Protein kinase C isoforms in human airway smooth muscle cells: activation of PKC-ζ during proliferation

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Carlin, Steve, Kenny X. F. Yang, Richard Donnelly and Judith L. Black. Protein kinase C isoforms in human airway smooth muscle cells: activation of PKC-ζ during proliferation. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L506–L512, 1999.—Protein kinase C (PKC) is implicated in the regulation of smooth muscle contractility and growth. We have previously described the pattern of isoform expression of PKC in canine airway smooth muscle. This study identified the isoforms present in human cultured airway smooth muscle cells and also addressed the question of whether mitogenesis in these cells is associated with changes in a specific isoform, PKC-ζ. Western blot analysis revealed the presence of PKC-α, -β1, -βII, -δ, -η, and -ζ of the novel group; and PKC-ɛ, -µ, and -γ of the atypical group. There was a significant increase in density of the Western blot for PKC-ζ in cells proliferating in response to 10% fetal bovine serum (FBS) to 372 ± 115% of control values (P < 0.05; n = 3 patients in the cytosolic fraction. Platelet-derived growth factor (PDGF) produced increases in PKC-ζ in both the cytosolic and membrane fractions to 210 ± 49 and 443 ± 227%, respectively, of control values (P < 0.05; n = 4 patients). There was no change in expression of PKC-α, -βI, -βII, -δ, -ε, -η, -ζ, or -γ in response to the same stimuli. PGE2 (1 µM) added to the cells 30 min before PDGF reduced incorporation of [3H]thymidine from 5,580 ± 633 (SE) to 3,980 ± 126 dpm (P < 0.05; n = 3 patients) and, in addition, reduced expression of PKC-ζ in the membrane fraction as determined by Western blotting from 266 ± 66 to 110 ± 4% of control values (P < 0.05; n = 3 patients). PKC-ζ activity in stimulated cells (10% FBS), as assessed by immunoprecipitation and phosphorylation of glycogen synthase peptide, was ~3-fold greater than that in unstimulated cells, and the amount of PKC-ζ protein correlated with isoenzyme activity (r² = 0.91; P < 0.02; n = 4 patients). In conclusion, this study 1) provides the first description of which isoforms of PKC are present in human cultured airway smooth muscle cells and 2) shows that proliferation of these cells is associated with upregulation of PKC-ζ. Whether activation of PKC-ζ is a primary or secondary event in airway smooth muscle cell proliferation remains to be determined.

Protein kinase C isoforms; atypical protein kinase C; bronchial muscle cells; hyperplasia

PROTEIN KINASE C (PKC) is a key regulatory enzyme involved in the regulation and cross talk between signal transduction pathways associated with various cellular functions. Specifically, in the airways, PKC is important in regulating smooth muscle contractility and growth. It has been shown that PKC phosphorylates a number of key contractile proteins in airway smooth muscle (ASM) (23), and PKC activation appears to be more involved in the sustained rather than in the initial phase of ASM contraction. In human isolated airways, stimulation of PKC produces both contraction (25, 29) and relaxation, and Yang and Black (30) previously reported that contractile responses are dependent on extracellular Ca²⁺ influx, whereas the relaxation phase involves stimulation of Na⁺-K⁺-ATPase.

PKC has also been implicated in the mitogenic response of a number of different cell types including vascular smooth muscle cells (14), fibroblasts (11), and, more recently, ASM cells (21). For example, activation of PKC by phorbol esters stimulates proliferation of porcine and human ASM (20, 22). Moreover, inhibitors of PKC such as Ro-31–8220 and Ro-31–7549 reduce the proliferative response of rabbit ASM to fetal calf serum (9) and the proliferative response of bovine pulmonary arterial adventitial fibroblasts (5).

PKC is not a single protein kinase but rather a family of multiple isoenzymes with different biochemical characteristics, substrate specificities, and cofactor requirements (10). The various isoforms of PKC, which vary in their tissue distribution and function, have been classified into three main groups (19): group A, the conventional calcium-dependent isoenzymes (PKC-α, -βI, -βII, and -γ); group B, the calcium-independent (novel) isoenzymes (PKC-δ, -ε, -η, and -ι); and group C, the atypical isoenzymes (PKC-ζ, -µ, -ι, and -λ).

Donnelly et al. (5) first described the PKC isoforms present in ASM using canine tissue. They found that PKC-βI and -βII from the conventional group, PKC-δ, -ε, and -ι from the novel group, and PKC-ζ from the atypical group were all present, whereas PKC-α, -γ, and -η were absent.

Togashi et al. (27) followed this with experiments performed in porcine ASM and reported some differences and some similarities in isoform expression in this species. Webb et al. (28) examined homogenates of human trachealis and peripheral lung and found some differences in the expression of isoforms both between these regions of the airways and in the results obtained by Northern and Western blotting. Thus it has become apparent that significant species and tissue differences exist in the expression of PKC isoforms in ASM, and so one of the aims of the present study was to identify the isoforms present in human cultured ASM. In addition, because evidence is emerging that different isoforms of PKC have specific functions and, in particular, that PKC-ζ might be important in mitogenic signal transduction in a number of different cell types (2), a second aim of this study was to address the question of whether mitogenesis in human cultured ASM cells is associated with changes in PKC-ζ.

METHODS

Human ASM culture. Primary cultures of human ASM were established as reported previously by our group (13).
The protocol was approved by the Human Ethical Review Committee of The University of Sydney (Australia). Briefly, macroscopically normal human lung was obtained from patients undergoing lung transplantation or partial resection (n = 4 with emphysema, n = 1 with bronchiectasis, n = 1 with cystic fibrosis, n = 1 with bronchopulmonary dysplasia, n = 1 with pulmonary hypertension, and n = 5 with primary carcinoma). Large bronchi (5- to 15-mm internal diameter) were dissected from the surrounding parenchyma and dipped in 70% (vol/vol) ethanol in water to kill any surface organisms. ASM bundles, viewed with a dissecting microscope, were dissected from the bronchi and placed in tissue flasks containing 1% Fungizone, 1% penicillin-streptomycin, and 10% fetal bovine serum (FBS). The smooth muscle cells grew to confluence in a humidified CO2 incubator in 16–24 days and were passed in 175-cm² flasks at 7- to 10-day intervals. Pure populations of smooth muscle cells were confirmed by the presence of positive staining for α-smooth muscle actin.

Cell preparation. Cells from passages 4–7 were seeded at a density of 10⁴ cells/cm², and the medium was changed from DMEM with 10% FBS to DMEM with 1% FBS for 24 h. It has previously been shown with flow cytometry that >80% of cells are in the G0/G1 phase of the cell cycle (K. Hawker, unpublished observations) after this treatment. Cells were then incubated in DMEM with 10% FBS or DMEM with 1% FBS plus platelet-derived growth factor (PDGF) at 40 ng/ml (13) for 24 h. The cells were then washed in PBS and homogenization buffer containing 30 mM Tris·HCl, 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine, 5 mg/ml of leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, and 0.0003% bromphenol blue, heated at 100°C for 2 min, and cooled to room temperature. The samples were subjected to SDS-PAGE (10% wt/vol), as described by Laemmli (16), with a Mini-PROTEAN II electrophoresis system (Bio-Rad, Sydney, Australia). Crude extracts of rat brain were loaded onto adjacent lanes as positive controls. The following high-molecular-mass protein markers were also loaded onto each gel: 20-kDa myosin, 116-kDa β-galactosidase, 97-kDa phosphorylase b, 66-kDa albumin, and 45-kDa ovalbumin. Electrophoretic separation was carried out in 1% SDS, 25 mM Tris, and 200 mM glycine (pH 8.4) at 200 V for 45–60 min at room temperature with a Bio-Rad model 1000/500 power supply. A polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA) was activated in 20% methanol for 1 min. The proteins were then transferred to the membrane in 25 mM Tris, 192 mM glycine, and 20% methanol (vol/vol; pH 8.3) at 100 V and 250 mA for 1 h at 4°C. At the conclusion of the transfer process, the molecular-mass standards were cut off from the membrane, stained with 0.1% amino black in 2% acetic acid for 5 min, and then destained in 40% methanol and 10% acetic acid for 10 min. The membrane was incubated for 12 h at 4°C in a Tris-buffered saline-Tween 20 solution (TBS-T) containing 10 mM Tris, 0.5 M NaCl, and 0.5% Tween 20, pH 7.4, with 5% (wt/vol) nonfat dried milk. After the blocking step, the membrane was rinsed in TBS-T with 1% nonfat dried milk, pH 7.4, and divided into strips. Isoform-specific primary antibodies for all PKC isoforms were diluted 1:2,000 in rinsing solution. Each strip was incubated with one primary antibody for 2 h on a rocking platform and washed four times in rinsing solution. All strips were then incubated with secondary antibody (horseradish peroxidase-conjugated IgG fraction of goat anti-rabbit IgG) diluted 1:20,000 in TBS-T. The membrane strips were then washed in TBS-T and developed with an enhanced chemiluminescence detection kit before exposure onto Kodak-X-Omat film. The fluorescence ratio values were calculated for the molecular-mass standards and the bands of interest on each gel, and the apparent molecular masses were obtained from a linear plot of the fluorescence ratio versus molecular mass for the standard markers. Blots were quantified by computerized densitometry (Molecular Dynamics). The identity of each band detected on the gel was confirmed by experiments in which the primary antibody was blocked with the appropriate peptide.

Effect of PGE₂ on PKC-ζ expression: [3H]thymidine incorporation. The effect of PGE₂ on cell proliferation was estimated by measuring the incorporation of [methyl-3H]thymidine ([3H]TdR) as previously described (13). Briefly, cells were grown in 96-well plates, and 5 h before the end of a growth period, 1 µCi of [3H]TdR (specific activity 20 Ci/mmol) was added to each well containing 100 µl of the test solution before the plates were returned to the incubator. Stock solutions of [3H]TdR were diluted in Hanks’ balanced salt solution to obtain 1 µCi/10 µl. Ten microliters of this solution were added to each well containing 100 µl of the test solution. Thus the final concentration of [3H]TdR was 1 µCi/110 µl. After the 5-h incubation period, the cells were harvested with distilled water onto Whatman GF-C glass fiber filters with a Harvard cell harvester. The filters were washed five times with distilled water to remove unbound [3H]TdR. The glass fibers were allowed to dry and were then transferred to 5-ml plastic scintillation vials that contained 4 ml of scintillant. The vials were mixed on a vortex and, after a 24-h equilibration period, were counted on a beta emission counter for 5 min/sample. PKC-ζ enzyme activity. PKC-ζ was isolated by immunoprecipitation (26) from cells (from 2 separate patients) exposed to 100 µM PGE₂ (288 µM stimulated and 39 µM control) FBS. Cytosolic and membrane fractions (500 µg of total protein) were added to 100 µl of PBS (Ca²⁺ free) and 2.5 µl of anti-PKC-ζ antibody (Santa Cruz Biotechnology). After overnight incubation at 4°C, 40 µl of protein A-Sepharose 4B were added, and the samples were mixed at 4°C for 2 h. The Sepharose beads were then washed twice with 1 ml of PBS by pelleting at low speed and removing the supernatant, resuspended in 150 µl of PBS, and centrifuged, and the supernatant was removed. For the kinase assay (24), samples were mixed in 25 mM HEPES (pH 7.4), 10 mM magnesium acetate, 20 mM glycogen synthase (GS) peptide PKC substrate (15), 250 µM EGTA, and 2 g/l of a phosphatidylyserine (PS) suspension, then warmed to 37°C. Control samples with no substrate peptide were run as well as PK controls with substrate but no PS. The assay was started by the addition of [γ-32P]ATP (1 µCi/tube) in 100 µM ATP, and the samples were incubated at 37°C for 30 min. The assay was stopped by the addition of 75 mM phosphoric acid, and the samples were spotted onto Whatman P81 cation-exchange chromatography paper that was then washed three times in 75 mM phosphoric acid and dried. Radioactivity was measured by scintillation counting.

Materials. The following were purchased from Sigma (St. Louis, MO): ATP, PS, PGE₂ benzadine, leupeptin, soybean trypsin inhibitor, phenylmethylsulfonyl fluoride, and Triton X-100. Molecular-mass markers, horseradish peroxidase-
Western blot analysis of cells from three patients revealed the consistent presence of PKC-α, βI, βII, γ, δ, ε, η, ζ, θ, μ, and i. These isoforms were compared in stimulated and unstimulated cells by subtracting the counts per minute observed in the absence of the GS peptide substrate from those observed in its presence. Increases in the counts per minute in the membrane and cytosolic fractions are expressed as multiples of the difference and were correlated with the amount of PKC-ζ protein obtained from Western blotting in corresponding samples with linear regression analysis.

RESULTS

Expression of PKC isoforms in human ASM cells. Western blot analysis of cells from three patients revealed the consistent presence of PKC-α (81 kDa), -βI (79 kDa), and -βII (80 kDa) of the conventional isoforms; PKC-δ (76 kDa), -ε (79 kDa), and -η (80 kDa) of the novel group; and PKC-ζ (74 kDa), -μ (100 kDa), and -i (75 kDa) of the atypical group. Detection of the isoforms was not influenced by patient diagnosis because their presence was consistent across all patient groups. The results of the immunoblots are shown in Fig. 1.

Changes in PKC isoforms in proliferating cells. In the presence of 10% FBS, which has previously been shown to cause a 17-fold increase in cell number (13), there was an approximately threefold increase (372 ± 115%) in the expression of PKC-ζ as observed by the density compared with that in control cells (100%) incubated in 1% FBS (P < 0.05; n = 3; Fig. 2). PDGF (40 ng/ml) also produced significant increases in PKC-ζ expression in both the cytosolic and membrane fractions (210 ± 49 and 443 ± 227%, respectively; P < 0.05, n = 4; Fig. 2).

In contrast, there was no significant change in PKC-α expression in either the membrane or cytosolic fraction in the presence of 10% FBS or PDGF (Fig. 3) or in the isoforms βI, βII, ε, δ, θ, μ, or i. The signal for PKC-δ was very weak in the cytosol despite a strong signal in the rat brain standard and in the membrane was 66 ± 8% of the control value in the presence of PDGF (P > 0.05; n = 3).

PKC-ζ enzyme activity. PKC-ζ activity in the cytosolic and membrane fractions of cells stimulated with 10% FBS was 2.6- and 3.8-fold (patient 1) and 3.4- and 2.3-fold (patient 2), respectively, greater than that in unstimulated control cells (1% FBS). Corresponding values for the amount of PKC-ζ protein as measured by Western blotting were 4.8- and 5.6-fold (patient 1) and 7.5- and 3.9-fold (patient 2) greater. There was a significant correlation for the relationship between the
increase in kinase activity and the increase in expression of PKC-ζ protein for the two membrane and two cytosolic fractions \((r^2 = 0.903; P < 0.01; n = 4)\). The values obtained in the two sets of assay controls, i.e., those that omitted GS substrate and those that omitted PS, did not differ significantly from each other.

**Effect of PGE \(_2\) on cell proliferation and PKC-ζ.** PGE \(_2\) decreased both the proliferative response of the cells to PDGF and the amount of PKC-ζ as measured in the membrane fraction by immunoblotting (Fig. 4, left and right, respectively). \([3H]TdR\) incorporation in response to PDGF was 5,580 ± 633 (SE) dpm compared with that in the presence of 1% FBS (186 ± 5 dpm; \(P < 0.05; n = 3\)). PGE \(_2\) significantly reduced the count to 3,980 ± 126 dpm \((P < 0.05; n = 3)\).

PDGF again increased the amount of PKC-ζ in the membrane fraction to 266 ± 66% of the control value \((P < 0.05; n = 3)\), and PGE \(_2\) almost completely abolished this increase \((110 ± 4% of the control value; P < 0.05; n = 3)\). PGE \(_2\) did not decrease the amount of PKC-ζ in the cytosol \((171 ± 11% of the control value vs. 157 ± 16% for PDGF alone; P > 0.05; n = 3)\).

**DISCUSSION**

This study represents, to the best of our knowledge, the first description of the pattern of PKC isoforms expressed in human cultured ASM. Isoenzymes from all three groups (conventional, novel, and atypical) were present, and the pattern of expression was similar but not identical to that which Donnelly et al. (5) first described in homogenates of canine ASM. The main difference between the two species was the presence of PKC-α in human but not in canine tissue. In other respects, the isoforms detected in the two species were qualitatively identical, but this study did not permit...
quantitative comparisons of the relative amounts of each isoform expressed. PKC-ζ and PKC-δ, ε, η, and θ of the novel isoforms; and PKC-ζ, -µ, and -ε from the atypical group were present. There was no evidence of the neuronal isoform PKC-γ. Our results show some similarities to those of Webb et al. (28), who studied homogenates of both human trachealis and peripheral lung. These investigators used Western and Northern blotting to detect PKC isoforms, and the major difference between their findings and those of the present study lies in the detection of the muscle-specific isoform PKC-θ. The reason that we detected this isoform and Webb et al. did not may reflect the fact that we studied cells in culture as opposed to those in tissue homogenates. This is unlikely, however, because Donnelly et al. (5) also found the PKC-θ isoform in homogenates of canine trachealis. Togashi et al. (27), using porcine trachealis, also found expression of PKC-α and, in addition to those reported in canine tissue, two other members of the atypical group, τ/λ and µ. They did not report the presence of PKC-θ.

The presence of different isoforms of PKC strongly suggests a complex multifunctional role for PKC in ASM, and it seems possible that different isoforms may be involved in the pathways regulating ASM contraction and proliferation. Indeed, previous work by Rossetti et al. (25) and Standaert et al. (26) has demonstrated a role for PKC in the contraction of human isolated airways, and it is likely that the contractile responses are associated with the calcium-dependent group of PKC isozymes. It was of interest, therefore, to find that the expression and isoenzyme activity of a calcium-independent isoform, PKC-ζ, were upregulated in cells that were stimulated to proliferate with either FBS or PDGF. Evidence that this effect was specific for PKC-ζ was provided by the fact that, under similar conditions, no other PKC isoforms were upregulated during mitogenic stimulation.

An association between PKC activation and ASM cell proliferation has been established in previous experiments in which PKC inhibitors have been shown to reduce the proliferative response (5, 9). The present data extend the observations obtained with nonselective PKC inhibitors by associating the proliferative response in ASM with a selective increase in the expression and isoenzyme activity of an individual PKC isoform, PKC-ζ, but these data do not provide direct information on whether PKC-ζ activation is a primary initiating event or a secondary consequence of ASM growth. It would clearly be desirable to evaluate the effects of a selective inhibitor of PKC-ζ on cell proliferation, but although specific antagonists for other PKC isoforms are becoming available (12), a selective pharmacological blocker of PKC-ζ has not been developed, and there are still concerns about the specificity of alternative techniques, such as the use of antisense oligonucleotides, for blocking PKC signaling. Nevertheless, the possibility that PKC-ζ may be involved in the initiation of cell division is supported by previous work (2).

The results obtained in the kinase assay after immunoprecipitation with anti-PKC-ζ antibody demonstrated that mitogenic stimulation of the ASM cells is associated with not only an increase in the amount of PKC-ζ protein but also an increase in the kinase activity of the isoform. Moreover, there was a positive correlation between the increase in enzyme activity and the increased amount of protein expressed in the cytosolic and membrane fractions. We chose GS peptide as our substrate in the kinase activity assay because, although alternative substrates are sometimes preferred (15), PKC-ζ has a good affinity for GS peptide (15), and this was certainly confirmed by our findings in the present experiments. It is unlikely that the activity of any other PKC isoform confounded our immunoprecipitation results because the addition of EGTA would have excluded activity of any calcium-dependent isoforms, and in the absence of any PKC activators such as phorbol esters, there would be minimal activation of the novel group of isoforms. Moreover, in the kinase assay, we used the same concentration of PKC-ζ antibody as that shown to be extremely specific for PKC-ζ in the Western blot experiments.

Others have attributed specific cellular functions to specific PKC isoforms (4, 18), especially PKC-ζ. For example, activation of PKC-ζ is not only necessary but is sufficient by itself to activate maturation in oocytes and stimulate growth in fibroblasts (2). In addition, stimulation of vascular smooth muscle by angiotensin results in activation of PKC-ζ (17), whereas in 3T3/L1 cells, insulin activates PKC-ζ, -α, and -β, but only PKC-ζ is required for glucose transport (1). Furthermore, PKC-ζ is particularly abundant in fetal tissues and adult rat liver, an organ that retains regenerative capacity, and this would be consistent with the notion that PKC-ζ plays a contributory role in cell proliferation (7). Conversely, PKC-δ inhibits proliferation in both vascular smooth muscle cells (6) and fibroblasts (8, 17).
The association between increased activity of PKC-ζ and cell proliferation in human ASM is consistent with other evidence suggesting that an increase in intracellular calcium is not an essential requirement for mitogenesis (19). The fact that PKC-ζ is an isoform that is activated independently of calcium and that conventional calcium-dependent PKC isoforms were not increased in this system would support the hypothesis that there can be some dissociation between increased calcium levels and proliferation as previously reported in other experiments (20).

The association of individual PKC isoforms with specific biological functions raises the intriguing possibility of the use of selective inhibition of these isoforms to achieve targeted abolition of unwanted pathological effects. Indeed, selective inhibition of PKC-β with a novel, orally active inhibitor ameliorates vascular dysregulation in a rat model of diabetes (12). It is therefore conceivable that, if further evidence were available linking human ASM proliferation with specific PKC isoforms, new pharmacological or antisense approaches could be used to inhibit the ASM hyperplasia that is an unwanted feature of asthmatic airways.

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