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Identification of the Protein Kinase C Isoenzymes in Human Lung and Airways Smooth Muscle at the Protein and mRNA Level

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ABSTRACT. The protein kinase C (PKC) isoenzymes expressed by human peripheral lung and tracheal smooth muscle resected from individuals undergoing heart–lung transplantation were identified at the protein and mRNA level. Western immunoblot analyses of human lung identified multiple PKC isoenzymes that were differentially distributed between the soluble and particulate fraction. Thus, PKCα, PKCβ_{II}, PKCε, and PKCζ were recovered predominantly in the soluble fraction whereas the η isoform was membrane-associated together with trace amounts of PKCα and PKCε. PKCβ_1-like immunoreactivity was occasionally seen although the intensity of the band was uniformly weak. Immunoreactive bands corresponding to PKCs γ, δ, or θ were never detected. Reverse transcription-polymerase chain reaction (RT-PCR) of RNA extracted from human lung using oligonucleotide primer pairs that recognise unique sequences in each of the PKC genes amplified cDNA fragments that corresponded to the predicted sizes of PKCα, PKCβ_I, PKCβ_{II}, PKCε, PKCζ, and PKCη (consistent with the expression of PKC isoenzyme protein) and, in addition, mRNA for PKCδ; PCR fragments of the expected size for the supposedly muscle-specific isoform, PKCθ, or the atypical isoenzyme, PKCλ, were never obtained. The complement and distribution of PKC isoforms in human trachealis were similar, but not identical, to human lung. Thus, immunoreactive bands corresponding to the α, β_I, β_{II}, ε, and ζ isoenzymes of PKC were routinely labelled in the cytosolic fraction. In the particulate material PKCα, PKCε, PKCα, PKCη, and PKCμ were detected by immunoblotting. With the exception of PKCζ, RT-PCR analyses confirmed the expression of the PKC isoforms detected at the protein level and, in addition, identified mRNA for PKCδ. Collectively, these data clearly demonstrate the expression of multiple PKC isoenzymes in human lung and tracheal smooth muscle, suggesting that they subserve diverse multifunctional roles in these tissues. BIOCHEM PHARMACOL **54**;1:199–205, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. human lung; human tracheal smooth muscle; human trachealis; protein kinase c; PKC mRNA; PKC multiplicity

Protein kinase C (PKC§) is a generic term used to describe the largest serine/threonine-directed kinase subfamily currently known [1]. Since the discovery of PKC in 1977 [2, 3], multiple isoenzymes have been identified unequivocally that differ in primary structure, cellular distribution, substrate specificity, inhibitor sensitivity and mechanism of activation [4, 5]. The PKC isoenzymes can be broadly divided into three main groups as outlined in Nishizuka [6]: Group A or conventional PKCs (cPKC) are dependent upon calcium, diglyceride and phospholipid for their activity and include PKCα, PKCβ_I, PKCβ_{II} and PKCγ; Group B or novel PKCs (nPKC) are calcium-independent of which the δ, ε, η and θ isoforms are members; and Group C or atypical PKCs (aPKC) that require only phospholipid for activation and are typified by PKCs ζ and λ (human equivalent of murine PKCι). In addition, recent evidence warrants the designation of a fourth group of PKCs which is based upon the finding that the catalytic domain of PKCμ (classified originally as a member of the nPKC family) is more closely related to Ca^{2+}/calmodulin-dependent protein kinases such myosin light chain kinase and contains signal and transmembrane moieties at its amino terminus that are absent in other PKC family members [7, 8].

Despite the continuing discovery of new PKC members and our increasing knowledge of their basic biochemistry

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§ *Abbreviations:* aPKC, atypical protein kinase C; ASM, airways smooth muscle; cPKC, conventional protein kinase C; ECL, enhanced chemiluminescence; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRPO, horseradish peroxidase; nPKC, novel protein kinase C; PKC, protein kinase; 4β-PMA, 4β phorbol myristate acetate; PS, phosphatidylserine; RT, reverse transcriptase; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS, sodium dodecyl sulphate; SSC, standard saline citrate; and TSM, tracheal smooth muscle.

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and molecular biology, relatively little is known of the expression and functional properties of these enzymes in intact cells and tissues. In airways smooth muscle, PKC has been implicated in a number of functional responses including the regulation of contractility [6, 9–16], and in cell growth and division [17]. However, these indications are based largely on indirect pharmacological evidence obtained with phorbol diesters and purportedly selective PKC inhibitors. The finding that most cells and tissues express multiple forms of PKC which have a discrete subcellular distribution strongly suggests that they subserve specific functional roles [18]. This necessitates, in the first instance, studies to identify the complement of enzymes expressed in the cell or tissue of interest before the specific function(s) of the individual enzymes can be studied. Recently, Donnelly and colleagues [19] described the PKC isoforms in canine ASM using Western immunoblot analysis and, in agreement with studies in vascular smooth muscle [20–22], confirmed the presence of multiple PKC isoforms. To our knowledge, however, nothing is known of the complement of PKC isoenzymes expressed in human lung parenchyma and airways smooth muscle particularly in relation to the more recent members (PKCs θ, λ, ζ and μ) of this rapidly expanding family of protein kinases. We have, therefore, determined at the protein and mRNA level using Western analysis and reverse transcription-polymerase chain reactions (RT-PCR) respectively the PKC isoforms expressed in human trachealis and lung parenchyma obtained from non-atopic, non-asthmatic adult subjects.

MATERIALS AND METHODS

Drugs and Analytical Reagents

The following drugs and analytical reagents were used: ECL Western blotting reagents, ECL Hybond paper, high molecular weight rainbow markers (14.3–200 kDa), donkey anti-rabbit HRPO-linked IgG, sheep anti-mouse HRPO-linked IgG and nitrocellulose paper were from Amersham International (Little Chalfont, Buckinghamshire, UK). AMV reverse transcriptase, dNTP, RNase inhibitor (RNAsin), oligo dT and T4 polynucleotide kinase were obtained from Promega (Southampton, Hampshire, UK). *Taq* polymerase was from Bioline (Finchley, UK) and rabbit polyclonal antibodies to PKCα, β_I, β_{II}, γ, δ, ε, ζ, η, θ, ζ and μ and corresponding competing peptides were purchased from Santa Cruz Biotechnology Inc/Autogen Bioclear Ltd. (Devizes, Wiltshire, UK). Tris-HCl SDS (10%) mini-gels and X-Omat, and PKCη standard were obtained from Bio-Rad, Kodak (Hemel Hempstead, Hertfordshire, UK) and Calbiochem (Nottingham, Nottinghamshire, UK) respectively. Oligonucleotide primers were kindly custom synthesised by Glaxo-Wellcome (Stevenage, Hertfordshire, UK). Organic solvents and all other reagents (AnalaR grade) were obtained from BDH or the Sigma Chemical Company (Poole, Dorset, UK).

Western Immunoblot Analysis

Lung and tracheal smooth muscle were obtained from four individuals undergoing heart-lung transplantation; there was no evidence of long term lung disease or airway inflammation in these subjects. Tissue was placed immediately into ice-cold oxygenated physiological salt solution and transported to the laboratory. Approximately 2 g of lung, 50 mg epithelium-denuded tracheal smooth muscle, 2 g rat quadriceps femoris (control for PKCθ) and 500 mg rat brain (control for all isoenzymes except PKCη) was pulverised under liquid nitrogen, suspended in 2 ml buffer A (10 mM MOPS - pH 7.2, 2 mM EDTA, 5 mM EGTA, 10 mM DTT) supplemented with the proteinase inhibitors benzamidine (2 mM), soybean trypsin inhibitor (10 μg/ml), bacitracin (100 μg/ml) and leupeptin (100 μM) and homogenised at full power for 10 s with a Polytron. The resulting homogenate was centrifuged at 100,000 g for 60 min and the supernatant filtered through glass wool. The remaining particulate material was sonicated (3 × 5 min) in buffer A in the presence of proteinase inhibitors, frozen in liquid nitrogen, pulverised in a mortar and pestle and re-centrifuged as described above.

Aliquots of the supernatant and solubilised particulate material were diluted (50:50) in buffer B (62.5 mM Tris-HCl - pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.5% w/v bromophenol blue) and denatured by boiling for 5 minutes. For the immunodetection of PKC isoenzymes, denatured protein was loaded onto 10% SDS ready gels and run at 200 mA for 40 minutes at 25°C. Proteins were transferred onto Hybond nitrocellulose paper in buffer C (193 mM glycine, 50 mM Tris base, 0.03% SDS, 20% v/v methanol) at 200 mA for 3 hours at 25°C and the nitrocellulose membranes were subsequently blocked overnight at 4°C in buffer D (10 mM Tris-base, 150 mM NaCl, 5% w/v non-fat milk) and then incubated for 1 hour at 25°C with the appropriate rabbit polyclonal anti-PKC isoform-specific IgG (diluted 1:500 in buffer D). Membranes were washed (5 × 5 minutes) at 25°C in buffer D (excluding non-fat milk) and incubated for a further 60 min with a donkey anti-rabbit HRP-linked IgG (diluted 1:6000 in buffer D supplemented with 0.05% v/v Tween 20). Filters were washed thoroughly (5 × 5 minute washes in buffer D excluding non-fat milk) and antibody-labelled proteins were detected by ECL, photographed and developed.

Detection of PKC isoform mRNA by RT-PCR

Total RNA was extracted from approximately 250 mg frozen human lung and epithelium-denuded tracheal smooth muscle according to the single step extraction procedure of Chomczynski and Sacchi [23] and purity assessed by A_{260}/A_{280} spectrophotometry. One microgram of RNA was reversed transcribed using AMV Reverse Transcriptase according to the manufacturer's instructions and RT-generated cDNAs encoding the α, β_I, β_{II}, δ, ε, η,

TABLE 1. Primers and conditions used for RT-PCR

Gene	GenBank accession number(s)	Deoxyoligonucleotide sequences	Coordinates for PCR product in human cDNA sequence	Product size (base pairs)	Annealing temperature
PKCα	X52479	forward: 5′-CGA CTG TCT GTA GAA ATC TGG-3′	742 to 762	443	58° for 30 s
		reverse: 5′-CAC CAT GGT GCA CTC CAC GTC-3′	1185 to 1165		
$PKC\beta_I$	X06318	forward: 5′-CTG TGG AAC TGA CTC CCA CTG-3′	2134 to 2154	404	60°C for 30 s
	M27545	reverse: 5′-ATA CTG AAG CAT TTT GGT ATC-3′	2538 to 2518		
$PKC\beta_{II}$	X07109	forward: 5′-GAC CGG TTT TTC ACC CGC CA-3′	2108 to 2123	309	60°C for 30 s
	M27546	reverse: 5′-CCA TCT CAT AGA GAT GCT CC-3′	2418 to 2393		
PKCδ	X72972	forward: 5′-AAA GGC AGC TTC GGG AAG GT-3′	1127 to 1146	260	58° for 30 s
	L07860/L07861	reverse: 5′-TGG ATG TGG TAC ATC AGG TC-3′	1376 to 1358		
PKCε	X72974	forward: 5′-AGC TTG AAG CCC ACA GCC TG-3′	59 to 78	464	58° for 30 s
		reverse: 5′-CTT GTG GCC GTT GAC CTG ATG-3′	523 to 503		
PKCζ	X72973	forward: 5′-GTC CTC CCA GAT GGA GCT GGA AG-3′	187 to 209	359	54° for 30 s
		reverse: 5′-GAA GGC ATG ACA GAA TCC AT-3′	546 to 527		
PKCλ	L18964	forward: 5′-TAT AAT CCT TCA AGT CAT G-3′	663 to 679	547	56° for 30 s
	D28579	reverse: 5′-TTA CAC ATG CCG TAG TCA GT-3′	1208 to 1189		
PKCθ	L07032	forward: 5′-CTA TCA ATA GCC GAG AAA CCA TG-3′	659 to 681	418	54° for 30 s
		reverse: 5′-CTC ATC CAA CGG AGA CTC CC-3′	1074 to 1058		
PKCη	M55284	forward: 5′-ACG GTG AGC GTG GAC CAG GT-3′	302 to 321	642	58° for 30 s
		reverse: 5′-GAT CGC AGA ATG TTG GCA C-3′	944 to 926		
GAPDH	J04038	forward: 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′		598	58° for 30 s
		reverse: 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′			

θ, ζ and λ PKC gene sequences were amplified by PCR using specific primers (Table 1) designed from the reported human sequences deposited with the GenBank data base (see [24] and references therein). To confirm the integrity of each RNA sample, PCR analysis of the GAPDH gene was routinely performed using primers synthesised from sequences described in Maier and colleagues [25]. PCR amplification was conducted in a reaction volume of 25 μl using a Hybaid OmniGene thermal cycler (Hybaid, Teddington, Middlesex) and 0.5 U *Taq* polymerase set for 35 cycles at a denaturing temperature of 94°C for 30 seconds, specific annealing temperature (Table 1) and an extension temperature of 72°C for 1 min. PCR products were subsequently size fractionated on 1.5% agarose gels, stained with ethidium bromide and visualised under UV light.

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

RESULTS AND DISCUSSION

Western Immunoblot Analysis of PKC Isoforms

Representative immunoblots of the PKC isoforms present in human lung and tracheal smooth muscle are shown in Figure 1. Using isotype-selective polyclonal antibodies, immunoreactive bands corresponding to conventional (PKCα, $PKC\beta_I$, $PKC\beta_{II}$), novel (PKCε) and atypical (PKCζ) PKC isoforms were detected in the soluble fraction

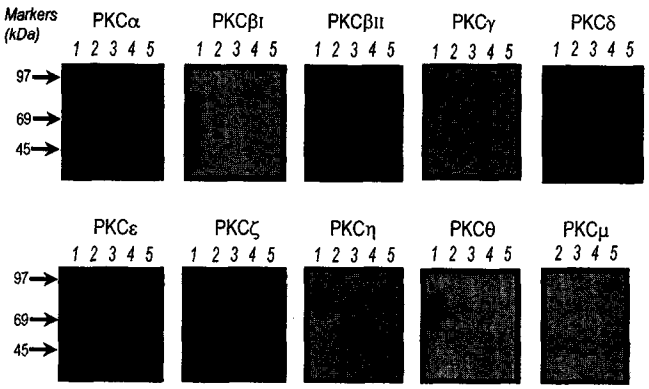

FIG. 1. Western immunoblot analysis of PKC isoenzymes in human trachealis and lung. Twenty to 100 μg of the 100,000 × g supernatant or solubilised particulate material from human lung and tracheal smooth muscle were denatured and subjected to electrophoresis on 10% SDS polyacrylamide gels. Rat brain was loaded as a positive control for all isoforms with the exception of PKCη, PKCθ, and PKCμ, for which recombinant PKCη, rat skeletal muscle, and human lung were used, respectively. Proteins were transferred to nitrocellulose and probed with the appropriate rabbit polyclonal anti-PKC isoform-specific IgG and extensively washed. Antibody-labelled proteins were finally detected by ECL, photographed, and developed. The position of 97, 69, and 45 kDa markers is indicated in each blot. See Materials and Methods for further details. Data are representative of four experiments using tissue from three different donors. Key: *1*, Positive control; *2*, Soluble fraction—trachealis; *3*, Particulate fraction—trachealis; *4*, Soluble fraction—lung; *5*, Particulate fraction—lung.

TABLE 2. Protein and mRNA for PKC isoforms in lung and airways smooth muscle

PKC isoenzyme	Human trachealis		Canine trachealis*	Bovine trachealis*		Human lung		Rat lung*
	Protein	mRNA	Protein	Protein	mRNA	Protein	mRNA	Protein
PKCα	+	+	−	+	ND	+	+	+
PKCβ_I	±	+	+	+	ND	+	+	−
PKCβ_{II}	+	+	+	+	ND	+	+	−
PKCγ	−	ND	−	−	ND	−	ND	−
PKCδ	−	+	+	+	+	−	+	+
PKCε	+	+	+	+	+	+	+	+
PKCζ	+	−	+	−	+	+	+	+
PKCη	±	+	−	−	ND	+	+	ND
PKCθ	−	−	+	+	−	−	−	ND
PKCλ	ND	−	ND	ND	ND	ND	−	ND
PKCμ	−	ND	ND	ND	ND	+	ND	ND

* = Data taken from [19, 24].
ND = Not determined.
− = Isoenzyme not detected.
± = borderline expression.

of both tissues. PKCα and PKCε were also found in the particulate fraction of human lung and tracheal smooth muscle together with the novel isoform, PKCη. Human lung, unlike tracheal smooth muscle, also expressed PKCs ζ and μ (an established source of this isoenzyme [8]) in the particulate fraction. No evidence was obtained for PKCγ, δ and θ in any experiment (Fig. 1; Table 2). The unequivocal identification of immunoreactive bands as PKCs was suggested from experiments using rat brain (PKCs α, β_I, β_{II}, γ, δ, ε), skeletal muscle (PKCθ) and recombinant PKCη as positive controls (Fig. 1), and by employing antibodies pre-adsorbed with the peptides used as immunogens. In these latter experiments immunoreactive bands were not detected with blocked antibodies for any PKC isoenzyme in trachealis, lung or the positive controls (data not shown).

Intriguingly, the complement of PKC isoforms is not invariant between species (Table 2). Thus although PKCs β_I, β_{II} and ε have been detected immunologically in bovine, canine and human trachealis [19, 24, this study] marked differences are apparent. For example, whereas PKCα is abundantly expressed in human (this study) and bovine [24] airways smooth muscle, it is not found in canine trachealis [19]. Conversely, we were unable to detect PKCδ in human trachealis at the protein level despite unequivocal identification of this isoform in bovine [24] and canine [19] airways. Similarly, the purportedly muscle-specific isoform, PKCθ [26], is present in canine [19] and bovine trachealis [24] but not in the equivalent human tissue (this study) while the contrary pattern is seen for PKCη (Table 2), a novel member of the PKC superfamily that, in laboratory animals, is abundantly expressed in lung, skin and in epithelial cells lining the oesophagus, stomach, large and small intestines, trachea and bronchi [27].

The profile of PKCs in human lung was also at variance with the isoforms expressed in other species (Table 2). In adult rat lung for example, PKCs α, δ and ε are present (albeit at low levels) and PKCβ is apparently absent or below the level of detection [28]. These findings contrast with human lung where PKCβ_{II} is abundantly expressed and PKCδ is absent. The reason(s) for these discrepancies is not immediately clear but a number of possibilities are worthy of consideration that are not mutually exclusive. One obvious explanation is a *bona fide* difference in PKC isoenzyme expression between species which would question the validity of using laboratory animals as models of human airways (at least when PKC is the subject of interest). Alternatively, the disparity could relate to differences in the "age" of the tissue since PKC isoform expression differs markedly with ontogeny [18, 28–31]. Finally, it is conceivable that the complement of PKCs is, indeed, invariant across human, canine, bovine and rat respiratory tissues but the absolute amount of mRNA translated between species differs such that certain isoforms may be present at a level too low to be detected immunologically. Indeed, some evidence to support this contention was the unequivocal identification of mRNA transcripts for PKC isoenzymes that were not detected at the protein level (see below).

Detection of PKC Isoforms by RT-PCR

The presence of mRNA for the PKC isoforms expressed by human lung and tracheal smooth muscle was determined by RT-PCR using primer pairs (Table 1) designed to recognise unique sequences in the human PKC genes (Fig. 2). Using tissue from three different subjects, staining of gels for PCR products from human tracheal smooth muscle total RNA revealed amplified cDNA fragments that corresponded to the predicted sizes of PKCα (443 bp), PKCβ_I (404 bp), PKCβ_{II} (309 bp), PKCδ (260 bp), PKCε (464 bp) and PKCη (642 bp). Messenger RNA for PKCθ and PKCλ was not detected in any sample after 35 cycles of amplification (Table 2). Identical results were obtained with human lung

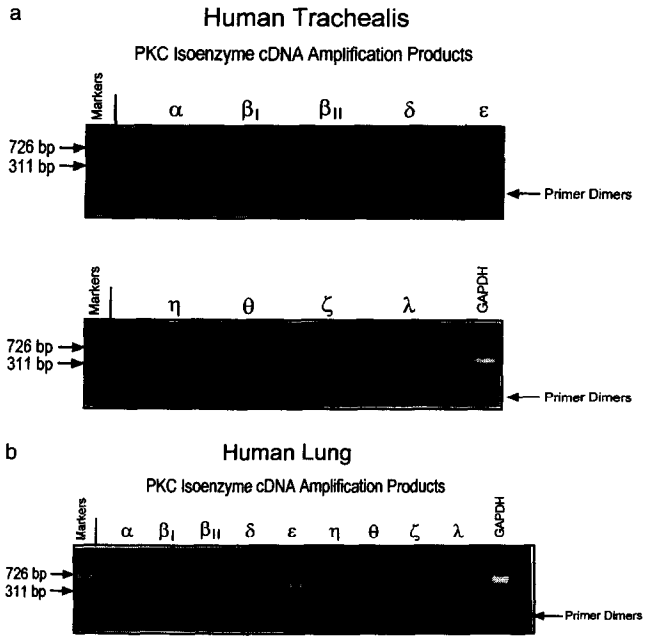

FIG. 2. Qualitative RT-PCR analysis of PKC mRNAs in human trachealis and lung. RT-PCR was performed using RNA from human lung and tracheal smooth muscle, the products subjected to electrophoresis on 1.5% agarose gels and visualised with ethidium bromide. RT-PCR product sizes for PKCα, PKCβ$_I$, PKCβ$_{II}$, PKCδ, PKCε, PKCζ, PKCη, PKCθ, and PKCλ were 443 bp, 404 bp, 309 bp, 260 bp, 464 bp, 359 bp, 642 bp, 418 bp, and 547 bp, respectively. The left hand lane shows molecular weight markers (0.5 μg 1 kb ladder, Gibco). Data are representative of three experiments using total RNA extracted from tissue from three different donors. See Materials and Methods for further details.

total RNA together with a 359 bp PCR product corresponding to PKCζ mRNA (Fig. 3).

Unexpectedly, comparison of the RT-PCR and western analyses results identified a number of inconsistencies. For example, mRNA for PKCζ was never detected in any specimen of human tracheal smooth muscle after 35 cycles of amplification despite unequivocal identification of this isoenzyme at the protein level. This finding is consistent with an observation made by Assender and colleagues [18] who failed to detect PKCε protein in vascular smooth muscle despite unambiguously identifying PKCε mRNA transcripts. The reason for these discrepancies is not clear but might reflect stable expression and low turnover of product associated with a limited translation rate of PKC message. Another anomaly was the finding that PKCδ was not detected at the protein level in either lung or tracheal smooth muscle whereas PCR products of the correct size were reproducibly seen. Again, the reason for this inconsistency is unclear but can be explained by a low or nominal translation rate of PKCδ mRNA, the generation of unstable protein, or the restricted expression of PKCδ in a minor population of cells.

Collectively, these data suggest that human lung and airways smooth muscle have the potential to express multiple PKC isoenzymes which, given their differences in sub-cellular location and mode of regulation, implies that they subserve distinct and divergent functional roles. In particular, evidence for the participation of Ca^{2+}-dependent and/or independent isoforms in the regulation of airways smooth muscle tone is provided by a number of studies which demonstrate that phorbol diesters are invariably spasmogenic [6–14] while inhibitors of PKC suppress agonist-induced tension generation and maintenance [8, 14]. There is increasing evidence that PKCs may modulate smooth muscle contractility through their ability to phosphorylate a number of contractile and cytoskeletal proteins including myosin light chain kinase, caldesmon, calponin, desmin, synemin and filamin [32–40].

The potential for airways smooth muscle cells to express PKCζ and PKCδ suggests that these isoforms may play a role in the regulation of mitogenesis. Thus, activation of PKCδ by phorbol diesters in Chinese hamster ovary cells markedly suppresses cell growth [41]. In contrast, the activation of *ras*-mediated maturation of *Xenopus* oocytes [42] and DNA synthesis in mouse fibroblasts [43] have a requirement for activated PKCζ, and Diaz-Meco and colleagues [44] have provided evidence that this isoform participates in the regulation of NFκB-dependent gene transcription through its ability to phosphorylate and, thereby, inactivate IκBα. If PKCζ or other isoforms are ubiquitous regulators of cell growth and division then these findings may be of particular relevance to respiratory diseases such as chronic severe asthma where airway hypertrophy and hyperplasia are characteristic features. Indeed, we have previously demonstrated that selective PKC inhibitors (Ro 31-8220, Ro 31-7549) and down-regulation of PKC with 4β-PMA effectively suppress fetal calf serum-induced proliferation of rabbit cultured tracheal myocytes indicating that PKC positively regulates mitogenesis [17]. Furthermore, Adamo and colleagues [45] reported that PKC activity and the proportion of enzyme present in the particulate fraction of cultured cells (an index of activation) is significantly increased during proliferation when compared to cells that are mitotically quiescent.

In conclusion, the experiments described herein are the first to identify the PKC isoforms in human trachealis and lung tissue at the protein and mRNA level and clearly demonstrate the expression of multiple (and presumably multi-functional) isoenzymes the pattern of which differs, apparently, between species.

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