Different pathways of calcium sensitization activated by receptor agonists and phorbol esters in vascular smooth muscle

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1 It has been shown that receptor agonists and activators of protein kinase C, phorbol esters, increase Ca^{2+} sensitivity of contractile elements in vascular smooth muscle. To discover if protein kinase C is involved in the agonist-mediated Ca^{2+} sensitization, we examined the effects of receptor agonists in the rat isolated aorta in which protein kinase C activity had been diminished by pretreatment with phorbol 12-myristate 13-acetate for 24 h.

2 In the aorta with protein kinase C activity, a high concentration (1 μM) of 12-deoxyphorbol 13-isobutyrate induced contraction and a low concentration (100 nM) potentiated high K^+ -induced contraction. In addition, prostaglandin $\text{F}_{2\alpha}$ induced greater contractions than high K^+ at a given cytosolic Ca^{2+} level. The maximally effective concentrations of noradrenaline and endothelin-1 also induced greater contraction than high K^+ . In the aorta without protein kinase C activity, the contraction induced by 12-deoxyphorbol 13-isobutyrate and its potentiation of the high K^+ -induced contraction were abolished. However, prostaglandin $\text{F}_{2\alpha}$, noradrenaline and endothelin-1 still induced a greater contraction than high K^+ .

3 In the aorta without protein kinase C activity, noradrenaline, endothelin-1 and prostaglandin $\text{F}_{2\alpha}$, but not 12-deoxyphorbol 13-isobutyrate, induced contractions in the presence of the Ca^{2+} channel blocker, verapamil, or in the absence of external Ca^{2+} , by increasing Ca^{2+} sensitivity.

4 In the permeabilized preparations, inhibition of protein kinase C activity abolished the effect of potentiation of the Ca^{2+} -induced contraction by 12-deoxyphorbol 13-isobutyrate although the potentiation of the contraction by prostaglandin $\text{F}_{2\alpha}$ did not change.

5 These results suggest that there are two pathways for Ca^{2+} sensitization in rat aorta; a protein kinase C-dependent pathway which is activated by phorbol esters, and a protein kinase C-independent pathway which is activated by receptor agonists.

Keywords: Protein kinase C; cytosolic Ca^{2+} level; receptor agonists; Ca^{2+} sensitization; vascular smooth muscle (rat aorta)

Introduction

Although smooth muscle contraction is induced by increasing the cytosolic Ca^{2+} level ($[\text{Ca}^{2+}]_i$) and phosphorylating the myosin light chain (MLC), receptor agonists cause greater contraction than high K^+ -depolarization at a given $[\text{Ca}^{2+}]_i$ (For review see Karaki, 1989; Somlyo & Himpens, 1989). In muscle permeabilized with bacterial α -toxin, receptor agonists potentiate Ca^{2+} -induced contraction in the presence of GTP (Nishimura *et al.*, 1988; Kitazawa *et al.*, 1989). Since Ca^{2+} sensitization induced by receptor agonists is accompanied by an increase in MLC phosphorylation in intact and permeabilized smooth muscle preparations (Rembold & Murphy, 1988; Suematsu *et al.*, 1991; Kitazawa *et al.*, 1991; Hori *et al.*, 1992), it has been suggested that an altered balance between the activities of MLC kinase and MLC phosphatase results in the Ca^{2+} sensitization of MLC phosphorylation (Stull *et al.*, 1990; Gong *et al.*, 1992). In addition, the existence of a regulatory mechanism that is not dependent on MLC phosphorylation is also proposed and this mechanism may also be involved in the Ca^{2+} sensitization in smooth muscle (Hori *et al.*, 1992).

Activators of protein kinase C, phorbol esters, also induce contraction and increase Ca^{2+} sensitivity in intact and permeabilized smooth muscle preparations (Nishimura & van Breemen, 1989; Anabuki *et al.*, 1990; Singer 1990; Sato *et al.*, 1992). Since various receptor agonists activate inositol phosphate metabolism to generate the endogenous activator of protein kinase C, diacylglycerol (Berridge, 1984; Nishizuka 1984), it is possible that activation of protein kinase C is

involved in Ca^{2+} sensitization mediated by the receptor agonists. In the present experiments, this possibility was examined in rat isolated aorta in which protein kinase C activity had been abolished. For this purpose, we treated the muscle with phorbol ester because such treatment has been shown to decrease protein kinase C activity by decreasing the amount of protein kinase C molecules in the cells (Young *et al.*, 1987). An account of some of these results has been published in abstract form (Sato & Karaki, 1992).

Methods

Muscle preparations

Male Wistar rats (250–300 g) were killed by a sharp blow on the neck and exsanguination. The thoracic aorta was isolated and cut into helical strips. The endothelium was removed by gently rubbing the intimal surface with a finger moistened with normal physiological salt solution (PSS) which contained (mM): NaCl 136.9, KCl 5.4, CaCl_2 1.5, MgCl_2 1.0, NaHCO_3 23.8, EDTA 0.01 and glucose 5.5. High K^+ solution was made by substituting 72.7 mM NaCl with equimolar KCl. These solutions were saturated with 95% O_2 and 5% CO_2 mixture at 37°C to maintain the pH at 7.4.

Muscle strips were stimulated by the maximally effective concentrations of noradrenaline (1 μM ; Sato *et al.*, 1988), endothelin-1 (30 nM; Sakata *et al.*, 1989), 12-deoxyphorbol 13-isobutyrate (DPB; 1 μM ; Sato *et al.*, 1992) or KCl (72.7 mM; Karaki *et al.*, 1986). A low concentration of DPB (100 nM) was also used to modulate high K^+ -induced contractions.

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Pretreatment with phorbol ester

To decrease protein kinase C activity, thoracic aortic strips were incubated in Dulbecco's modified Eagle medium with 10% foetal calf serum containing penicillin-streptomycin (GIBCO, New York, U.S.A.) for 24 h at 37°C in a CO₂ incubator in the presence or absence of 3 µM phorbol 12-myristate 13-acetate (TPA). At the end of the incubation, tissues were washed with normal PSS for more than 60 min to remove TPA from the surface of the tissue. In a preliminary experiment, we also used 3 µM DPB and found that the effect of DPB was less than that of TPA. For this reason, we used TPA to decrease protein kinase C activity in the present experiments.

Measurement of protein kinase C activity

Cytosolic and membrane fractions were prepared from rat aorta as described previously (Haller *et al.*, 1990). After a 20 min incubation with test agents, muscle strips were quickly frozen in liquid nitrogen and crushed. The muscle powder was dissolved in buffer containing (mM): Tris-HCl (pH 7.4) 20, sucrose 300, EDTA 5, EGTA 10, phenylmethylsulphonyl fluoride 0.3, benzamidine 10 and 0.3% β-mercaptoethanol. The suspension was centrifuged at 100,000 g at 4°C for 60 min. The supernatant was used as cytosolic fraction. The precipitate was re-suspended in the same buffer containing 0.1% Triton X-100 and kept on ice for 30 min. The suspension treated with Triton X-100 was centrifuged at 30,000 g for 20 min at 4°C. The supernatant was used as the membrane fraction.

Protein kinase C activity was measured by a commercial assay system (RPN 77A, Amersham Japan, Tokyo, Japan). The cytosolic and membrane fractions were dissolved in a reaction medium containing: Tris-HCl (pH 7.5) 50 mM, calcium acetate 1 mM, magnesium acetate 15 mM, L-α-phosphatidyl-L-serine 0.67 mol %, TPA 2 µg ml⁻¹, synthetic peptide 75 µM, sodium azide 0.05% w/v and dithiothreitol 2.5 mM. The reaction was started by adding 50 µM ATP containing 0.2 µCi [γ-³²P]-ATP. After treatment at 25°C for 15 min, the reaction was stopped and the radioactivity in the synthetic peptide was counted with a liquid scintillation spectrometer. In some experiments, calcium acetate, TPA and L-α-phosphatidyl-L-serine were removed from the reaction medium to inhibit the protein kinase C activity and to determine the background level of the measuring system.

Simultaneous measurement of force and [Ca²⁺]_i

[Ca²⁺]_i was measured simultaneously with muscle force by use of the fluorescent Ca²⁺ indicator, fura-2, as reported previously (Sato *et al.*, 1988). The muscle strips (2 mm wide and 7 mm long) were loaded with 5 µM acetoxymethyl ester of fura-2 for 4–5 h at room temperature (23–25°C). The muscle strip was illuminated alternately (48 Hz) with 340 nm and 380 nm light and 500 nm emission was detected with a fluorimeter (CAF-100, JASCO, Tokyo, Japan). The ratio of 500 nm fluorescence excited at 340 nm and 380 nm (R340/380) was used as an indicator of [Ca²⁺]_i. Absolute Ca²⁺ concentration was not calculated because the dissociation constant of fura-2 in cytoplasm may be different from that obtained *in vitro* (Karaki, 1989). Contractile force was measured by a force displacement transducer under a resting force of 10 mN.

Permeabilized muscle

Permeabilized muscle preparations were made by treating the TPA-pretreated muscle strips (1 mm wide and 4 mm long) with *Staphylococcus aureus* α-toxin (80 µg ml⁻¹). Relaxing solution contained; potassium propionate 130 mM, MgCl₂ 4 mM, Na₂ATP 2 mM, Tris maleate (pH 6.8) 20 mM, EGTA 2 mM, carbonyl cyanide p-trifluoromethoxyphenylhydrazone

(FCCP) 1 µM, creatine phosphate 5 mM, creatine phosphokinase 10 U ml⁻¹ and leupeptine 1 µg ml⁻¹. Free Ca²⁺ concentration was changed by adding an appropriate amount of CaCl₂. The apparent binding constant of EGTA for Ca²⁺ was considered to be 10⁶ M⁻¹. Force of contraction was measured by an isometric transducer under a resting force of 5 mN. Intracellular Ca²⁺ stores were depleted by adding 10 µM ionomycin for 20 min (Nishimura *et al.*, 1988).

Materials

Prostaglandin F_{2α}, verapamil and FCCP (Sigma Chemical, St. Louis, MO, U.S.A.), TPA and DPB (Funakoshi, Tokyo, Japan), noradrenaline bitartrate, dithiothreitol and β-mercaptoethanol (Wako Pure Chemicals, Tokyo, Japan), cremophor EL (Nacalai Tesque, Kyoto, Japan), forskolin (Nihon Kayaku, Tokyo, Japan), GTP (Boehringer Mannheim Corp. New York, NY, U.S.A.), endothelin-1 (donated by Ciba-Geigy Japan, Takarazuka, Japan), fura-2/AM (donated by Dojindo Laboratories, Kumamoto, Japan), and α-toxin (donated by Dr Iwao Kato, Chiba University) were used.

Results

Preparations without protein kinase C activity

Figure 1 shows the effect of DPB on the protein kinase C activity in rat aorta. Protein kinase C activity was expressed as pmol ATP incorporated in synthetic peptide per mg of smooth muscle protein per min (pmol mg⁻¹ min⁻¹). In the muscle without TPA-pretreatment (control), protein kinase C activity in the cytosolic fraction was higher than that in the membrane fraction. After incubation with 1 µM DPB for 20 min, protein kinase C activity in the cytosolic fraction decreased and that in the membrane fraction increased, suggesting that DPB activated protein kinase C. In the muscle pretreated with TPA for 24 h, protein kinase C activities in both fractions were reduced to a level similar to that

Figure 1 Effect of 12-myristate 13-acetate (TPA)-pretreatment on protein kinase C activity in rat aorta. Muscle strips without (control) or with TPA-pretreatment were incubated with 1 µM 12-deoxyphorbol 13-isobutyrate (DPB) for 20 min. Protein kinase C activity was shown by pmol ATP incorporated into synthetic peptide per mg of smooth muscle protein per min (pmol mg⁻¹ min⁻¹). In some experiments, protein kinase C activity was measured in the absence of Ca²⁺, TPA and lipid (Ca and lipid-free) to determine the background level of the measurement. Open and closed columns show cytosolic and membrane fractions, respectively. Each column represents mean of 4–8 experiments and s.e.mean is shown by vertical bar. **Significantly different from the value in resting muscle with *P* < 0.01 using Student's *t* test.

Figure 2 Changes in $[\text{Ca}^{2+}]_i$ (upper trace; indicated by R340/380) and contractile force (lower trace) in the muscle pretreated with vehicle (control; a) or phorbol 12-myristate 13-acetate (TPA) (b) KCl 72.7 mM; 12-deoxyphorbol 13-isobutyrate (DPB) 100 nM; prostaglandin $\text{F}_{2\alpha}$ 30 μM ; verapamil 10 μM ; EGTA 4 mM; forskolin 1 μM . 100% represents the levels of $[\text{Ca}^{2+}]_i$ stimulated by 72.7 mM KCl. Traced from typical results out of 4 experiments.

Figure 3 $[\text{Ca}^{2+}]_i$ force relationship in the muscle pretreated with vehicle (control; open symbol) or phorbol 12-myristate 13-acetate (TPA) (closed symbol): ($\square$, $\blacksquare$) 72.7 mM KCl; ($\circ$, $\bullet$) 30 μM prostaglandin $\text{F}_{2\alpha}$. (1) In normal solution; (2) in the presence of 10 μM verapamil; (3) in the presence of 10 μM verapamil and 4 mM EGTA. 100% represents the levels of contractile force and $[\text{Ca}^{2+}]_i$ stimulated by 72.7 mM KCl. Each point represents mean $\pm$ s.e.mean of 4–6 experiments.

obtained in the absence of Ca^{2+} , TPA and lipid (background level). In these preparations, DPB was ineffective. These results suggest that treatment with TPA for 24 h abolished protein kinase C activity in the aorta.

Effects of prostaglandin $\text{F}_{2\alpha}$ and other agonists

Figure 2 shows the effects of DPB and prostaglandin $\text{F}_{2\alpha}$ on $[\text{Ca}^{2+}]_i$ and contractions in the muscles pretreated with vehicle (control) or TPA for 24 h. KCl (72.7 mM) induced sustained increases in contractile force and $[\text{Ca}^{2+}]_i$ in both types of preparations. DPB (100 nM) changed neither resting force nor

Figure 4 Effects of maximally effective concentrations of 12-deoxyphorbol 13-isobutyrate (DPB, 1 μM), noradrenaline (NA: 1 μM) and endothelin-1 (ET-1: 30 nM) on the contractile force in the muscle pretreated with vehicle (control; open column) or phorbol 12-myristate 13-acetate (TPA) (closed column). Experiments were done as shown in Figure 2 except that $[\text{Ca}^{2+}]_i$ was not measured. 100% (dotted line) represents the level of contractile force stimulated by maximally effective concentration of KCl (72.7 mM). Each column represents mean with s.e.mean of 4–6 experiments. * and **: Significantly different from control muscles with $P < 0.05$ and 0.01, respectively, using Student's t test. #: Significantly greater than high K^+ -induced contraction in control muscles with $P < 0.05$ using Student's t test. Verapamil: 10 μM . EGTA: 4 mM.

$[\text{Ca}^{2+}]_i$ in control and in TPA-pretreated preparations ($n = 4$ each, data not shown). In control muscles, the same concentrations of DPB potentiated the high K^+ -induced contraction without changing $[\text{Ca}^{2+}]_i$ (Figure 2a). In the TPA-pretreated muscles, in contrast, 100 nM DPB was ineffective (Figure 2b).

Prostaglandin $\text{F}_{2\alpha}$ (30 μM) also increased $[\text{Ca}^{2+}]_i$ and force in the preparations pretreated with vehicle (control) or TPA (Figure 2). Prostaglandin $\text{F}_{2\alpha}$ induced greater force than high K^+ at a given $[\text{Ca}^{2+}]_i$ in both types of preparations (Figure 3). Verapamil (10 μM) inhibited the high K^+ -stimulated increase in $[\text{Ca}^{2+}]_i$ and force to their respective resting levels (Figure 3). The same concentration of verapamil inhibited the prostaglandin $\text{F}_{2\alpha}$ -stimulated $[\text{Ca}^{2+}]_i$ to a level near a resting level. However, the prostaglandin $\text{F}_{2\alpha}$ -induced contraction was only partially inhibited (Figures 2 and 3). Sequential addition of EGTA (4 mM) further decreased $[\text{Ca}^{2+}]_i$ and force although a part of the developed force was not inhibited in both types of preparations. Contraction resistant to EGTA was inhibited by forskolin without changing $[\text{Ca}^{2+}]_i$ (Figures 2 and 3). These results are similar to those reported previously in rat aorta (Hori *et al.*, 1992; Sato *et al.*, 1992). The $[\text{Ca}^{2+}]_i$ force relationship (Figure 3) indicated that prostaglandin $\text{F}_{2\alpha}$ shifted this relationship to a lower Ca^{2+} level compared to that obtained in the presence of high K^+ , irrespective of the pretreatment with TPA.

Figure 4 shows the effects of DPB, noradrenaline and endothelin-1 on contractile force in the aorta pretreated with vehicle (control) or TPA. The experiment was done as shown in Figure 2 except that $[\text{Ca}^{2+}]_i$ was not measured. In control preparations, DPB (1 μM) induced a sustained contraction greater than that induced by high K^+ . DPB-induced contraction was partially inhibited by the addition of 4 mM EGTA. In control preparations, maximally effective concentrations of noradrenaline (1 μM ; see Methods) and endothelin-1 (30 nM; see Methods) also induced contractions greater than those induced by 72.7 mM K^+ . Addition of 10 μM verapamil partially inhibited the contractions induced by these receptor agonists. Sequential addition of EGTA further decreased contractile force although a part of the contraction was not inhibited.

In TPA-pretreated preparations, in contrast, 1 μM DPB did not induce contraction in the presence or absence of external Ca^{2+} . However, the contractions to noradrenaline and endothelin-1 in the absence or presence of EGTA and/or verapamil were not modified by the pretreatment with TPA (Figure 4).

Figure 5 Ca^{2+} -induced contraction in permeabilized aorta pretreated with vehicle (control) or phorbol 12-myristate 13-acetate (TPA). (a) Trace of typical result, one of four experiments. Numbers indicate the concentrations of Ca^{2+} in $-\log M$ (pCa); GTP 100 μM ; prostaglandin $\text{F}_{2\alpha}$ 10 μM ; 12-deoxyphorbol 13-isobutyrate (DPB) 1 μM . (b) Summary of the results in (a). Open column: control. Closed column: TPA-pretreated. Each column represents the mean with s.e. mean of 4 experiments. 100% represents the level of contraction induced by 10 μM Ca^{2+} . *Significantly different from the contraction induced by 1 μM Ca^{2+} (pCa6) with $P < 0.05$ using Student's *t* test.

Permeabilized preparation

As shown in Figure 5, addition of Ca^{2+} induced contraction in the permeabilized aorta pretreated with vehicle (control). This contraction was potentiated by 10 μM prostaglandin $\text{F}_{2\alpha}$ in the presence of 100 μM GTP. DPB (1 μM) had a similar effect to prostaglandin $\text{F}_{2\alpha}$ in the vehicle-treated preparations. In the permeabilized aorta pretreated with TPA, addition of Ca^{2+} also induced contraction. In TPA-pretreated preparations, the potentiation of the Ca^{2+} -induced contraction by DPB was abolished. However, 10 μM prostaglandin $\text{F}_{2\alpha}$ still potentiated the Ca^{2+} -induced contractions in the TPA-pretreated preparations. Thus, DPB showed similar effects to prostaglandin $\text{F}_{2\alpha}$ only in control preparations.

Discussion

To obtain preparations without protein kinase C activity, we treated the rat isolated aorta with tissue culture medium containing TPA for 24 h. In the preparations pretreated with TPA, there was no protein kinase C activity and the translocation of protein kinase C activity from cytosol to membrane by DPB was abolished. It has been reported that suppression of protein kinase C activity by phorbol esters is due to a decrease in protein kinase C molecules resulting from an

increased rate of degradation, with no change in the amount of mRNA or rates of synthesis of protein kinase C (Young *et al.*, 1987).

In a previous paper (Sato *et al.*, 1992), we have shown that DPB induced greater contraction and greater myosin light chain (MLC) phosphorylation than in preparations stimulated by high K^+ for a given increase in $[\text{Ca}^{2+}]_i$ and that DPB induced contraction in the absence of external Ca^{2+} which is accompanied by an increase in neither $[\text{Ca}^{2+}]_i$ nor MLC phosphorylation. These results suggest that DPB has dual effects, increasing $[\text{Ca}^{2+}]_i$ and Ca^{2+} sensitivity of contractile elements. It is also suggested that Ca^{2+} sensitization is due not only to the increase in Ca^{2+} sensitivity of MLC phosphorylation but also to the activation of a mechanism that is not dependent on the increase in MLC phosphorylation. In the present experiments, we found that DPB potentiated the high K^+ -induced contraction without changing $[\text{Ca}^{2+}]_i$ in intact muscle and potentiated the Ca^{2+} -induced contraction in permeabilized muscle. All of the effects of DPB were abolished by pretreatment with TPA. These results suggest that the contraction and increase in Ca^{2+} sensitivity induced by DPB are due to activation of protein kinase C.

It has been shown that receptor agonists such as noradrenaline, prostaglandin $\text{F}_{2\alpha}$ and endothelin-1 caused greater contractions than those induced by high K^+ at a given $[\text{Ca}^{2+}]_i$ in rat aorta (Hori *et al.*, 1992). In permeabilized arterial muscles, it has also been reported that receptor agonists such as phenylephrine, noradrenaline and endothelin-1 enhance Ca^{2+} -induced contraction in the presence of GTP (Kitazawa *et al.*, 1989; 1991; Nishimura *et al.*, 1989; 1992; Anabuki *et al.*, 1990; Hori *et al.*, 1992). These receptor agonists induced contraction in the presence of the Ca^{2+} channel blocker, verapamil, or in the absence of external Ca^{2+} (with EGTA), and these contractions were not accompanied by an increase in MLC phosphorylation (Hori *et al.*, 1992). From these results, it has been suggested that receptor agonists have similar effects to phorbol esters: (1) increasing $[\text{Ca}^{2+}]_i$ and MLC phosphorylation, (2) increasing Ca^{2+} sensitivity of MLC phosphorylation, and (3) increasing Ca^{2+} sensitivity by activating a mechanism that is not dependent on MLC phosphorylation (Hori *et al.*, 1992).

Although receptor agonists and phorbol esters have similar effects in increasing Ca^{2+} sensitivity, we found that pretreatment with TPA selectively inhibited the effects of phorbol esters. After TPA-treatment, prostaglandin $\text{F}_{2\alpha}$ still induced greater contraction than high K^+ at a given $[\text{Ca}^{2+}]_i$. The maximally effective concentrations of noradrenaline and endothelin-1 also induced greater contractions than high K^+ . In addition, these agonists induced contractions in the absence of external Ca^{2+} . In the permeabilized muscle without protein kinase C activity, prostaglandin $\text{F}_{2\alpha}$ potentiated Ca^{2+} -induced contraction. These results suggest that Ca^{2+} sensitization induced by receptor agonists is not attributable to the activation of protein kinase C.

A difference between receptor agonists and phorbol esters has been reported using protein kinase C inhibitors. For example, calphostin C selectively inhibited the contraction induced by phorbol ester without changing the contraction induced by endothelin-1 (Shimamoto *et al.*, 1992) and calphostin C did not inhibit Ca^{2+} sensitization induced by acetylcholine in permeabilized guinea-pig stomach (Oishi *et al.*, 1992). In contrast, it has also been shown that various protein kinase C inhibitors inhibited the contraction and Ca^{2+} sensitization induced by receptor agonists (Heaslip & Sickels, 1989; Nishimura *et al.*, 1992; Murray *et al.*, 1992; Katsuyama & Morgan, 1993). This discrepancy may be explained as follows. The first possibility is that these could be a problem with the selectivity of the protein kinase C inhibitors; some protein kinase C inhibitors inhibit kinases other than protein kinase C (Garland *et al.*, 1987; Jefferson & Schulman, 1988), resulting in non-selective inhibition of smooth muscle contractions. The second possibility is that different agonists may

activate different signal transduction pathways. Murray *et al.* (1992) reported that contractions induced by phorbol 12, 13-dibutyrate and 5-hydroxytryptamine but not those induced by prostaglandin $\text{F}_{2\alpha}$ were inhibited by protein kinase C inhibitors, sphingosine and H-7, in rat basilar artery. The third possibility is that these are tissue differences. Although the increases in Ca^{2+} sensitivity in rat aorta due to prostaglandin $\text{F}_{2\alpha}$ and endothelin-1 did not seem to be mediated by protein kinase C (present results), protein kinase C inhibitors selectively inhibited Ca^{2+} sensitization induced by prostaglandin $\text{F}_{2\alpha}$ and endothelin-1 in permeabilized rabbit mesenteric artery (Nishimura *et al.*, 1992), rat basilar artery (Murray *et al.*, 1992) and ferret aorta (Katsuyama & Morgan, 1993).

From these results, we suggest that there are two pathways of Ca^{2+} sensitization in smooth muscle, a protein kinase C-dependent pathway and a protein kinase C-independent pathway. Although we did not determine whether receptor agonists activate protein kinase C in rat aorta, present results

suggest that this is not the case. Thus, it seems likely that phorbol esters selectively activate the protein kinase C-dependent pathways whereas receptor agonists selectively activate a protein kinase C-independent pathway in rat aorta. In other smooth muscles, however, some receptor agonists may generate enough diacylglycerol to activate protein kinase C to a level similar to that activated by phorbol esters and in turn also activate the protein kinase C-dependent pathway. Further studies are necessary to clarify the mechanisms of protein kinase C-dependent and protein kinase C-independent pathways of Ca^{2+} sensitization in smooth muscle.

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