Effect of Phorbol Esters on Cytosolic Ca\(^{2+}\) Level, Myosin Phosphorylation and Muscle Tension in High K\(^{+}\)-Stimulated Bovine Tracheal Smooth Muscle

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ABSTRACT—To determine the role of protein kinase C (PKC) in bovine tracheal smooth muscle contractility, we examined the effects of phorbol esters on cytosolic Ca\(^{2+}\) level ([Ca\(^{2+}\)]\(_i\)), myosin light chain (MLC) phosphorylation and contractile force in intact muscle and contraction in a permeabilized preparation. In intact muscle, 12-deoxyphorbol 13-isobutyrate (DPB, 1\(\mu\)M) increased the force without changing [Ca\(^{2+}\)]\(_i\). High K\(^{+}\) (72.7 mM) induced sustained contraction with sustained increase in [Ca\(^{2+}\)]\(_i\). In the muscle stimulated by high K\(^{+}\), 50 nM DPB increased the contractile force without changing [Ca\(^{2+}\)]\(_i\), and 1\(\mu\)M DPB increased the contractile force with decreasing [Ca\(^{2+}\)]\(_i\). Thus DPB shifted the [Ca\(^{2+}\)]\(_i\)/force relationship for high K\(^{+}\) to the lower [Ca\(^{2+}\)]\(_i\) in a concentration-dependent manner. In permeabilized muscle, DPB did not induce contraction in the absence of Ca\(^{2+}\) (<<0 nM), but shifted the Ca\(^{2+}\)/force relationship to the lower Ca\(^{2+}\) levels. In the muscle stimulated with high K\(^{+}\), DPB (50 nM and 1\(\mu\)M) increased MLC phosphorylation and force without changing the MLC phosphorylation/force relationship. DPB (1\(\mu\)M) increased PKC activity estimated by the translocation from the cytoplasm to the membrane. These results suggest that DPB increases the Ca\(^{2+}\) sensitivity of MLC phosphorylation via the activation of PKC. Furthermore, DPB at higher concentration has an inhibitory effect on stimulated [Ca\(^{2+}\)]\(_i\).

Keywords: Protein kinase C, Phorbol ester, Myosin phosphorylation, Cytosolic Ca\(^{2+}\) level, Tracheal smooth muscle

Cytosolic Ca\(^{2+}\) level ([Ca\(^{2+}\)]\(_i\)) is the primary regulator of myosin phosphorylation and contraction in smooth muscle (1, 2). It was found that high K\(^{+}\) and receptor agonists induce sustained increments of [Ca\(^{2+}\)]\(_i\), and contractile force, and receptor agonists induce greater contraction (3–5) and greater myosin light chain (MLC) phosphorylation (6, 7) than high K\(^{+}\) at a given [Ca\(^{2+}\)]\(_i\), suggesting that receptor agonists increase the Ca\(^{2+}\) sensitivity of contractile elements.

Nishimura et al. (8) and Kitazawa et al. (9) have shown that \(\alpha\)-adrenoceptor agonists modulate the Ca\(^{2+}\) sensitivity of contractile elements via GTP-binding protein (G-protein) in arterial muscle permeabilized with Staphylococcus aureus \(\alpha\)-toxin or \(\beta\)-escin. Receptor agonists stimulate phospholipase C via G-protein followed by activation of phosphatidylinositol turnover. During the activation, two intracellular second messengers are formed; one is inositol-1,4,5-trisphosphate (IP\(_3\)) that releases Ca\(^{2+}\) from intracellular Ca\(^{2+}\) pools, and the other is diacylglycerol (DG) that activates protein kinase C (PKC) (10). Phorbol esters have effects similar to DG and are widely used to activate PKC.

In tracheal smooth muscle, Park and Rasmussen (11) have shown that phorbol ester increases contractions induced by a Ca\(^{2+}\) ionophore and a Ca\(^{2+}\) channel activator. Menkes et al. (12) have reported that phorbol esters relaxed the resting and carbachol-stimulated tracheal muscle, although it potentiated the high K\(^{+}\)-induced contraction. However, little is known about the effects of phorbol esters on the relationship between [Ca\(^{2+}\)]\(_i\), and muscle force or [Ca\(^{2+}\)]\(_i\), and MLC phosphorylation in tracheal smooth muscle. Recently, we have shown that phorbol esters changed the relationship between [Ca\(^{2+}\)]\(_i\), and force in the presence of high K\(^{+}\) in rat aorta, suggest-
ing that the Ca\(^{2+}\) sensitivity of contractile elements was increased (5, 13). In the present experiments, to determine if activation of PKC increases Ca\(^{2+}\) sensitivity also in tracheal smooth muscle, we examined the effects of phorbo1 esters on [Ca\(^{2+}\)], MLC phosphorylation, contractile force and PKC activity in bovine tracheal smooth muscle.

**MATERIALS AND METHODS**

Freshly excised bovine tracheae were obtained from a local abattoir. The smooth muscle was excised from cartilage, and the epithelium and connective tissues were removed. The smooth muscles were cut into small strips (approximately 1-mm-wide and 5-mm-long) and placed in a normal physiological salt solution (PSS), which contained: 136.9 mM NaCl, 5.4 mM KCl, 1.5 mM CaCl\(_2\), 1.0 mM MgCl\(_2\), 23.9 mM NaHCO\(_3\), 5.5 mM glucose and 0.01 mM EDTA. High K\(^+\) solution was made by substituting NaCl with equimolar KCl. These solutions were saturated with a 95% O\(_2\) and 5% CO\(_2\) mixture at 37°C and pH 7.4. Muscle force was recorded isometrically with a force displacement transducer. Each muscle was attached to a holder under a resting force of 5 mN and equilibrated for 60–90 min in a 10-ml muscle bath. The high K\(^+\) (72.7 mM)-induced response was used as the reference response (100%).

[Ca\(^{2+}\)]\(_i\) was measured simultaneously with muscle contraction as reported by Ozaki et al. (5, 14) and Sato et al. (15). Muscle strips were loaded with fura-2/AM for >4 hr at room temperature (23–25°C). After the fura-2-loading, muscle strips were washed with normal PSS in a tissue bath at 37°C for 30–40 min to remove uncleaved fura-2/AM. One end of the muscle was connected to a force displacement transducer to monitor the mechanical activity. The muscle strips were illuminated alternatively at 340 and 380 nm (48 Hz) and the amount of 500 nm fluorescence for F340 and F380 (n = 6). The absolute Ca\(^{2+}\) concentration was not calculated in this experiment because the dissociation constant of fura-2 for Ca\(^{2+}\) in the smooth muscle cytoplasm may be different from that in vitro (16). Instead, we used the ratio of F340/F380 as an indicator of [Ca\(^{2+}\)]\(_i\), as has been reported (5, 14, 15).

Permeabilized muscle was prepared with Staphylococcus aureus \(\alpha\)-toxin as described by Nishimura et al. (8) and Kitazawa et al. (9). A thin bundle (0.1-mm-wide and 2-mm-long) of muscle tissue was prepared and treated with \(\alpha\)-toxin (10 \(\mu\)g protein/ml) for 10 min in a solution containing 130 mM K-propionate, 4 mM MgCl\(_2\), 4 mM Na\(_2\)ATP, 20 mM Tris maleate (pH 6.8), 2 mM creatine phosphate, 10 U/ml creatine phosphokinase, 2 mM EGTA and an appropriate amount of CaCl\(_2\). The permeabilized muscle was mounted in a small chamber at room temperature (23–25°C), and the mechanical activity was monitored. The apparent binding constant used for the Ca\(^{2+}\)-EGTA complex was assumed to be 10\(^{7}\)/M.

For determination of MLC phosphorylation, we used two dimensional electrophoresis (IEF-SDS PAGE). Small muscle strips (approximately 1-mm-wide and 10-mm-long) were frozen in acetone-dry ice. Muscle strips were crushed and mixed with the buffer containing 8.5 M urea, 2% nonidet P-40, 2% pharmalyte (mixture of 4 vol. of pH 4–6.5 pharmalyte and 1 vol. of pH 3–10 pharmalyte), 40 mM dithiothreitol (DTT) and 1% sodium dodecyl sulfate (SDS). The sample was then centrifuged for 20 min at 5,000 \(\times\) g and the supernatant was analyzed. Two dimensional electrophoresis was performed as described by O’Farrel (17). Proteins were visualized by the silver staining method. The optical density of the unphosphorylated and phosphorylated MLC was measured by densitometry.

The PKC activity was measured with a commercially available kit (Amersham-Japan, Tokyo). The protein content of each sample was measured immediately following the assay of PKC activity with a protein assay kit (Bio-Rad Lab., Richmond, VA, USA). PKC activity is expressed as pmol phosphate transferred min\(^{-1}\) mg\(^{-1}\) protein.

The following drugs and chemicals were used: verapamil hydrochloride (Sigma Chemical Co., St. Louis, MO, USA); ethyleneglycol bis(8-aminethyl ether)-N,N',N'',N'-tetraacetic acid (EGTA), ethylene diamine tetraacetic acid (EDTA), fura-2/AM (Dojindo Laboratories Kumamoto); 12-deoxyphorbol 13-isobutyrate (DPB), phorbol 12,13-dibutyrate (PDB), 4\(\alpha\)-phorbol 12,13-dibutyrate (4\(\alpha\)-PDB) (Funakoshi, Tokyo); cremophor EL (Nacalai Tesque, Kyoto) and ionomycin (Hoechst-Japan, Tokyo). \(\alpha\)-Toxin, isolated from Staphylococcus aureus, was supplied by Dr. Iwao Kato, University of Chiba.

Results of the experiments are expressed as the mean \(\pm\) S.E.M. Student’s \(t\)-test was used for statistical analyses of the results.

**RESULTS**

*The effects of phorbol ester on [Ca\(^{2+}\)]\(_i\) and muscle force*

A low concentration of DPB (10 nM) had no effect on [Ca\(^{2+}\)]\(_i\) and muscle force in fura-2 loaded bovine tracheal smooth muscle (n = 2). DPB (50 nM) induced a small sustained contraction (31.8 \(\pm\) 20.2% of control, n = 3) without changing [Ca\(^{2+}\)]. Sequential addition of a higher
Fig. 1. Effect of DPB on \([\text{Ca}^{2+}]\) (upper trace, indicated by \(F_{340}/F_{380}\)) and contractile force (lower trace) in bovine tracheal smooth muscle. After the effect of 72.7 mM KCl was examined, 50 nM and 1 \(\mu\)M DPB were cumulatively added.

Fig. 2. Effect of DPB on \([\text{Ca}^{2+}]\) (upper trace, indicated by \(F_{340}/F_{380}\)) and contractile force (lower trace) in bovine tracheal smooth muscle stimulated with high K\(^+\) (72.7 mM). After the effect of 72.7 mM KCl became stable, 50 nM (A) and 1 \(\mu\)M (B) DPB were added. After the addition of DPB, 10 \(\mu\)M verapamil and 4 mM EGTA were sequentially added.
concentration of DPB (1 μM) induced a greater contraction (154.3±35.8%, n=5) without changing [Ca^{2+}]. EGTA (4 mM) inhibited the contraction due to 1 pM DPB to 103.9±15.5% (n=5) with a decrease in [Ca^{2+}], below the resting level (Fig. 1).

High K^+ (72.7 mM)-induced sustained increases in [Ca^{2+}] and force. Addition of DPB (50 nM) increased the contractile force without changing [Ca^{2+}]. DPB (1 pM), on the other hand, increased the contractile force with decreasing [Ca^{2+}], (Fig. 2: A and B). In the presence of high K^+ with or without 50 nM or 1 pM DPB, 1 and 10 pM verapamil was cumulatively added, resulting in decreases of [Ca^{2+}] and muscle force. Figure 3 shows the [Ca^{2+}]/force relationship obtained from these experiments. DPB (50 nM and 1 μM) was demonstrated to shift the [Ca^{2+}]/force relationship due to high K^+ to lower [Ca^{2+}], in a concentration-dependent manner.

The effect of phorbol esters on permeabilized muscle

The effects of DPB on bovine tracheal smooth muscle permeabilized with α-toxin are shown in Fig. 4. In the absence of Ca^{2+} (<<10 nM), DPB (10 μM) had no effect on the contractile force (n=6) (Fig. 4A). Addition of 0.3 μM Ca^{2+} induced a small sustained contraction that was enhanced by DPB (10 μM) (Fig. 4B). Cumulative addition of Ca^{2+} (10 nM–30 μM) induced a graded contraction, and the Ca^{2+}/force curve obtained is shown in Fig. 4C. The EC_{50} for Ca^{2+} was 6.0±0.02 (−log M) (n=4). The Ca^{2+}/force curve was shifted to the left by 10 μM DPB (EC_{50}: 6.3±0.07; P<0.01, n=4). Another active phorbol ester, PDB (10 μM), also shifted the Ca^{2+}/force curve to the left (EC_{50}: 6.4±0.03, P<0.01, n=4). On the other hand, 4α-DBP (10 μM), an inactive phorbol ester, did not affect the Ca^{2+}/force curve.

The effect of phorbol ester on MLC phosphorylation and MLC phosphorylation/force relationship

In normal PSS, DPB (1 μM) increased MLC phosphorylation from 21.4±2.40% (n=5) to 39.0±1.7% (P<0.01, n=4). MLC phosphorylation was also increased by 72.7 mM K^+ to 37.8±1.0% (P<0.01, n=4).
In the presence of high K⁺, 50 nM and 1 μM DPB increased MLC phosphorylation to 47.8±0.6% (n=3) and 42.4±0.3% (n=3), respectively. Figure 5 shows the [Ca²⁺]/MLC phosphorylation relationships obtained from these experiments. DPB (50 nM and 1 μM) was shown to shift the [Ca²⁺]/MLC phosphorylation relationship due to high K⁺ to lower [Ca²⁺], in a concentration-dependent manner.

The relationship between MLC phosphorylation and muscle force shown in Fig. 6 indicates that DPB does not change the MLC phosphorylation/force relationship.

The effect of phorbol ester on PKC activity

Figure 7 shows the changes in cytosolic and particulate PKC activities. After exposure of muscle strips to 1 μM DPB for 10 min, PKC activity in the cytosolic fraction was significantly decreased. DPB also significantly increased the activity in the particulate fraction. The total
PKC activity (cytosol plus particle) did not change before and after exposure to DPB.

DISCUSSION

Present study demonstrated that DPB at a higher concentration (1 \mu M) induced a sustained contraction with no increase in [Ca^{2+}]_i. This contraction was associated with a significant increase in MLC phosphorylation. DPB also shifted the [Ca^{2+}]/force curve for high K^+ to lower [Ca^{2+}], in a concentration-dependent manner. In \alpha-toxin permeabilized muscle, DPB decreased the threshold Ca^{2+} concentration (from 0.3 to 0.1 \mu M). Furthermore, it was also demonstrated that DPB activated PKC activity by measuring the translocation of PKC activity to the membrane fraction. These results suggest that 1) DPB induces tracheal smooth muscle contraction by an MLC phosphorylation-dependent and Ca^{2+}-less-dependent manner and 2) DPB increases the Ca^{2+} sensitivity of contractile elements through activation of PKC.

Since smooth muscle contraction is regulated by the increase in [Ca^{2+}], and the subsequent MLC phosphorylation (1, 2, 18), Ca^{2+} sensitization may be due to the modulation of either the relationship between [Ca^{2+}] and MLC phosphorylation or the relationship between MLC phosphorylation and force development. DPB (50 nM and 1 \mu M) augmented MLC phosphorylation induced by high K^+ with decreasing [Ca^{2+}]. Thus, the [Ca^{2+}]/MLC phosphorylation relationship was shifted to the upper-left by DPB (Fig. 5). This result suggests that the Ca^{2+} sensitization by DPB may largely be due to increased MLC kinase activity and/or decreased phosphatase activity. On the other hand, DPB did not change the MLC phosphorylation/force relationship, as has been demonstrated in vascular smooth muscle (7). Kamm et al. (18) also have shown that PDB (1 \mu M) increased MLC phosphorylation from 9% to 22% in the same tissue. It was also shown that only 25% of the MLC phosphorylation is due to MLC kinase (at serine-19 and 75%) is due to PKC (at serine-1 and -2). These results suggest that the phosphorylated MLC observed in the present study contains the MLC kinase (at serine-19) and 75% is due to PKC (at serine-2). These results suggest that PKC may inhibit the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels. On the other hand, it has been shown that in ionomycin-stimulated uterine smooth muscle, DPB decreased [Ca^{2+}], and muscle force even in the presence of verapamil (19), suggesting that DPB decreases [Ca^{2+}] through the activation of Ca^{2+} pumping mechanisms. The activation of the plasma membrane Ca^{2+} pump by PKC (20) should also be taken into account for the DPB-induced decrease in [Ca^{2+}], in tracheal smooth muscle.

In \alpha-toxin permeabilized muscle, DPB did not induce contraction in the absence of Ca^{2+} (pCa<8). In contrast, in intact tissue, DPB induced a sustained contraction in Ca^{2+}-free solution. These results suggest that a trace amount of Ca^{2+}, which may be remaining in the cell even after the prolonged depletion of external Ca^{2+}, is necessary for inducing DPB-contraction in intact muscle.

In summary, it is suggested that activation of PKC by DPB has dual effects on tracheal smooth muscle stimulated by high K^+: it increases Ca^{2+} sensitivity of MLC phosphorylation to augment smooth muscle contraction and decreases the stimulated [Ca^{2+}].

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