Identification of PKC isoforms expressed in human bronchial smooth muscle cell

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Abstract

The PKC family has been implicated in a wide range of biological response in a number of different cellular systems. Although the functions of PKC have been vigorously studied using pharmacological tools, there is little information about the expression pattern of its isoforms in human bronchial smooth muscle cells (hBSMCs). In the present study, RT-PCR and immunoblot analyses were carried out to detect all the PKC isoforms expressed in cultured normal hBSMCs. The RT-PCR analyses revealed that mRNAs of the known PKC isoforms except for PKCγ and θ were detected in the hBSMCs. Among them, protein expressions of the isoforms except for PKCη and µ were also detected by immunoblottings. Thus, the present study clearly demonstrated the expression of multiple PKC isoforms, i.e., PKCα, β, δ, ε, ζ and ι in hBSMCs.

Key words: PKC isoform, bronchial smooth muscle cell

Introduction

Protein kinase C (PKC) is a generic term used to describe the largest serine/threonine-directed kinase subfamily currently known. Since the discovery of PKC in 1977 (Takai et al., 1977; Inoue et al., 1977), multiple isoenzymes have been identified unequivocally that differ in primary structure, cellular distribution, substrate specificity, inhibitor sensitivity and mechanism of activation (Stabel and Parder, 1991; Hug and Sarre, 1992). The PKC isoenzymes can be broadly divided into three main groups. The conventional PKCs (cPKC) α, βI, βII, and γ have four conserved regions (C1–C4) and five variable regions. The C1 region contains binding site for diacylglycerol (DAG) or phorbol esters. The C2 region contains the binding site for Ca²⁺. The C3 and C4 regions contain binding site for ATP, PKC substrates and some PKC antagonists. The PKC molecule folds to bring the ATP binding site into proximity with the substrate-binding site. The novel PKCs (nPKC) δ, ε, η and θ lack the C2 region and therefore do not require Ca²⁺ for its activation. The atypical PKCs (aPKC) ζ and λ (human equivalent of PKCι) have only one cysteine-rich zinc finger-like motif and are dependent on phosphatidylserine, but are not affected.
by diacylglycerol (DAG), phorbol esters or Ca\textsuperscript{2+}. In addition, previous evidence warrants the designation of the forth group of PKCs which is based upon the finding that the catalytic domain of PKC\textsubscript{\(\mu\)} is more closely related to Ca\textsuperscript{2+}/calmodulin-dependent protein kinases such myosin light chain kinase (MLCK) and contains signal and transmembrane moieties at its amino terminus that are absent in other PKC family members (see review; Salamanca and Khalil, 2005; Johannes et al., 1994).

Despite the continuing discovery of new PKC members and our increasing knowledge of their biochemistry and molecular biology, relatively little is known of the expression and functional properties of these enzymes in intact cells. In airway smooth muscle, PKC has been implicated in a number of functional responses including the regulation of contractility (Rossetti et al., 1995; Hakonarson and Grunstein, 1998; Sakai et al., 2005; 2007), and in cell growth and division (Hirst et al., 1995). However, these indications are based largely on indirect pharmacological evidence obtained with phorhol esters and purportedly selective PKC inhibitors. The finding that most cells and tissues express multiple isoforms of PKC which have a discrete subcellular distribution strongly suggests that they subserves specific functional role (Kiley and Parker, 1995). This necessitates, in the first instance, studies to identify the complement of enzymes expressed in the cell of interest before the specific functions of the individual enzymes can be studied. Previously, Webb et al. (1997a) described the PKC isoforms in human lung and tracheal smooth muscle using RT-PCR and Western blot analysis. To our knowledge, however, nothing is known of the complement of PKC isoforms expressed in human bronchial smooth muscle. Therefore we have determined, at the protein and mRNA level using Western blot and reverse transcription-polymerase chain reaction (RT-PCR) respectively, the PKC isoforms expressed in cultured human bronchial smooth muscle.

**Materials and methods**

**Human bronchial smooth muscle cells**

Primary cultured human bronchial smooth muscle cells (hBSMC) were purchased from Cambrex Bio Science Walkersville, Inc. and maintained in smooth muscle cell growth media (Cambrex Bio Science Walkersville, Inc.) at 37°C in humidified air containing 5% CO\textsubscript{2}. The cells were carefully characterized by the manufacturer with specific markers to confirm their selective smooth muscle phenotype and to exclude any contamination with other cell types. The cells were grown in smooth muscle basal medium (SmBM) supplemented with 5% FBS, insulin (5 ng/ml), EGF (10 ng/ml; human recombinant), FGF (2 ng/ml; human recombinant), gentamicin (50 ng/ml), and amphotericin-B (50 ng/ml). HBSMC were subcultured into clear-based 6-well plates. After the cells from the 6th to 8th passages became confluent, the cells were cultured in FBS free SmBM for 24 hours, and used for Western blotting and RT-PCR.

**RT-PCR analyses**

Total RNA was isolated from hBSMC by using TRI REAGENT (Sigma-Aldrich) and stored at −85°C until use. Rat brain samples were used as positive control. cDNAs were prepared from the total RNA (1 \(\mu\)g) by reverse transcriptase reaction in a total volume of 50 \(\mu\)l reaction buffer
containing 10 mM Tris-HCl, pH 8.3, 50 mM MgCl₂, 1 mM dNTP mixture, 1 U/ml RNase
inhibitor, 2.5 μM random hexamers, and 0.25 U/ml avian myeloblastosis virus reverse
transcriptase. The reaction mixture was incubated for 5 min at 25°C followed by 60 min at 42°C
to initiate the synthesis of the cDNAs. Reverse transcriptase was inactivated at 70°C for 5 min.
Then the RT reaction mixture (1 μl) was added by 1 μl of 1 μM forward primer, 1 μl of 1 μM
reverse primer, 3 μl of 2X PCR Master Mix (0.05 U/μl Taq DNA polymerase, 4 mM MgCl₂ and
0.4 mM of each dNTP; Fermentas Life Science) in a total volume of 20 μl. RT-generated cDNAs
encoding the gene sequences of PKCs isoforms were amplified by PCR using specific primers
(Table 1), which were designed from published sequences. The thermal cycle profile used in
the present study was 1) denaturing for 3 min at 95°C, 2) annealing primers for 15 sec at 55°C,
and 3) extending the primers for 1 min at 72°C. The PCR amplifications were performed for 35
cycles. The PCR mixtures were subjected to electrophoresis on 2% agarose gel with DNA
molecular weight standard marker (100 bp DNA ladder, Takara Bio Inc.) and visualized by
ethidium bromide staining.

**Immunoblot analysis**

To confirm the protein expression of PKC isoforms, Western blot was performed with the
lysate of hBSMC. Briefly, the samples (10 μg of total protein per lane) were subjected to 7.5%
sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were
then electrophoretically transferred to a PVDF (polyvinylidene difluoride) membrane. After
blocking with 5% skim milk, the PVDF membrane was incubated with primary antibody (mouse
anti-PKCα, β, γ, δ, ε, η, θ, ζ, ι antibody, BD Biosciences; 1:2000 dilution and rabbit anti PKD/
PKCμ antibody, Cell Signaling; 1:1000 dilution). Then the membrane was incubated with
horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (1:2500 dilution;
Sigma-Aldrich) or donkey anti-rabbit IgG (1:2500) and detected by an enhanced
chemiluminescence system (ECL system; Amersham).

**Immunocytochemistry**

The hBSMCs were fixed in 10% formaldehyde. For the immunohistochemical examination,
the hBSMCs were incubated with each antibody as the primary antibody by using a streptavidin-
biotin immunoperoxidase method as described previously (Chiba et al., 2006; 2008) with a minor
modification. In brief, before immunostaining, rehydrated sections were pretreated by incubation
in 0.5% Triton-X 100 in PBS for 10 min for permeabilization and were immersed in 0.3% hydrogen
peroxide in 100% methanol for 30 min to remove endogenous peroxidase activity. The pretreated
hBSMCs were washed with PBS and incubated sequentially at room temperature in the following
solutions: (1) primary antibody (diluted 1:100 in PBS containing 1% skim milk) overnight, (2)
biotinylated goat anti-mouse IgG (diluted 1:200 in PBS containing skim milk) for 30 min, and (3)
avidin-biotinylated peroxidase complexes (Vector Laboratory, Inc.) in PBS for 30 min. The
bound peroxidase activity was visualized by incubation with 0.7 mg/ml 3,3-diaminobenzidine-
0.02% H₂O₂ in 60 mM Tris buffer (pH 7.5). The hBSMCs were rinsed in PBS after each step of
immunostaining procedure. Finally, the hBSMCs were counterstained with Hematoxylin QS
(Vector Laboratories, Inc.), dehydrated, and mounted in permanent mounting medium.
Results and discussion

Figure 1 shows that the mRNAs for the PKC isoforms were expressed in cultured human bronchial smooth muscle cells (hBSMCs) determined by RT-PCR analysis. The PCR amplifications were performed for 35 cycles. The predicted size (see Table 1) of the bands for respective PKC isoform were detected. Photographs are the representatives of three independent experiments, respectively. Lane M: molecular weight marker (100 bp DNA ladder).

Table 1. PCR primers for PKC in human

| Subfamily | Subtype | Accession number | Primer Deoxyribonucleotide sequences | Predict size (base pairs) |
|-----------|---------|-----------------|-------------------------------------|--------------------------|
| cPKC      | PKCα    | NM_002737       | forward 5’CGACTGTCTGTAGAATCTGG3’    | 444                      |
|           |         |                 | reverse 5’CACCATGTCAGCCACTCCAGCTG3’ |                          |
| PKCβI     |         | NM_002737       | forward 5’TTGTGATGGAGTATGTAAGGGG3’  | 490                      |
|           |         |                 | reverse 5’CCTGGGTTTGTGTTAGGCTG3’    |                          |
| PKCβII    | X07109  |                 | forward 5’GACCGGTTCACCCGCACTAG3’    | 433                      |
|           |         |                 | reverse 5’GCCATTTTCCTCCCACTTGG3’    |                          |
| PKCγ      | NM_002739|                | forward 5’TGATGGGAGATGAGGGG3’       | 347                      |
|           |         |                 | reverse 5’GAAATCACTTTGTCAGGCTG3’    |                          |
| nPKC      | PKCδ    | L07860          | forward 5’CACCACTTTCCAGAAGAAGG3’    | 358                      |
|           | PKCe    | NM_005400       | forward 5’AGCTTGAGCTCCCTTGGG3’      | 465                      |
|           |         |                 | reverse 5’CTTGTGACCCCTTCAGCGG3’     |                          |
| PKCζ      | NM_006255|                | forward 5’CTCATCAAGGAGCCGCGG3’      | 480                      |
|           |         |                 | reverse 5’CTCATCCACAGGACGCTGCC3’    |                          |
| aPKC      | PKCζ    | NM_002744       | forward 5’CGATGGGAGATGAGGGG3’       | 532                      |
|           | PKCι    | NM_002740       | forward 5’GCAGAAGATCGTGGTGGATG3’    | 548                      |
|           |         |                 | reverse 5’ATCACTTCCAGTTGAGCCG3’     |                          |
| PKD       | PKCζ    | NM_002742       | forward 5’CATTGGCCAGAGTCTTCCCG3’    | 134                      |
|           | PKCι    |                 | reverse 5’TCAAGGTCATAGATGAGACC3’    |                          |
PKC isoforms in human bronchial muscle

(433 bp), PKCδ (358 bp), PKCε (465 bp), PKCη (480 bp), PKCζ (532 bp), PKCι (548 bp) and PKCµ (134 bp), although PKCγ (347 bp) and PKCθ (419 bp) were not detected.

Representative immunoblots of the PKC isoforms expressed in hBSMCs are shown in Fig. 2. The rat brain sample was used as positive control (lane: PC) for all PKC isoforms. Photographs are the representatives of three experiments, respectively. hB; human bronchial smooth muscle cells.

Fig. 2. Protein expression of PKC isoforms in human bronchial smooth muscle cells determined by immunoblot analysis. Rat whole brain was used as positive control (lane: PC) for all PKC isoforms. Photographs are the representatives of three experiments, respectively. hB; human bronchial smooth muscle cells.

(433 bp), PKCδ (358 bp), PKCε (465 bp), PKCη (480 bp), PKCζ (532 bp), PKCι (548 bp) and PKCµ (134 bp), although PKCγ (347 bp) and PKCθ (419 bp) were not detected.

Representative immunoblots of the PKC isoforms expressed in hBSMCs are shown in Fig. 2. The rat brain sample was used as positive control. Using isotype-selective antibodies, immunoreactive bands corresponding to PKC isoforms of α (82 kDa), β (80 kDa), δ (79 kDa), ε (90 kDa), ζ (76 kDa) and ι (74 kDa) were detected in samples of hBSMCs. The bands of PKC γ (80 kDa), η (80 kDa), θ (82 kDa) and µ (115 kDa) were not detected in hBSMCs samples. To visually confirm the expression of PKC isoform in hBSMC, immunocytochemistry of PKC α, β, ε, ζ and ι isoforms was performed (Fig. 3). PKCε and PKCζ isoforms and smooth muscle α-actin were strongly stained by DAB. However, PKC α, β, δ and ι isoforms were weakly stained (data not shown).
Interestingly, although PKC β and ε isoforms have been detected immunologically in bovine (Webb et al., 1997b), canine (Donnelly et al., 1995), human tracheal smooth muscles (Webb et al., 1997a) and human bronchial smooth muscle (current study), the complement of PKC isoforms differs among airway smooth muscles. For example, whereas PKCα is abundantly expressed in human bronchial (current study), tracheal (Webb et al., 1997a), bovine tracheal (Webb et al., 1997b) smooth muscles, it is not found in canine trachealis (Donnelly et al., 1995). Conversely, PKCθ is present in canine (Donnelly et al., 1995) and bovine tracheal smooth muscles (Webb et al., 1997b) but not in human tracheal (Webb et al., 1997a) and bronchial smooth muscles (current study).

Unexpectedly, the comparison of the results on RT-PCR and immunoblot analyses revealed the inconsistency. mRNAs for PKCη and PKCμ were not reflected at the protein level in hBSMCs, whereas PCR products of the correct size were reproducibly observed. The reason for the inconsistency is unclear, but might be explained by a low or nominal translation rate of PKCη and PKCμ mRNAs, the generation of unstable protein, or the restricted expression of PKCη and PKCμ in a minor population of hBSMCs.

Collectively, these data suggest that human bronchial smooth muscle has the potential to express multiple PKC isoforms. PKC is a key regulatory enzyme involved in the regulation and cross-talk between signal transduction pathways associated with various cellular functions. In particular, evidence for the participation of PKC in the regulation of airway smooth muscle contraction is provided by a number of studies (Rossetti et al., 1995; Hakonarson and Grunstein, 1998; Sakai et al., 2005; 2007). Although the role of each PKC isoform in agonist-induced

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**Fig. 3.** Immunocytochemistry of PKC isoforms in human bronchial smooth muscle cells. A represents control (no 1st antibody treatment). B, C and D represent PKCε, ζ and smooth muscle α-actin in human bronchial smooth muscle cells. Original magnification: ×80. Bar=50 μm.
bronchial smooth muscle contraction is unclear, PKC can phosphorylate a number of key contractile proteins in airway smooth muscle. PKC activation appears to be more involved in the sustained rather than the initial phases of airway smooth muscle contraction (Park and Rasmussen, 1985). In vascular smooth muscle, Shirasawa et al. (2003) suggested that PKCε could modulate phenylephrine-induced contraction via calcium-independent pathways. Therefore, PKCε might be also involved in agonist-induced contraction of bronchial smooth muscle, as well as that of vascular smooth muscle. PKCε lacks the C2 region that contains the binding site for Ca\textsuperscript{2+} and therefore do not require Ca\textsuperscript{2+} for its activation. Thus, PKCε may play an important role in the Ca\textsuperscript{2+}-independent contraction of bronchial smooth muscle, i.e., Ca\textsuperscript{2+} sensitization.

The activation of ras-mediated maturation of Xenopus oocytes (Dominguez et al., 1992) and DNA synthesis in mouse fibroblasts (Berra et al., 1993) have a requirement for activation of PKCζ, and Diaz-Meco et al., (1994) have provided evidence that this isoform participates in the regulation of NFκB-dependent gene transcription through its ability to phosphorylate and, thereby, inactivate IκBα. The potential for airway smooth muscle cells to express PKCζ thus suggests that this isoform might play a role in the regulation of mitogenesis. If PKC is 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.

In conclusion, the present study described herein is the first to identify the PKC isoforms in human bronchial smooth muscle at the protein and mRNA levels and to clearly demonstrate the expression of multiple isoforms.

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References

Berra, E., Diaz-Meco, M.T., Dominguez, I., Munioio, M.M., Sanz, L., Lozano, J., Chapkin, R.S. and Moscat, J. (1993). Protein kinase C zeta isoform is critical for mitogenic signal transduction. Cell 74: 555–563.

Chiba, Y., Goto, K., Hirahara, M., Sakai, H. and Misawa, M. (2008). Glucocorticoids ameliorate antigen-induced bronchial smooth muscle hyperresponsiveness by inhibiting upregulation of RhoA in rats. J. Pharmacol. Sci. 106: 615–625.

Chiba, Y., Kurotani, R., Kusakabe, T., Miura, T., Link, B.W., Misawa, M. and Kimura, S. (2006). Am. J. Respir. Crit. Care Med. 173: 958–964.

Diaz-Meco, M.T., Dominguez, I., Sanz, L., Dent, P., Lozano, J., Munioio, M.M., Berra, E., Hay, R.T., Sturgill, T.W. and Moscat, J. (1994). zetaPKC induces phosphorylation and inactivation of I kappa B-alpha in vitro. EMBO J. 13: 2842–2848.

Dominguez, I., Diaz-Meco, M.T., Munioio, M.M., Berra, E., Garcia de Herreros, A., Cornet, M.E., Sanz, L. and Moscat, J. (1992). Evidence for a role of protein kinase C zeta subspecies in maturation of Xenopus laevis oocytes. Mol. Cell Biol. 12: 3776–3783.
Donnelly, R., Yang, K., Omary, M.B., Azhar, S. and Black, J.L. (1995). Expression of multiple isoenzymes of protein kinase C in airway smooth muscle. *Am. J. Respir. Cell Mol. Biol.* **13**: 253–256.

Hakonarson, H. and Grunstein, M.M. (1998). Regulation of second messengers associated with airway smooth muscle contraction and relaxation. *Am. J. Respir. Crit. Care Med.* **158**: S115–S122.

Hirst, S.J., Webb, B.L., Giembycz, M.A., Barnes, P.J. and Twort, C.H. (1995). Inhibition of fetal calf serum-stimulated proliferation of rabbit cultured tracheal smooth muscle cells by selective inhibitors of protein kinase C and protein tyrosine kinase. *Am. J. Respir. Cell Mol. Biol.* **12**: 149–161.

Hug, H. and Sarre, T.F. (1993). Protein kinase C isoenzymes: divergence in signal transduction? *Biochem. J.* **291**: 329–343.

Inoue, M., Kishimoto, A., Takai, Y. and Nishizuka, Y. (1977). Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent protease from rat brain. *J. Biol. Chem.* **252**: 7610–7616.

Johannes, F.J., Prestle, J., Eis, S., Oberhagemann, P. and Pfizenmaier, K. (1994). PKC_{\mu} is a novel, atypical member of the protein kinase C family. *J. Biol. Chem.* **269**: 6140–6148.

Kiley, S.C. and Parker, P.J. (1995). Differential localization of protein kinase C isozymes in U937 cells: evidence for distinct isozyme functions during monocyte differentiation. *J. Cell Sci.* **108**: 1003–1016.

Park, S. and Rasmussen, H. (1985). Activation of tracheal smooth muscle contraction: synergism between Ca^{2+} and activators of protein kinase C. *Proc. Natl. Acad. Sci. USA.* **82**: 8835–8839.

Rossetti, M., Savineau, J.P., Crevel, H. and Marthan, R. (1995). Role of protein kinase C in nonsensitized and passively sensitized human isolated bronchial smooth muscle. *Am. J. Physiol.* **268**: L966–L971.

Sakai, H., Hirano, T., Takeyama, H., Chiba, Y. and Misawa, M. (2005). Acetylcholine-induced phosphorylation of CPI-17 in rat bronchial smooth muscle: the roles of Rho-kinase and protein kinase C. *Can. J. Physiol. Pharmacol.* **83**: 375–381.

Sakai, H., Chiba, Y. and Misawa, M. (2007). Role of Rho kinase in endothelin-1-induced phosphorylation of CPI-17 in rat bronchial smooth muscle. *Pulm. Pharmacol. Ther.* **20**: 734–739.

Salamanca, D.A. and Khalil, R.A. (2005). Protein kinase C isoforms as specific targets for modulation of vascular smooth muscle function in hypertension. *Biochem. Pharmacol.* **25**: 1537–1547.

Shirasawa, Y., Rutland, T.J., Young, J.L., Dean, D.A. and Joseph, B.N. (2003). Modulation of protein kinase C (PKC)-mediated contraction and the possible role of PKC epsilon in rat mesenteric arteries. *Front. Biosci.* **8**: 133–138.

Stabel, S. and Parker, P.J. (1991). Protein kinase C. *Pharmacol. Ther.* **51**: 71–95.

Takai, Y., Kishimoto, A., Inoue, M. and Nishizuka, Y. (1977). Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. I. Purification and characterization of an active enzyme from bovine cerebellum. *J. Biol. Chem.* **252**: 7603–7609.

Webb, B.L., Lindsay, M.A., Seybold, J., Brand, N.J., Yacoub, M.H., Haddad, E.B., Barnes, P.J., Adcock, I.M. and Giembycz, M.A. (1997a). Identification of the protein kinase C isoenzymes in human lung and airways smooth muscle at the protein and mRNA level. *Biochem. Pharmacol.* **54**: 199–205.

Webb, B.L., Lindsay, M.A., Barnes, P.J. and Giembycz, M.A. (1997b). Protein kinase C isoenzymes in airway smooth muscle. *Biochem. J.* **324**: 167–175.