Involvement of multiple PKC isoforms in phorbol 12,13-dibutyrate-induced contraction during high K+ depolarization in bronchial smooth muscle of mice

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Abstract

In airway smooth muscle, protein kinase C (PKC) has been implicated in a number of functional responses including the regulation of contractility. However, the exact role of PKC on bronchial smooth muscle (BSM) contraction is still unclear. In the present study, to determine the role of PKC activation in the BSM contraction, the effects of phorbol 12,13-dibutyrate (PDBu, a direct PKC activator) on BSM tone were examined in the absence and presence of K+-induced depolarization stimulation. The force development was not evoked by treatment with 1 μM PDBu alone. However, a strong contraction was induced by PDBu during high K+ stimulation. The contraction induced by PDBu during high K+ stimulation was significantly abolished by pretreatment with nicardipine, an L-type voltage dependent Ca2+ channel blocker. In RT-PCR analysis, mRNAs of PKCα, β, γ, δ, ε, η and θ isoforms were detected in mouse BSM. Gö6976 (an inhibitor of PKCs α and β) and rottlerin (an inhibitor of PKCδ) significantly but partially inhibited the PDBu-induced BSM contraction during K+ stimulation. GF109203X (an inhibitor of PKCs α, β, γ, and ε) completely inhibited the PDBu-induced contraction during K+ stimulation. In conclusion, it is suggested that the PDBu-induced BSM contraction is dependent on an increase in cytosolic Ca2+. Furthermore, it is possible that both cPKC and nPKC(s) participate in the PDBu-induced contraction of mouse BSM during K+ stimulation.

Key words: protein kinase C, bronchial smooth muscle, contraction, phorbol 12,13-dibutyrate

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 unequivocally identified, with differences in their primary structure, cellular distribution, substrate specificity, inhibitor sensitivity and
mechanism of activation (Stabel and Parder, 1991; Hug and Sarre, 1993). The PKC isoenzymes can be broadly divided into three main groups. The conventional PKCs (cPKC) α, β and γ have four conserved regions (C1–C4) and five variable regions. The C1 region contains the binding site for diacylglycerol (DAG) or phorbol esters (such as phorbol 12,13-dibutyrate: PDBu). The C2 region contains the binding site for Ca\(^{2+}\). The C3 and C4 regions contain the 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 Ca\(^{2+}\) for its activation. The atypical PKCs (aPKC) ξ and λ (human equivalent of PKC\(^{\lambda}\)) have only one cysteine-rich zinc finger-like motif and are dependent on phosphatidylserine, but are not affected by DAG, phorbol esters or Ca\(^{2+}\) (see review; Salamanca and Khalil, 2005).

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; 2008; 2009). However, the exact role of PKC on BSM contraction is still unclear. In the present study, to determine the role of PKC activation in BSM contraction, the effects of PDBu, a direct PKC activator, on BSM tone were examined in the absence and presence of K\(^{+}\)-induced depolarization stimulation.

### Materials and Methods

#### Animals

Male BALB/c mice (10 weeks of age, specific pathogen-free) were used. All experiments were done according to the Guiding Principles for the Care and Use of Laboratory Animals approved by the Animal Care Committee of Hoshi University (Tokyo, Japan).

#### Functional studies

Mice were killed by exsanguination from the abdominal aorta under urethane anesthesia (1.6 g/kg, i.p.), and the lungs and airways from just below the larynx removed immediately. A 3-mm length (approx.) of the left main bronchus (0.5 mm in diameter) was isolated, and the epithelium removed by gently rubbing with sharp tweezers (Chiba et al., 2008; 2009). The resultant tissue ring preparation was then suspended in a 5-ml organ bath by two stainless steel wires (0.2 mm in diameter) that were passed through the lumen. For all preparations, one end was fixed to the bottom of the organ bath while the other was connected to a force-displacement transducer (TB-612T; Nihon Kohden, JAPAN) for the measurement of isometric force. A resting tension of 0.5 g was applied. The buffer solution contained modified Krebs-Henseleit solution with the following composition (in mM): 118.0 NaCl, 4.7 KCl, 2.5 CaCl\(_2\), 1.2 MgCl\(_2\), 25.0 NaHCO\(_3\), 1.2 KH\(_2\)PO\(_4\), and 10.0 glucose. The buffer solution was maintained at 37°C and oxygenated with 95% O\(_2\)-5% CO\(_2\). After the equilibration period, the contraction induced by 60 mM K\(^{+}\) was observed. After this contraction had reached a plateau, 1 \(\mu\)M phorbol 12,13-dibutyrate (PDBu) was applied. The preparations were pretreated with nicardipine (an L-type voltage-dependent Ca\(^{2+}\) channel blocker)
and one of the PKC inhibitors (either Gö6976, GF109203X or rottlerin) 30 min before 60 mM K⁺ application. In other experiments, after the preparation had reached a plateau following PDBu contraction during high K⁺ stimulation, nicardipine (0.01, 0.1 and 1 μM) was cumulatively applied. All the experiments were performed in the presence of atropine and indomethacin (both 1 μM).

**RT-PCR analyses**

Total RNA was isolated from mouse bronchial tissue by using TRI Reagent (Sigma-Aldrich) and stored at –85 °C until use. Mouse brain samples were used as a positive control. cDNAs were prepared from the total RNA (1 μg) using the reverse transcriptase reaction in a total volume of 50 μL 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, and then for 60 min at 42°C to initiate the synthesis of the cDNAs. The reverse transcriptase was inactivated at 70°C for 5 min. To a 1 μL aliquot of the RT reaction mixture was added 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 1.2% agarose gel with DNA molecular weight standard marker (100 bp DNA ladder, Takara Bio Inc., JAPAN) and visualized by ethidium bromide staining.

**Table 1.** PCR primers for cPKCs and nPKCs

| Isoform | Assession number | Primer Deoxyribonucleotide sequences | Product size (base pairs) |
|---------|------------------|--------------------------------------|--------------------------|
| PKCα    | NM_011101        | forward: 5’-TGAATCCTCACGGAGAATGCT-3’<br>reverse: 5’-GGTTGCTTTCCTGTGCTGAA-3’ | 325                      |
| PKCβ    | NM_00855         | forward: 5’-AAGGAAGCAGGGGCAATGGA-3’<br>reverse: 5’-AGTTACATTGCCTCTGCCCCCT-3’ | 223                      |
| PKCγ    | NM_011102        | forward: 5’-GCTGTTAGAGATGGTGCCAG-3’<br>reverse: 5’-GAGATTACATGACAGGGCA-3’ | 467                      |
| PKCδ    | NM_01103         | forward: 5’-GCTGGAAGAGGAGGAGGCAC-3’<br>reverse: 5’-CGGAGGGGGGTGGGCTG-3’ | 415                      |
| PKCε    | NM_01104         | forward: 5’-ATCAAAATCTGCGAGGCGG-3’<br>reverse: 5’-CGATCGGAGGCGGTGAAAGAC-3’ | 241                      |
| PKCη    | NM_00856         | forward: 5’-CGGCCTCCTCTTAAAAGAG-3’<br>reverse: 5’-GATTGACTGCCTTCGCAATGC-3’ | 415                      |
| PKCθ    | NM_00859         | forward: 5’-TGTTGAAGACAGAAAATGGCAG-3’<br>reverse: 5’-TGTTGAGGGAAGGAGGTC-3’ | 404                      |
| GAPDH   | NM_008084        | forward: 5’-CCATCAGCTCCACTGAAAC-3’<br>reverse: 5’-TACCCCTGGAGGAGCATGGAG-3’ | 469                      |
Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of differences were determined using the unpaired Student’s t-test or a one-way ANOVA with Bonferroni/Dunn’s test.

Results

Force development of BSM preparations did not occur following treatment with 1 μM PDBu alone. However, in the presence of high K⁺ the contraction reached 0.20 ± 0.03 g. The force was markedly enhanced by the further application of 1 μM PDBu (380.20 ± 21.72% of 60 mM high K⁺ alone, \( P<0.001 \)) (Fig. 1A). The contraction induced by high K⁺, which changes the K⁺ equilibrium potential to activate L-type Ca²⁺ channels, is regulated solely by augmentation of the cytosolic Ca²⁺ concentration (Karaki et al., 1991). Taken together, this finding suggests that an augmented cytosolic Ca²⁺ is necessary to develop the contraction produced by PKC. To ascertain the effect of augmented cytosolic Ca²⁺ on the PDBu-induced bronchial contraction, we investigated the effect of nicardipine, an L-type Ca²⁺ channel inhibitor, on the PDBu-induced contraction during high K⁺ stimulation. We found that the contraction induced by PDBu during high K⁺ stimulation was abolished by pretreatment with 1 nM nicardipine (\( P<0.001 \), Fig. 1A). This finding is consistent with our hypothesis. To further test the necessity for an augmented cytosolic Ca²⁺ to produce the PDBu-induced bronchial contraction, the effect of post-treatment with nicardipine on the PDBu-induced augmentation of high K⁺-induced contraction was examined. The PDBu-induced contraction during high K⁺ stimulation was concentration-dependently inhibited by application of nicardipine (Fig. 1, B and C).

Previously, Webb et al. (1997) described the existence of 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 species of PKC isoforms expressed in mouse bronchial smooth muscle. Therefore, we have determined PKC isoforms expressed in mouse bronchial smooth muscle at the mRNA level using the reverse transcription-polymerase chain reaction (RT-PCR). Figure 2 shows that the mRNAs for the PKC isoforms were expressed in mouse bronchial smooth muscles as determined by RT-PCR using the primer pairs (Table 1) which were designed to recognize unique sequences in the mouse PKC genes. The mRNAs for all the PKC isoforms were detected in the samples after 35 cycles of amplification. The staining of gels for PCR products from the total RNA of mouse bronchial tissues revealed amplified cDNA fragments that corresponded to the predicted sizes of PKCα (325 bp), PKCβ (223 bp), PKCγ (467 bp), PKCδ (415 bp), PKCe (241 bp), PKCη (415 bp) and PKCθ (404 bp).

Figure 3 shows the effects of treatment with the three PKC inhibitors, viz. Gö6976 (an inhibitor of PKCs δ and β), GF109203X (an inhibitor of PKCs α, β, γ, δ and ε) and rottlerin (an inhibitor of PKC δ) on the PDBu-induced contraction during high K⁺ stimulation. These three PKC inhibitors significantly inhibited the PDBu-induced augmentation of force during K⁺ stimulation in the mouse BSM. The PDBu-induced contraction during K⁺ stimulation was abolished by 3 μM GF109203X, although the contraction partially remained at the higher concentrations of Gö6976 (3 μM) or rottlerin (30 μM).
In the present study, PDBu (a direct PKC activator) alone did not cause contraction. However, PDBu induced a strong bronchial smooth contraction when the muscle was exposed to high K⁺ stimulation. The PDBu-induced contraction during K⁺ stimulation was completely inhibited by the L-type voltage-dependent Ca²⁺ channel blocker, nicardipine.

PKC is a key regulatory enzyme involved in the regulation and cross-talk between signal transduction pathways associated with various cellular functions. PKC is also important for regulating smooth muscle contractility in the airways. Indeed, agonist-induced bronchial smooth muscle contractions were inhibited by PKC inhibitors (Sakai et al., 2008; 2009).

**Discussion**

In the present study, PDBu (a direct PKC activator) alone did not cause contraction. However, PDBu induced a strong bronchial smooth contraction when the muscle was exposed to high K⁺ stimulation. The PDBu-induced contraction during K⁺ stimulation was completely inhibited by the L-type voltage-dependent Ca²⁺ channel blocker, nicardipine.

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It is generally known that the development of force is induced by PD Bu alone in vascular smooth muscle. The activation of PKC by the addition of PDBu induces a contraction and inhibits the myosin light chain phosphatase (MLCP) of vascular smooth muscle (Itoh et al., 1993; Masuo et al., 1994). However, PDBu did not induce any contraction of mouse bronchial smooth muscle. We suggest that the cytosolic Ca^{2+} concentration in bronchial smooth muscle at the basal level might
be lower than that in vascular smooth muscle. Therefore, the contraction might occur under conditions where cytosolic Ca^{2+} is augmented. In the present study, force development occurred following treatment with PDBu during high K^{+} stimulation.

An inhibitor protein specific for MLCP was isolated from pig aorta smooth muscle extracts and was named CPI-17 (Eto et al., 1995). Expression of CPI-17 is highly restricted to smooth muscle cells (Woodsome et al., 2001). Phosphorylation of the Thr38 in CPI-17 converts it to a potent MLCP inhibitor with an IC50 of \( \sim 5 \) nM (Eto et al., 1995; 1997). Phospho-CPI-17 enhances both the myosin phosphorylation and contraction of permeabilized arterial smooth muscle (Li et al., 1998). The expression pattern of CPI-17 in six different smooth muscle preparations (femoral artery, aorta, portal vein, urinary bladder, vas deferens and trachea) correlates with the extent of their PKC-induced contraction, implying that CPI-17 is a key to the PKC-mediated agonist-induced smooth muscle contraction (Woodsome et al., 2001). In addition, we previously reported that both acetylcholine and endothelin-1 induced phosphorylation of CPI-17 through activation of PKC in rat bronchial smooth muscle (Sakai et al., 2005; 2007).

In the current study, the PDBu-induced force development during K^{+} stimulation was inhibited by Gö6976. However, the contraction was not completely inhibited by using even 3 \( \mu \)M Gö6976. It has been reported that the IC50 of Gö6976 for PKC\( \alpha \) and \( \beta \) are 2 and 6 nM respectively (Martiny-Baron et al., 1993). Incomplete inhibition was also observed with 30 \( \mu \)M rottlerin (IC50 for PKC\( \delta \); 3–6 \( \mu \)M) (Gschwendt et al., 1994). But the PDBu-induced contraction during K^{+} stimulation was abolished by GF109203X (IC50 for PKC\( \alpha \), \( \beta \), \( \gamma \), \( \delta \) and \( \epsilon \); 8, 18, 21 and 132 nM, respectively) (Martiny-Baron et al., 1993). As well as these cPKCs, PKC\( \gamma \) might also be involved in the PDBu-induced contraction during K^{+} stimulation, since the response of PDBu in the presence of K^{+}-induced depolarization stimulation seems to be dependent on intracellular Ca^{2+}. Therefore, multiple PKCs (i.e., PKC\( \alpha \), \( \beta \), \( \gamma \), \( \delta \) and \( \epsilon \)) might be involved in the PDBu-induced contraction during K^{+} stimulation. Although the detailed mechanism is not clear, both PKC\( \delta \) and PKC\( \epsilon \) reportedly belong to the Ca^{2+}-independent PKC family, the novel PKC’s (nPKC). In a previous study, we showed an involvement of PKC\( \epsilon \) in the rat bronchial smooth muscle contraction induced by a contractile agonist (ref). However, further studies are needed to clarify the mechanism of PKC\( \delta \) and PKC\( \epsilon \) activation in the PDBu-induced contraction during K^{+} stimulation.

In conclusion, it is suggested that the PDBu-induced bronchial smooth muscle contraction is dependent on a cytosolic Ca^{2+} increase. Furthermore, it is possible that cPKC and nPKC(s) participate in the PDBu-induced smooth muscle contraction during K^{+} stimulation in mouse bronchial smooth muscle.

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