)/5 $\times$ Denhardt's solution (0.02% Ficoll/0.02% polyvinylpyrrolidone/0.002% BSA)/20 mM NaH₂PO₄/0.1% (w/v) SDS/sonicated salmon sperm DNA (250 μ g/ml)] and hybridized

at 65 °C overnight in the same buffer with ^{32}P -radiolabelled gene-specific internal oligonucleotide probes directed against a sequence internal to the specific PCR product of interest (Table 1). The filters were washed twice in $3 \times \text{SSC}/0.1\%$ SDS for 15 min at 65 °C, once with $1 \times \text{SSC}/0.1\%$ SDS for 30 min at 65 °C and, finally, twice with $0.1 \times \text{SSC}/0.1\%$ SDS for 30 min at 65 °C. Each filter was then exposed for 6–18 h to Kodak X-OMAT film with an intensifying screen and developed. Cyclic AMP phosphodiesterase 4A5 amplification fragments and genomic DNA from human lung were routinely used as negative and positive controls respectively.

To control for possible genomic contamination of DNA samples, all PCR reactions were also performed with 100 ng of genomic DNA, and test sample RNA was processed in parallel with the reverse-transcribed sample in the absence of RT. To guard against contamination by PCR products, water blanks were subjected to PCR in parallel with test samples.

RESULTS AND DISCUSSION

Complement of PKC isoenzymes expressed by BTSM

Representative immunoblots of the PKC isoenzymes present in BTSM are shown in Figure 1. Using isotype-selective polyclonal antibodies, strong immunoreactive bands were identified at 81 kDa corresponding to PKC α , PKC β_1 and PKC β_{11} . In some experiments, bands of approx. 81, 82 and 87 kDa were also revealed, corresponding to PKC δ , PKC θ and PKC ϵ respectively; no evidence for PKCs γ , η or ζ was obtained. The identification of immunoreactive bands as PKCs was suggested from experiments using rat brain (PKCs α , β_1 , β_{11} , γ , δ , ϵ), skeletal muscle (PKC θ) and recombinant PKC η as positive controls (Figure 1), and by employing antibodies pre-adsorbed with the peptides used as immunogens. In these latter experiments immunoreactive

Figure 1 Western immunoblot analysis of PKC isoenzymes in BTSM

The 100000 *g* supernatant (20–100 μg) or solubilized particulate material from BTSM was denatured and subjected to electrophoresis on SDS/polyacrylamide (10% gels). Rat brain (4–40 μg) was loaded as a positive control for all isoforms with the exception of PKC η (1 ng) and PKC θ (8–20 μg), for which recombinant PKC η and rat skeletal muscle were used respectively. Proteins were transferred on to nitrocellulose and probed with rabbit polyclonal anti-(PKC isoform)-specific IgG, extensively washed and incubated with a donkey anti-(rabbit HRP)-linked IgG. Antibody-labelled proteins were subsequently detected by enhanced chemiluminescence, photographed and developed. The position of 97, 69 and 45 kDa markers is indicated in each blot. See the Experimental section for further details. S, soluble fraction; P, particulate fraction; +, positive control; +S, positive control from soluble fraction; +P, positive control from particulate fraction.

Table 2 PKC isoenzymes expressed in tracheal smooth muscle

Human trachealis data were compiled from refs. [45,46]. Canine trachealis data were taken from ref. [47]. – Isoenzyme not detected; + isoenzyme detected; ND, not determined.

PKC isoform	Bovine trachealis	Human trachealis	Canine trachealis
PKC α	+	+	–
PKC β_1	+	+	+
PKC β_{11}	+	+	+
PKC γ	–	–	–
PKC δ	+	+	+
PKC ϵ	+	+	+
PKC ζ	+	+	+
PKC η	–	+	–
PKC λ	ND	ND	ND
PKC θ	+	–	+

*Isoenzyme detected at the mRNA level only.

bands were not detected with blocked antibodies for any PKC isoenzyme in BTSM or the positive controls (results not shown).

Reference to Figure 1 also shows that the localization of PKC isoenzymes varied between the cytosolic and the 100000 *g* membrane fraction. Thus whereas PKC α and PKC β_1 were predominantly soluble isoenzymes, PKC β_{11} , PKC δ and PKC θ were enriched in the particulate material and PKC ϵ was distributed approximately evenly between the two fractions. This pattern of PKC isoenzyme distribution is similar to many other tissues including adipocytes [43], fibroblasts [44] and eosinophils (M. A. Lindsay and M. A. Giembycz, unpublished results). Intriguingly, the complement of PKC isoforms expressed in BTSM at the protein level differed from that present in other species (Table 2). For example, whereas PKC α is present in BTSM (the present study) and human trachealis [45,46], it is apparently absent in canine airways [47]. Conversely, PKC ζ is abundantly expressed in canine trachealis [47] but was not detected by Western blot analysis in the present study. The reason(s) for these discrepancies is not immediately clear but at least two possibilities are worthy of consideration that are not mutually exclusive. One obvious explanation is a *bona fide* difference in PKC isoenzyme expression between species. Alternatively, the complement of PKCs might be uniform across human, canine and bovine airways, but the expression of protein might vary so that certain isoforms cannot be readily detected immunologically. Indeed, some evidence to support this latter contention was the unequivocal identification of mRNA transcripts for PKC ζ that was not confirmed at the protein level (see below).

Detection of PKC isoenzyme mRNA in BTSM by RT PCR and Southern blotting

The possible expression of mRNA for the PKC isoforms (PKCs δ , ϵ , ζ , η , λ , θ) that were absent or were not always identified immunologically, or for which antibodies were not commercially available, was determined by RT PCR using primer pairs designed to recognize unique sequences in the human PKC genes (see the Experimental section for details). PCR products of the expected size were not detected for PKC η and PKC λ mRNA, indicating that the primer pairs designed from the human, mouse and rabbit PKC cDNAs failed to recognize the homologous bovine proteins (results not shown). However, staining of

Figure 2 Representative ethidium bromide-stained agarose gels of PKC isoenzyme message amplification fragments in BTSM and confirmation of PCR product identity by Southern blotting

Total RNA was extracted from approx. 250 mg of BTSM, and 1 μ g was reverse-transcribed to generate cDNAs for the PKC genes using specific primers (Table 1). PCR was then performed (35 cycles) using reverse-transcribed cDNA, the products subjected to electrophoresis on 1.5% agarose gels and DNA subsequently visualized by ethidium bromide staining (a). RT-PCR product sizes for PKCs δ , ϵ and ζ were 260, 464 and 359 bp respectively. The left-hand lane shows Hinf1 ϕ X174 base pair markers. For Southern blotting (b), PCR products were transferred on to nylon membranes and hybridized with the appropriate 32 P-labelled detection oligonucleotide specific to the PKC isoenzyme sequence of interest (Table 1). Filters were then washed extensively and exposed to film. Cyclic AMP phosphodiesterase 4A amplification fragments and genomic DNA from human lung were routinely used as negative and positive controls respectively. See the Experimental section for further details.

ethidium bromide gels for PCR products amplified from total RNA, extracted from BTSM, routinely revealed cDNA fragments that corresponded to the predicted sizes of PKC δ (260 bp), PKC ϵ (464 bp) and, in contrast with the Western blot analysis results, PKC ζ (359 bp) (Figure 2a). Conversely, a PCR product corresponding to mRNA for the θ isoform of PKC was not detected in any sample after 35 cycles of amplification when compared with genomic DNA extracted from human lung, which acted as a positive control (results not shown). It is possible that since PKC θ was unequivocally identified at the protein level, these observations might reflect stable expression and a low turnover of product associated with a limited translation rate of PKC θ message.

To confirm the identity of the cDNAs amplified by PCR, Southern blots for BTSM were prepared and hybridized with radiolabelled oligonucleotide 'detection' probes specific to each isoform. As shown in Figure 2(b), Southern blotting of the PCR products verified the presence of PKC δ , PKC ϵ and PKC ζ mRNA. Collectively, these data suggest that BTSM has the potential to express at least seven PKC isoforms (α , β_1 , β_{II} , δ , ϵ , θ , ζ). The finding that PKC ζ was not detected at the protein level is somewhat surprising but can be explained by a low or nominal translation rate of PKC ζ mRNA, the generation of unstable protein, or the restricted expression of PKC ζ in a minor population of contaminating non-smooth muscle cells. In the former respect, it is intriguing that PKC ζ is intimately involved in cell maturation and DNA synthesis [48,49]. If a primary role of this isoenzyme is the regulation of mitogenesis in BTSM, then it is perhaps not surprising that its mRNA is not translated in

Figure 3 Identification of cPKC isoenzymes in BTSM by anion-exchange chromatography and Western blotting

The 45000 g supernatant from BTSM was applied to a column of Q-Sepharose, washed and eluted with a linearly increasing salt gradient (0–750 mM KCl). In (a) fractions were assayed for PKC activity in the presence of Ca^{2+} , phosphatidylserine (PS) and PDBu (●), PS and PDBu (○) and PS only (■) using ϵ -peptide as the substrate. In (b) PKC α , PKC β_1 and PKC β_{II} immunoreactivity for selected fractions spanning the chromatogram is shown, as assessed by immunoblotting. The position of 97 and 69 kDa markers is indicated in each blot. See legend to Figure 1 and the Experimental section for further details.

mitotically quiescent cells. It is important to emphasize that the discrepancies between the expression of mRNA and protein is not peculiar to BTSM. Indeed, these data are entirely consistent with a recent study performed by Assender et al. [50], who detected mRNA but not protein for PKC ϵ in vascular smooth muscle.

Anion-exchange chromatography of PKC isoenzymes in the soluble fraction of BTSM

Figure 3(a) shows a typical Q-Sepharose chromatogram of PKC activity from the cytosol of BTSM. Using ϵ -peptide as the substrate, one major peak (fractions 6–15) of Ca^{2+} /PDBu-dependent activity was resolved by the column eluting at 30 mM KCl, which was markedly reduced (by approx. 60%) when Ca^{2+} were omitted from the reaction cocktail, and was essentially abolished in the absence of both Ca^{2+} and PDBu. Qualitatively identical results were obtained when histone H1S was used as the substrate (results not shown). In addition, a much broader peak of activity (fractions 33–70) was eluted from the column at higher ionic strength (190–450 mM KCl), and did not require Ca^{2+} and PDBu for the phosphorylation of ϵ -peptide (Figure 3a) but, paradoxically, was dependent upon lipid cofactors (but not Ca^{2+}) when histone H1S was the substrate (results not shown).

To determine the identity of the PKC activities in each peak, column fractions were immunoblotted with polyclonal antibodies directed against PKCs α , β_1 and β_{II} . As shown in Figure 3(b), the cofactor-dependent and -independent peaks of activity apparently contained a mixture of PKC α , PKC β_1 and PKC β_{II} . For the purposes of this study the enzymes that comprised the constitutive activity were assigned PKC α' , PKC β_1' and PKC β_{II}' .

Figure 4 Identification of PKC ϵ in BTSM by anion-exchange chromatography and Western blotting

The 45000 *g* supernatant from BTSM was applied to a column of Q-Sepharose, washed and eluted with a linearly increasing salt gradient (0–750 mM KCl). In (a) fractions were assayed for PKC activity in the presence of phosphatidylserine (PS) and PDBu (●) using histone IIIS as the substrate. Panel (b) shows PKC ϵ immunoreactivity for selected fractions assessed by immunoblotting. The position of 97 and 69 kDa markers is indicated. See legend to Figure 1 and the Experimental section for further details.

(see below for rationale). The expression of a cofactor-independent cPKC was also recently reported by Allen et al. [51]. In that study, hydroxyapatite chromatography of rat brain supernatant yielded a peak of activity that was apparently PKC α but did not require Ca^{2+} or phorbol diester for activity when ϵ -peptide, ζ -peptide or myelin basic protein were the substrates, and bound more avidly to the exchange resin than *bona fide* PKC α [51] (see below).

In initial studies, PKC ϵ was not always identified due, presumably, to a low level of expression. However, in subsequent experiments bands of PKC ϵ -like immunoreactivity were detected when more protein (> 20 μg) was subjected to SDS/PAGE and coincided with PKC activity that eluted at approx. 180 mM KCl (Figure 4). PKC δ was never detected due to a low degree of expression in the cytosol (Figure 1).

Ability of regulated and constitutively active BTSM PKCs to bind [^3H]PDBu

The nature of the cofactor-dependent and -independent activities was investigated further by assessing their ability to bind [^3H]PDBu. As illustrated by the chromatogram in Figure 5, which was developed using a steeper salt gradient than those shown in Figures 3 and 4, the PKC isoenzymes comprising the first peak bound [^3H]PDBu in a manner that mirrored the phosphorylation of histone IIIS. This finding was therefore entirely concordant with that predicted from the cPKCs in BTSM, which had an absolute requirement for Ca^{2+} and phorbol diester for activity (Figure 3). In contrast, the second, constitutive peak of PKC activity behaved as if it was an atypical isoenzyme when ϵ -peptide was the substrate, in that it failed to bind [^3H]PDBu. Nevertheless, this observation was consistent with the phorbol diester-independence of ϵ -peptide phosphorylation that was catalysed by the isoenzymes present in these fractions (Figures 3a and 5a).

Figure 5 Binding of [^3H]PDBu and cofactor dependence of PKC isoenzymes resolved by anion-exchange chromatography

The 45000 *g* supernatant from BTSM was applied to a column of Q-Sepharose, washed and eluted with a linearly increasing salt gradient (0–550 mM KCl). In (a) fractions were assayed for PKC activity in the presence of Ca^{2+} , phosphatidylserine (PS) and PDBu (●) and PS only (○) using histone IIIS as the substrate. Panel (b) shows the binding of [^3H] 4 β -PDBu (■) to each column fraction. Note the inability of the 4 β -PDBu to bind to the cPKCs with constitutive activity. See the Results and Discussion section and the Experimental section for further details.

Hydroxyapatite chromatography of regulated and constitutively active BTSM PKCs

Fractions that comprised the regulated and constitutively active PKCs were pooled separately, concentrated, desalted and subjected to hydroxyapatite chromatography. Figures 6(a) and 7(a) show typical elution profiles of the Ca^{2+} /PDBu-dependent PKC activity using histone IIIS as the substrate. Two peaks of Ca^{2+} /PDBu-dependent activity were partially resolved by the column, eluting at approx. 60 and 100 mM potassium phosphate, and were mirrored exactly by the binding of [^3H]PDBu (Figure 6b). Western immunoblot analysis revealed that the first and second peaks of activity represented a mixture of PKC β_1 and PKC β_{11} , and PKC α and PKC β_1 respectively (Figure 7b). It is currently unclear why PKC β_1 eluted at two different ionic strengths, although it is possible that PKC α was contaminated with PKC β_1' , since approx. 20% of the activity was Ca^{2+} /PDBu-independent (results not shown). Unexpectedly, when the combination of PKC α' , PKC β_1' and PKC β_{11} was subjected to hydroxyapatite chromatography (Figure 8), a slightly different, but nevertheless reproducible, profile of activity was observed. Thus three discernible peaks of activity were resolved by the column eluting at 90, 130 and 150 mM potassium phosphate (Figure 8). Consistent with results obtained with regulated PKCs, the first peak of activity contained a mixture of PKC β_1 and PKC β_{11} isoenzymes and the third peak was PKC α (Figure 8).

Figure 6 Resolution of PKC isoenzymes by hydroxyapatite chromatography: $[\text{3H}]\text{PDBu}$ binding and cofactor dependence

Active fractions comprising Ca^{2+} /PDBu-regulated activity resolved from the Q-Sepharose step were pooled, concentrated and applied to a hydroxyapatite column. After extensive washing the column was eluted with a linearly increasing salt gradient (0–160 mM potassium phosphate buffer). In (a) fractions were assayed for PKC activity in the presence of Ca^{2+} , phosphatidylserine (PS) and PDBu (●) and PS only (○) using histone H1S as the substrate. Panel (b) shows the binding of $[\text{3H}]\text{4}\beta\text{-PDBu}$ (■) to each column fraction. See the Results and Discussion section and the Experimental section for further details.

However, histone kinase activity in the second peak of activity was recognized by the $\text{PKC}\beta_{\text{II}}$ antibody, but migrated as a 54 kDa protein on SDS/PAGE. These data indicate that limited proteolysis of the native $\text{PKC}\beta_{\text{II}}$ isoenzyme had occurred, which apparently arose during the processing of the sample since it was not detected by Western blot analysis following the initial anion-exchange chromatography step.

Sensitivity of regulated and constitutively active PKCs to bisindolylmaleimide PKC inhibitors

The bisindolylmaleimide derivative, GF 109203X, which is a selective inhibitor of cPKCs [51], was evaluated for its ability to inhibit the regulated and constitutively active cPKC isoenzymes resolved by hydroxyapatite chromatography (see Figures 6 and 7). At 10 μM ATP, GF 109203X inhibited $\text{PKC}\alpha/\beta_1$, $\text{PKC}\beta_1/\beta_{11}$, $\text{PKC}\alpha'$ and $\text{PKC}\beta_1'/\beta_{11}'$ equally with IC_{50} values of 2.9 ± 0.7 ($n = 3$), 4.3 ± 2.4 ($n = 3$), 0.9 ± 0.2 ($n = 3$) and 2.3 ± 0.9 nM ($n = 3$) respectively. These data are comparable to the potency of GF109203X reported by Toullec et al. [52] against the cPKC isoenzymes purified from rat brain.

Nature of the Ca^{2+} /PDBu-independent activities

The identification of protein kinases that: (i) were recognized by antibodies specific for the α , β_1 and β_{11} isoenzymes of PKC, (ii)

Figure 7 Resolution of cPKC isoenzymes in BTSM by hydroxyapatite chromatography and identification by Western blotting

Active fractions comprising Ca^{2+} /PDBu-regulated activity, resolved from the Q-Sepharose step, were processed as described in the legend to Figure 6. In (a) fractions were assayed for PKC activity in the presence of Ca^{2+} , phosphatidylserine and PDBu (●) using histone H1S as the substrate. In (b) $\text{PKC}\alpha$, $\text{PKC}\beta_1$ and $\text{PKC}\beta_{11}$ immunoreactivity for selected fractions spanning the chromatogram in panel (a) is shown, as assessed by Western blotting. See the Results and Discussion section and the Experimental section for further details.

were inhibited by GF 109203X to the same extent as *bone fide* cPKCs, (iii) phosphorylated ϵ -peptide in the absence of Ca^{2+} and PDBu, and (iv) bound relatively strongly to Q-Sepharose compared with native cPKCs, prompts speculation on the nature of these activities. The Western blot analysis results presented in Figures 1 and 3(b) unambiguously exclude the possibility that the constitutive activity is attributable to $\text{PKC}\zeta$. Equally, limited proteolysis of cPKCs or the presence of the atypical isoenzyme $\text{PKC}\lambda$, which is a 67 kDa protein, were considered unlikely to account for these novel activities, since $\text{PKC}\alpha'$, $\text{PKC}\beta_1'$ and $\text{PKC}\beta_{11}'$ migrated as approx. 81 kDa proteins on SDS/PAGE in a manner that was indistinguishable from the behaviour of their Ca^{2+} /PDBu-regulated counterparts (see Figure 3b). This conclusion was supported further by the observation that histone kinase activity retained an absolute requirement for lipid co-factors (see above). Another consideration was that PKCs α' , β_1' and β_{11}' were phosphorylated forms of the same enzyme, but this too was discounted as phosphorylation does not render cPKCs independent of Ca^{2+} and PDBu. Taken together, these data therefore suggest that PKCs α' , β_1' and β_{11}' represent modified, but not proteolysed, forms of their respective native enzymes that retain antibody immunoreactivity and sensitivity to GF 109203X, but have lost their sensitivity to Ca^{2+} and PDBu when ϵ -peptide is used as the substrate. A similar conclusion was reached by Allen et al. [51] for a constitutively active form of $\text{PKC}\alpha$, designated $\text{PKC}\alpha'$, identified in rat brain, based upon the findings that (i) $\text{PKC}\alpha'$ was inhibited by the pseudosubstrate peptide inhibitor 19-31 in $\text{PKC}\alpha$, (ii) autophosphorylation of $\text{PKC}\alpha'$ was not stimulated by Ca^{2+} and PDBu, unlike the native enzyme, (iii) $\text{PKC}\alpha'$ was of the same mass as $\text{PKC}\alpha$ on SDS/PAGE and (iv) two-dimensional phosphopeptide maps of autophosphorylated $\text{PKC}\alpha$ and $\text{PKC}\alpha'$ were identical.

Figure 8 Resolution of constitutively active PKC isoenzymes in BTSM by hydroxyapatite chromatography, and identification by Western blotting

Active fractions comprising constitutive cPKC activity, resolved from the Q-Sepharose step, were pooled, concentrated and applied to a column of hydroxyapatite. After extensive washing, the column was eluted with a linearly increasing salt gradient (60–250 mM potassium phosphate). In (a), fractions were assayed for PKC activity in the presence of Ca^{2+} , phosphatidylserine and PDBu (●) using histone H1S as the substrate. In (b) PKC α , PKC β_1 and PKC β_{11} immunoreactivity for selected fractions spanning the chromatogram in (a) is shown, as assessed by Western blotting. See the Results and Discussion section and the Experimental section for further details.

A survey of the literature suggests that one possible explanation for the appearance of PKCs α' , β_1' and β_{11}' in the present study was enzyme oxidation. Indeed, PKC is susceptible to oxidative modification at both its regulatory and catalytic domains [51,53–55], which, depending on the specific conditions employed, can result in either inhibition of catalytic activity or the generation of a constitutively active form of the enzyme. The similarity between cPKCs and PKCs α' , β_1' and β_{11}' is in keeping with this hypothesis and is the subject of a current investigation.

CONCLUSIONS

The experiments described herein clearly demonstrate that BTSM can express multiple (and presumably multi-functional) PKC isoenzymes. The identification also of Ca^{2+} /PDBu-independent cPKCs, is consistent with data reported by Allen et al. [51], who have proposed that expression of constitutively active PKC α in rat brain is due to selective regulatory site oxidation of the native enzyme that involves the deletion of the auto-inhibitory domain from the active site of the protein. Although the functional consequences of PKC oxidation are unexplored, it is tempting to speculate that as one or more of these isoenzymes is apparently involved in the regulation of airway smooth muscle contractility and mitogenesis, it might have significant implications in disease states associated with oxidative stress such as asthma.

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