Cdc42 Contributes to Phorbol Ester-Induced Ca^{2+} -Independent Contraction of Pulmonary Artery Smooth Muscle

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ABSTRACT. We determined the contribution of the Rho family of low molecular GTP-binding proteins to phorbol ester-induced contraction in swine pulmonary artery smooth muscle. In Ca^{2+} -free medium containing 1 mM EGTA, 12-deoxyphorbol 13-isobutyrate (DPB, 1 μM), a protein kinase C (PKC) activator, elicited sustained contractions, which were not inhibited by treatment with verapamil, a voltage-dependent Ca^{2+} channel antagonist, and Y27632, a Rho-associated kinase inhibitor. Immunoblot analysis showed three PKC isoforms (α , ϵ , and ζ) and two Rho GTPases (RhoA and Cdc42) in both cytosolic and the membrane fractions from quiescent strips. DPB (1 μM) significantly induced PKC α and ϵ to translocate from the cytosolic to the membrane fraction in Ca^{2+} -free medium. DPB also elicited the translocation of Cdc42, but not RhoA to the membrane fraction. Similarly, in the experiment for measurement of Rho GTPase activity by pull-down assay, DPB (1 μM) significantly increased the activity of Cdc42 in Ca^{2+} -free medium. Norepinephrine (NE, 10 μM) stimulated the redistribution of RhoA from the cytosolic to the membrane fraction in swine pulmonary artery smooth muscle. In contrast, NE did not alter the subcellular distributions of Cdc42 and the PKC isoforms. These results indicate that phorbol ester evokes PKC-mediated Ca^{2+} -independent contraction via a Rho GTPase pathway, especially Cdc42, in smooth muscle from swine pulmonary arteries.

KEY WORDS: Ca^{2+} -independent contraction, Cdc42, protein kinase C, pulmonary artery smooth muscle.

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Smooth muscle contraction is primarily regulated by an increase in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) and subsequent phosphorylation of the 20-kDa myosin light chain (MLC). It has been revealed, however, that the extent of contractions and MLC phosphorylation is not always paralleled by the changes in $[\text{Ca}^{2+}]_i$ [10]. The contractions and MLC phosphorylation induced by receptor agonists are higher than those caused by membrane depolarization. Therefore, in addition to the $[\text{Ca}^{2+}]_i$ -dependent MLC pathway, other mechanisms may contribute to smooth muscle contractions [15, 17]. Recently, it has generally been agreed that both Rho GTPases and protein kinase C (PKC) play an important role in the Ca^{2+} -independent regulation of smooth muscle contractions [7, 16, 32].

The Rho GTPases are a family of low molecular GTP-binding proteins, RhoA, Rac1, and Cdc42 being the main representatives. Rho GTPases are involved in the regulation of cell motility, actin assembly, and smooth muscle contractions [22]. In experiments with permeabilized smooth muscle strips, the addition of activated RhoA elevated muscle contractions at a given Ca^{2+} concentration [23]. Moreover, RhoA and Rho-associated kinase (ROCK) can inhibit the activation of MLC phosphatase, and subsequently enhance MLC phosphorylation [18, 35].

PKC is a family of serine/threonine kinases consisting of 12 isoforms distinguishable by their cofactor dependency

during activation. PKC might act as a major mediator of smooth muscle contractions [11, 16]. PKC activates several cellular signaling pathways, including ion channels and MLC [14, 20]. It has been reported that PKC inhibits myosin phosphatase, which could be the mechanism that underlies the PKC-mediated increase in Ca^{2+} -sensitivity [19].

PKC isoforms are found in pulmonary artery smooth muscle [5], and RhoA and ROCK expression have been demonstrated in the pulmonary artery [12, 21, 29]. In addition, phorbol ester, a potent PKC activator, increases muscle tone in the absence and presence of extracellular Ca^{2+} , and inhibitors of ROCK and PKC attenuate receptor agonist-induced contractions in the pulmonary artery [7, 26]. These results imply that Rho GTPases and PKC isoforms may be involved in pulmonary arterial contractions. It has been reported that Rho GTPase contributes to PKC-mediated actin reorganization in vascular smooth muscle cells [2]. Rho GTPase isoform Cdc42, known as a regulator in the formation and organization of actin structures, such as lamellipodia and filopodia in non-muscle cells [1], can be involved in the development of contractions induced by acetylcholine in tracheal smooth muscle [33]. Therefore, it can be assumed that Rho GTPase contributes to PKC-mediated contractile response in smooth muscle. However, the mechanisms regulating the interaction between Rho GTPases, especially Cdc42, and the PKC pathways in the contractions of swine pulmonary arteries have not been entirely elucidated.

In the present study, we focused on the potential roles of Rho GTPase during phorbol ester-mediated Ca^{2+} -independent

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dent contractions in smooth muscle from swine pulmonary arteries.

MATERIALS AND METHODS

Tissue preparation: Swine lungs were obtained from a local slaughterhouse and transported to the laboratory in an ice-cold physiological salt solution [PSS (in mM): NaCl 136.9; KCl 5.4; CaCl₂ 1.5; MgCl₂ 1.0; NaHCO₃ 23.8; glucose 5.5; ethylenediaminetetraacetic acid (EDTA) 0.01]. The pulmonary arteries, 3 mm in diameter, were dissected from the lung, and carefully cleaned of all visible parenchyma and connective tissue under a stereo microscope (SZ-ST, Olympus, Japan). Three-millimeter wide strips of artery were cut, and the endothelium was removed by gently rubbing the internal surface of the vessel with a cotton thread moistened with PSS.

Measurement of contraction: Each strip was attached to a holder with a resting tension of 10 mN. After equilibration for 20 min in PSS, strips were repeatedly exposed to a 70 mM K⁺ solution until the response became stable. The high concentration of K⁺ was prepared by replacing the NaCl in the PSS with an equimolar amount of KCl. These solutions were saturated with a mixture of 95% O₂ and 5% CO₂, at 37°C and pH 7.4. Muscle contraction was recorded isometrically with a force-displacement transducer (FT03, Grass, U.S.A.) connected to a polygraph system (RPS7C8, Grass).

Immunoblot analysis: Pulmonary artery strips were isolated and snap-frozen in liquid N₂ after treatment with various stimulants. Samples were then homogenized in sample buffer containing 200 mM Tris-HCl (pH 7.4), 300 mM sucrose, 10 mM ethylene glycol-bis[β -aminoethylether]-N,N,N',N'-tetraacetic acid (EGTA), 5 mM EDTA, 0.3% 2-mercaptoethanol, 0.3 mM phenylmethylsulfonyl fluoride, and 5 mM dithiothreitol (DTT). The homogenate was centrifuged at 100,000 $\times$ g for 60 min at 4°C (TL-100, Beckman, Germany) and the supernatant was collected as the cytosolic fraction. The resulting pellet was resuspended in sample buffer containing 1.0% Triton-X 100 for 30 min and centrifuged at 100,000 $\times$ g for 60 min; the supernatant was assumed to be the membrane fraction. The protein concentrations were determined using Bio-Rad DC protein assay reagents (Bio-Rad, U.S.A.), a colorimetric assay for protein based on the Lowry assay. Samples were diluted to 1:1 (vol:vol) with SDS sample buffer containing 40 mM Tris-HCl (pH 6.8), 8 mM EGTA, 4% 2-mercaptoethanol, 40% glycerol, 0.01% bromophenol blue, and 4% SDS, and were then boiled for 5 min.

Equal amounts (30–50 μ g/lane) of protein were separated on 10% SDS-polyacrylamide gels. Electrophoretically separated proteins were transferred to a nitrocellulose membrane (Amersham, U.S.A.). These were incubated for 1 min in phosphate-buffered saline with 0.1% Tween 20 and 5% non-fat dried milk, and subsequently incubated with individual antibodies diluted 1:1,000–5,000 overnight at 4°C. Following incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1:1,000) for 60 min, blots were devel-

oped using an enhanced chemiluminescence detection system (Amersham). Antibody-specific bands were quantified using an image analyzer (BioRad, U.S.A.).

Cdc42 pull-down assay: Strips were prepared as described for the immunoblot analysis. The strips were homogenized in pull-down sample buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 20 mM β -glycerophosphate, 10 mM NaF, 2 mM Na-orthovanadate, 5 mM DTT, and 5 mM protease inhibitors), and the lysates were centrifuged at 14,000 $\times$ g for 2 min at 4°C. Cleared lysates were incubated with glutathione-S-transferase-tagged Cdc42/Rac interactive-binding domain from PAK (GST-PBD) for 120 min at 4°C with gentle agitation. The collected beads were then washed with a washing buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 10 mM MgCl₂, 1% Triton X-100, 1 mM EDTA, 20 mg/ml each of leupeptin and aprotinin, and 1 mM PMSF). Rho GTPases bound to beads and supernatant were mixed to 1:1 (vol:vol) with SDS buffer and boiled for 5 min. Proteins were separated by 14% SDS-polyacrylamide gels. Further analysis was performed as described in the above immunoblot section with anti-Cdc42 antibody. In some experiments, the whole lysate from swine pulmonary arteries with 10 μ M GTP γ S (30 min) was also run in parallel as positive controls.

Materials: 12-Deoxyphorbol 13-isobutyrate (DPB) was purchased from Funakoshi (Japan). Polyclonal anti-PKC α , anti-PKC ϵ , anti-PKC ζ , anti-RhoA, anti-Cdc42, and anti-Rac1 antibodies were purchased from Santa Cruz Biotech (U.S.A.). Norepinephrine (NE), Y27632, verapamil, phenylmethylsulfonyl fluoride, and 2-mercaptoethanol were purchased from Sigma (U.S.A.). Triton-X 100 and DTT were purchased from Amersham. GST-PBD was purchased from Upstate (U.S.A.).

Statistical analysis: The results of the experiments were expressed as means $\pm$ standard error of the mean (SEM). Unpaired Student's *t*-tests were used to compare the data with the PRISM GraphPad software (Prism, U.S.A.), and values were considered significantly different at *P* < 0.05.

RESULTS

Ca²⁺-independent vascular contraction: DPB (1 μ M) gradually induced contractions in Ca²⁺-free medium containing 1 mM EGTA in swine pulmonary arteries (Fig. 1A). The DPB-induced contractions were retained for more than 60 min, and were not attenuated by treatment with verapamil (10 μ M and 30 μ M; Fig. 1B). These results imply that phorbol ester can induce muscle contractions mediated by Ca²⁺-independent mechanisms in swine pulmonary arteries.

Translocation of PKC isoforms by phorbol ester: In order to evaluate the expression of contractile proteins in swine pulmonary arteries, Rho GTPases and PKC isoforms were determined using specific antibodies. We focused on the PKC isoforms α , ϵ , and ζ , which are representative isoforms of the conventional, novel, and atypical types of PKC, respectively [16]. In the quiescent strips, PKC α , ϵ and ζ

Fig. 1. Effects of 12-deoxyphorbol 13-isobutyrate in smooth muscle from swine pulmonary arteries. (A) After the muscle strips were treated in Ca^{2+} -free medium containing 1 mM EGTA for 20 min, the muscle strips were contracted with 12-deoxyphorbol 13-isobutyrate (DPB, 1 μM). DPB elicited a sustained contraction for more than 60 min. (B) After DPB-induced contraction in Ca^{2+} -free medium was confirmed, muscle strips were exposed to verapamil (10 μM and 30 μM), a voltage-dependent Ca^{2+} channel inhibitor. Results are typical recordings obtained from 4 experiments.

were prominently found in both the cytosolic and membrane fractions (Fig. 2).

In order to determine the roles of PKC isoforms in DPB-induced contractile response, we attempted to detect the activities of the contractile proteins during stimulations, using immunoblot analysis with polyclonal antibodies. Because PKCs undergo changes in their subcellular distribution with extracellular stimuli [15, 26], the activities of the proteins were determined from the ratio of membrane expression relative to the total, cytosolic plus membrane fractions, expression. Treatment with DPB (1 μM) for 5 or 10 min significantly induced PKC α to translocate from the cytosolic to the membrane fraction in Ca^{2+} -free medium containing 1 mM EGTA (Fig. 2A). DPB (1 μM) also increased the translocation of PKC ϵ from the cytosolic to membrane fraction (Fig. 2B). Additionally, DPB did not evoke any changes in the subcellular distribution of PKC ζ (Fig. 2C).

Translocation of Rho GTPase by phorbol ester: To determine whether Rho GTPases influence PKC-mediated contractions, Rho GTPase activities were measured during phorbol ester-stimulations, using changes of the subcellular redistribution. In Ca^{2+} -free medium, tissue strips also contained the representative Rho GTPases RhoA and Cdc42 in both fractions (Fig. 3). However, under these conditions,

Fig. 2. Translocation of PKC isoforms induced by 12-deoxyphorbol 13-isobutyrate in smooth muscle from swine pulmonary arteries. Arterial strips were treated with 12-deoxyphorbol 13-isobutyrate (DPB, 1 μM) for 5 or 10 min in Ca^{2+} -free medium containing 1 mM EGTA, and then immunoblot analysis of PKC α (A), ϵ (B), and ζ (C) was performed. The immunoblots show representative results for the cytosolic (c) and membrane (m) fractions. One hundred percent represents the density of the membrane fraction relative to the total expression in the cytosolic plus membrane fractions in the quiescent state. The results shown are the mean $\pm$ SEM of 5 experiments. * Denotes a significant difference from results for the quiescent state.

Rac1 was not detectable in either fraction of swine pulmonary arteries ($n=3$). In Ca^{2+} -free medium, treatment with DPB (1 μM) did not induce RhoA to translocate from the cytosolic to the membrane fractions (Fig. 3A). In contrast, treatment with DPB (1 μM) for 5 or 10 min led to a significant increase in the translocation of Cdc42 from the cytosolic to membrane fraction (Fig. 3B).

Cdc42 activity during phorbol ester stimulation: To confirm the role of Rho GTPases on PKC-mediated Ca^{2+} -independent contractions, the effects of a ROCK inhibitor was tested. In Ca^{2+} -free medium, Y27632, a ROCK inhibitor, (1–10 μM) did not change DPB (1 μM)-mediated contractions (Fig. 4). It has been well established that active and GTP-bound forms of Cdc42 selectively bind to PAK and that the binding domain-tagged with GST can be precipitated by glutathione beads [1]. To clarify the effect of DPB

Fig. 3. Translocation of Rho GTPase induced by 12-deoxyphorbol 13-isobutyrate in swine pulmonary arteries. Strips were treated with 12-deoxyphorbol 13-isobutyrate (DPB, 1 μ M) for 5 or 10 min in Ca^{2+} -free medium containing 1 mM EGTA. Immunoblot analysis of RhoA (A) and Cdc42 (B) was performed on the cytosolic (c) and membrane (m) fractions. One hundred percent represents the density of the membrane fraction relative to total expression in the cytosolic plus membrane fractions in the quiescent state. The results are the mean $\pm$ SEM of 5 experiments. * Denotes a significant difference from results for the quiescent state.

on the Rho GTPase activity, the activity of Cdc42 was measured by pull-down assay. DPB 1 μ M for 5 or 10 min significantly increased the activity of Cdc42 in Ca^{2+} -free medium in swine pulmonary arteries (Fig. 5).

Translocation of PKC isoforms and Rho GTPases by NE: In order to evaluate the effects of a receptor agonist on PKC and Rho GTPase activity in swine pulmonary arteries, activities of PKC isoforms and Rho GTPases were measured during NE-stimulation in normal PSS. NE (10 μ M) did not induce any changes in the subcellular distributions of PKC α and ϵ in swine pulmonary arteries (Fig. 6A). Treatment with NE (10 μ M) significantly increased the distribution of RhoA to the membrane fraction (Fig. 6B). In contrast, NE did not change the subcellular distribution of Cdc42 in swine pulmonary arteries (Fig. 6A).

DISCUSSION

In the present study, we showed that the low molecular Rho GTPases RhoA and Cdc42, but not Rac1, were found in smooth muscle from swine pulmonary arteries. Furthermore, phorbol ester significantly increased the activities of Cdc42 and the PKC isoforms α and ϵ in Ca^{2+} -free medium. These results show that phorbol ester activates both PKC isoforms and Cdc42 in pulmonary artery smooth muscle, and that the activation of Cdc42 may contribute to phorbol ester-induced Ca^{2+} -independent contractions. Although it

Fig. 4. Effects of ROCK inhibitor on 12-deoxyphorbol 13-isobutyrate-induced contraction in smooth muscle from swine pulmonary arteries. After the muscle strips were incubated in Ca^{2+} -free medium containing 1 mM EGTA for 20 min, the muscle strips were treated with 12-deoxyphorbol 13-isobutyrate (DPB, 1 μ M) and Y27632 (1, 3, and 10 μ M), a ROCK inhibitor. Results are typical recordings obtained from 4 experiments.

Fig. 5. Increases in Cdc42 activity by 12-deoxyphorbol 13-isobutyrate in swine pulmonary arteries. Pulmonary arterial strips were treated in Ca^{2+} -free medium containing 1 mM EGTA for 20 min, and were stimulated with 12-deoxyphorbol 13-isobutyrate (DPB, 1 μ M) for 5 or 10 min. Extracts of muscle strips were incubated with GST-tagged PAK-binding domain (PBD), which binds to activated Cdc42. The active Cdc42/GST-PBD complexes were separated with glutathione affinity beads. The amounts of active Cdc42 were determined by immunoblot analysis with anti-Cdc42 antibody. (A) Representative immunoblot illustrating the active Cdc42 in DPB-stimulated strips. (B) Statistical results of levels of activated Cdc42 in DPB-stimulated strips. One hundred percent represents the density in the quiescent state. The results are the mean $\pm$ SEM of 4 experiments. * Denotes a significant difference from results for the quiescent state.

has been reported that RhoA and ROCK were detected in swine pulmonary arteries [1, 21], to our knowledge, this is the first result that directly determines the activity of Rho GTPases on PKC-mediated responses in the artery.

NE significantly activated RhoA, which is consistent with the findings of a previous study [8]. Receptor agonist-

Fig. 6. Translocation of PKC isoforms and Rho GTPases induced by norepinephrine in swine pulmonary arteries. Strips were treated with norepinephrine (NE, 10 μ M) for 5 or 10 min in normal PSS. (A) Immunoblot analysis of PKC α (upper panel), PKC ϵ (middle panel), and Cdc42 (lower panel) were performed on the cytosolic (c) and membrane (m) fractions in the quiescent state. (B) Statistical results of levels of activated RhoA in NE-stimulated strips. One hundred percent represents the density of the membrane fraction relative to total expression in the cytosolic plus membrane fractions in the quiescent state. The results are the mean $\pm$ SEM of 5 experiments. * Denotes a significant difference from results for the quiescent state.

induced contractions in pulmonary arteries were inhibited by treatment with ROCK inhibitor [13, 25, 28], implying that RhoA and ROCK contribute to agonist-mediated vasoconstriction. In contrast, the present and the previous results showed that another Rho GTPase isoform, Cdc42, did not influence agonist-mediated vasoconstriction [22]. Moreover, NE did not induce the subcellular redistribution of PKC isoforms. Previously, it has been proposed that Rho GTPases are individually regulated by different pathways [34]. RhoA activates ROCK, and thus stimulates the phosphorylation of MLC [23, 27]. Cdc42 and Rac1 activate another protein kinase, PAK, which can regulate levels of MLC phosphorylation resulting from changes of MLCK activity [3, 30]. Moreover, PAK also contributes to the activation of MAPK in smooth muscle [6]. Previously, we have reported that MAPK contributes to vascular contractions, which are not regulated by intracellular Ca^{2+} [24]. Therefore, it can be assumed that Cdc42 stimulated by phorbol ester increases the activation of PAK, and subsequently results in Ca^{2+} -independent MAPK activation. This pathway may contribute to phorbol ester-mediated Ca^{2+} -independent contractions in swine pulmonary artery smooth muscle. These results imply that RhoA contributes to NE-

induced contractions, and both the Cdc42 and PKC pathways are involved in the phorbol ester-induced response in pulmonary artery smooth muscle.

In the present study, phorbol ester elicited a tonic contraction in Ca^{2+} -free medium, which was strongly resistant to inhibition of the voltage-dependent Ca^{2+} channels. Similar properties of smooth muscle contractions have been demonstrated in intact and membrane-permeabilized muscle strips [28]. These results show that phorbol ester-induced muscle contractions can be mediated by Ca^{2+} -independent pathways, and that Rho/ROCK and/or PKC pathways may contribute to Ca^{2+} -independent contractions, which results in Ca^{2+} -sensitization in swine pulmonary arteries. In contrast, the present study showed that ROCK inhibitor failed to attenuate phorbol ester-mediated Ca^{2+} -independent contractions. Moreover, phorbol ester increased the activity of PKC isoforms and Cdc42. In contrast, the activation of RhoA was not evoked by stimulation with phorbol ester in swine pulmonary arteries. These results suggest that Ca^{2+} -sensitization induced by phorbol ester is regulated by the PKC/Cdc42 pathway, but not the Rho/ROCK pathway, in pulmonary arteries.

It has been suggested that Rho GTPases may be target molecules for the PKC pathway [2, 33], and this interaction may contribute to regulation of Ca^{2+} -independent contractions in pulmonary smooth muscle [35]. Recently an interrelationship between PKC activity and Rho GTPase has also been proposed in several other cell systems [4, 9, 31]. In vascular smooth muscle cells, Rho GTPase acts downstream of PKC in actin reorganization [2]. Recently, it has been reported that acetylcholine increased Cdc42 activity, and that a dominant negative mutant of Cdc42 attenuated contractile response to the receptor agonist in tracheal smooth muscle [33]. In the present study, phorbol ester significantly increased the activity of Cdc42 in swine pulmonary arteries. These results suggest that Cdc42 plays a critical role in regulation of Ca^{2+} -independent contractions mediated by PKC in swine pulmonary arteries. We propose that the interrelationship between PKC and Cdc42 may contribute to Ca^{2+} -sensitization in smooth muscle from swine pulmonary arteries.

In summary, phorbol ester induced sustained contractions of smooth muscle from the pulmonary artery by a Ca^{2+} -independent mechanism and increased the activities of PKC isoforms and Cdc42, but not RhoA. Therefore, we suggest that phorbol ester evokes PKC-mediated Ca^{2+} -independent contractions via a Rho GTPase pathway, especially Cdc42, in smooth muscle from swine pulmonary arteries.

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