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Role of protein kinase C in the endothelin-induced contraction in the rabbit saphenous vein

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Abstract

The role of protein kinase C in the endothelin-induced contraction was examined in the isolated rabbit saphenous vein in which endothelin-1, endothelin-3, sarafotoxin S6c and IRL 1620 (succinyl-[Glu^{9},$Ala^{11,15}$]endothelin-1-(8–21))-induced contraction at the threshold concentrations of 0.1–1 pM. A selective inhibitor of protein kinase C, 500 nM calphostin C (2-[12-[2-(benzyloxy)propyl]-3,10-dihydro-4,9-dihydroxy-2,6,7,11-tetramethoxy-3,10-dioxo-1-perylenyl]-1-methylethyl carbonic acid 4-hydroxyphenyl ester), shifted the concentration-response curves for these agonists to the right 7.4- to 109-fold. In the vein in which the endothelin ET_B receptor was desensitized, sarafotoxin S6c and IRL 1620 were ineffective whereas endothelin-1 and higher concentrations of endothelin-3 induced contractions by activating the endothelin ET_A receptor. Calphostin C (500 nM) shifted the concentration-response curves for endothelin-1 and endothelin-3 to the right more than 155-fold. Down-regulation of protein kinase C (by treatment with phorbol 12-myristate 13-acetate for 20 h) shifted the concentration-response curves for these agonists to the right before and after desensitization of the endothelin ET_B receptor 3.7- to 59-fold. In the permeabilized smooth muscle, Ca^{2+}-induced contraction was enhanced by endothelin-1, endothelin-3 and sarafotoxin S6c at concentrations much higher than those needed to induce contraction (threshold concentration was 3 nM). Calphostin C and down-regulation of protein kinase C shifted the concentration-response curves for endothelin-1 and endothelin-3 to the right and downwards without changing the effect of sarafotoxin S6c. In the permeabilized muscle in which the endothelin ET_B receptor was desensitized, endothelin-1 and endothelin-3 still augmented the Ca^{2+}-induced contraction. Calphostin C and down-regulation of protein kinase C shifted the concentration-response curves for endothelin-1 and endothelin-3 to the right and downwards. These results suggest that protein kinase C is involved in the contraction mediated by the endothelin ET_A and ET_B receptors; and Ca^{2+} sensitization mediated by the endothelin ET_A receptor is due to activation of protein kinase C whereas Ca^{2+} sensitization mediated by the endothelin ET_B receptor may be due not only to the activation of protein kinase C but also to other mechanisms.

Keywords: Endothelin; Endothelin receptor; Protein kinase C; Ca^{2+} sensitization; Smooth muscle, vascular

1. Introduction

Endothelin (Yanagisawa et al., 1988) binds to at least two types of receptors (Masaki et al., 1994), the endothelin ET_A (Arai et al., 1990) and the endothelin ET_B receptors (Sakurai et al., 1990). The endothelin ET_A receptor is selectively activated by endothelin-1 whereas the endothelin ET_B receptor is activated non-selectively by endothelin-1, endothelin-3, sarafotoxin S6c and IRL 1620. Both of these receptors mediate contraction in various types of smooth muscle (Rubanyi and Polokoff, 1994). Recently we have shown the co-existence of these two types of receptors both of which mediate contraction in the venous (Sudjarwo et al., 1994; Karaki et al., 1994) and tracheal smooth muscle (Yoneyama et al., 1995).

For arterial smooth muscle, it has been shown that stimulation of the endothelin ET_A receptor activates phospholipase C and increases the levels of inositol trisphosphate and diacylglycerol (Danthuluri and Brock, 1990) and these mobilize Ca^{2+} from the intracellular pool and activate protein kinase C, respec-

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tively. Because of the similarity between the slowly developing sustained contractions due to endothelin-1 and those due to phorbol esters in various vascular preparations, protein kinase C has been suggested to be involved in the endothelin-1-induced contraction (Griendling et al., 1989). The observation that the magnitude and duration of endothelin-1-induced contraction were reduced by the inhibitors of protein kinase C, staurosporine (Ohlstein et al., 1989; Danthuluri and Brock, 1990; Nishimura et al., 1992) and calphostin C (Shimamoto et al., 1992), also suggested the possible involvement of protein kinase C in the sustained component of endothelin-1-induced contraction.

Recently, in contrast, it has been demonstrated that the endothelin ET_B receptor, that mediates contraction in the swine pulmonary vein, is not coupled to Ca^{2+} release (Sudjarwo et al., 1995). It has also been shown that the contraction induced by endothelin-1 in the porcine coronary artery, that also contains the contractile endothelin ET_B receptor (Ihara et al., 1991), was linked to opening of the voltage-dependent Ca^{2+} channels through an inositol trisphosphate-independent pathway (Kasuya et al., 1992). These observations suggest that, unlike the smooth muscle endothelin ET_A receptor (Resink et al., 1990; Sudjarwo et al., 1995) or the endothelial ET_B receptor (Emori et al., 1990; Sudjarwo et al., 1992), the smooth muscle endothelin ET_B receptor has a signaling system that is independent of the inositol trisphosphate/Ca^{2+} release pathway. To further clarify the signal transduction pathway coupled to the endothelin ET_A and ET_B receptors in smooth muscle, we examined the effects of a selective inhibitor of protein kinase C, calphostin C (Kobayashi et al., 1989), and down-regulation of protein kinase C on the contraction of rabbit saphenous vein that has both endothelin ET_A and ET_B receptors.

2. Materials and methods

2.1. Tissue preparation and solution

Male New Zealand rabbits (2.5–3 kg) were killed by a sharp blow on the neck and exsanguination. The lateral saphenous vein was isolated, had fat and connective tissues carefully cleaned off and was cut into rings 2–3 mm wide. The endothelium was removed by gently rubbing the intimal surface with a glass rod moistened with oxygenated physiological salt solution that contained (mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.0, $NaHCO_3$ 23.8, ethylenediamine tetraacetic acid (EDTA) 0.01, and glucose 5.5. High-K^+ solution was made by replacing 72.4 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.

2.2. Muscle tension

The ring preparations were mounted on two parallel stainless steel wires in a tissue bath of 2.5 ml capacity. One wire was anchored to a stationary support and the other to a force-displacement transducer (Orientec, Tokyo, Japan) connected to an amplifier (Yokogawa, Tokyo, Japan). A resting tension of 10 mN was applied and the preparations were equilibrated for 1 h. The responses to 72.4 mM KCl were repeatedly measured at intervals of 30 min until a steady response was obtained. Endothelins and sarafotoxin S6c were added cumulatively. KCl was also added cumulatively to make final concentrations of 11.5, 21.5, 31.5, 41.5 and 51.5 mM. Concentration of a stimulant needed to induce a half-maximum contraction (EC_{50}) was calculated from the concentration-response relationship.

2.3. Desensitization of the endothelin ET_B receptor

The endothelin ET_B receptor was desensitized by treatment with 300 nM sarafotoxin S6c for 30 min followed by a wash with normal solution for 30 min for both intact and permeabilized preparations (Sudjarwo et al., 1994). Desensitization of the endothelin ET_B receptor was confirmed by the complete inhibition of the effect of subsequent application of sarafotoxin S6c or IRL 1620.

2.4. Down-regulation of protein kinase c

Protein kinase C activity was down-regulated by treatment with 1 μM phorbol 12-myristate 13-acetate in Dulbecco's modified Eagle medium with 10% fetal calf serum and penicillin/streptomycin (GIBCO, New York, USA) for 20 h at 37°C (Hori et al., 1993). Some of the preparations were treated with a solution without phorbol ester and were used as vehicle controls. The down-regulation of protein kinase C activity was assessed by determining whether the contraction induced by 12-deoxyphorbol 13-isobutyrate was completely inhibited.

2.5. Permeabilized smooth muscle

Helical strips of the rabbit saphenous vein (1 mm wide and 4 mm long) were permeabilized with 80 μg/ml *Staphylococcus aureus* α-toxin. The relaxing solution contained: potassium propionate 130 mM, $MgCl_2$ 4 mM, Na_2ATP 5 mM, Tris maleate (pH 6.8) 20 mM, EGTA 2 mM, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone 1 μM, creatine phosphate 2 mM, and creatine phosphokinase 10 U/ml. The free Ca^{2+} concentration was changed by adding an appropriate amount of $CaCl_2$. The apparent binding constant of EGTA for Ca^{2+} was considered to be 10^6

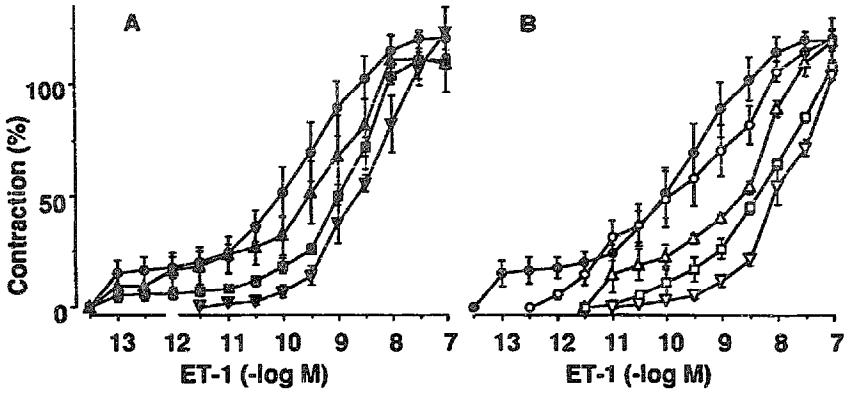

Fig. 1. Effects of calphostin C on the concentration-response curves for endothelin-1 in the rabbit saphenous vein. (A) Before desensitization of the endothelin ET_B receptor. ●: Control. ▲, ■ and ▼: Calphostin C 100, 300 and 500 nM, respectively. (B) After endothelin ET_B receptor desensitization. ●: Same control as (A). ○: After receptor desensitization. △, □ and ▽: Calphostin C 100, 300 and 500 nM, respectively. Calphostin C was added 60 min before the cumulative addition of endothelin-1. 100% represents the magnitude of the contraction induced by 72.4 mM KCl. Each point represents the mean of four to eight experiments and S.E.M is shown by a vertical bar.

Table 1
Concentration needed to induce a half maximum contraction (EC_{50}) for endothelin-1, endothelin-3, sarafotoxin S6c and IRL 1620 in the isolated rabbit saphenous vein

Condition	EC_{50} −log M ± S.E.M.	n
Endothelin-1 (control)	9.70 ± 0.30	8
+ 100 nM calphostin C	9.17 ± 0.36	4
+ 300 nM calphostin C	8.74 ± 0.10 [d]	4
+ 500 nM calphostin C	8.25 ± 0.16 [d]	4
+ 300 nM STXc [a]	10.02 ± 0.37	4
+ 300 nM STXc [a] + 100 nM calphostin C	8.39 ± 0.04 [d,e]	4
+ 300 nM STXc [a] + 300 nM calphostin C	8.08 ± 0.06 [d,e]	4
+ 300 nM STXc [a] + 500 nM calphostin C	7.81 ± 0.13 [d,e]	4
Endothelin-1 (vehicle control) [c]	9.31 ± 0.35	8
+ 1 μM TPA [b]	8.68 ± 0.16 [d,f]	4
+ 300 nM STXc [a]	8.78 ± 0.16 [d,f]	4
+ 300 nM STXc [a] + 1 μM TPA [b]	8.21 ± 0.03 [d,f]	4
Endothelin-3 (control)	9.35 ± 0.12	8
+ 100 nM calphostin C	9.29 ± 0.23	4
+ 300 nM calphostin C	8.74 ± 0.13 [d]	4
+ 500 nM calphostin C	8.48 ± 0.11 [d]	4
+ 300 nM STXc [a]	7.54 ± 0.14 [d]	4
+ 300 nM STXc [a] + 100 nM calphostin C	7.18 ± 0.11	4
+ 300 nM STXc [a] + 300 nM calphostin C	< 7 [d,e]	4
+ 300 nM STXc [a] + 500 nM calphostin C	< 7 [d,e]	4
Endothelin-3 (vehicle control) [c]	8.62 ± 0.13 [d]	8
+ 1 μM TPA [b]	7.93 ± 0.17 [d,f]	4
+ 300 nM STXc [a]	7.74 ± 0.11 [d,f]	4
+ 300 nM STXc [a] + 1 μM TPA [b]	< 7 [d,f]	4
Sarafotoxin S6c (control)	11.83 ± 0.10	8
+ 100 nM calphostin C	11.88 ± 0.08	4
+ 300 nM calphostin C	10.04 ± 0.40 [d]	4
+ 500 nM calphostin C	9.79 ± 0.20 [d]	4
Sarafotoxin S6c (vehicle control) [c]	11.25 ± 0.19 [d]	8
+ 1 μM TPA [b]	9.48 ± 0.12 [d,e]	4
IRL 1620 (control)	9.33 ± 0.28 [d]	4
+ 100 nM calphostin C	8.74 ± 0.16 [d]	4
+ 300 nM calphostin C	8.39 ± 0.05 [d]	4
+ 500 nM calphostin C	8.42 ± 0.10 [d]	4
IRL 1620 (vehicle control) [c]	8.79 ± 0.03 [d]	8
+ 1 μM TPA [b]	7.26 ± 0.09 [d,f]	4

[a] Endothelin ET_B receptor was desensitized by pretreatment with 300 nM sarafotoxin S6c (STXc). [b] Protein-kinase C was down-regulated by pretreatment with 1 μM phorbol 12-myristate 13-acetate for 20 h. [c] Vehicle control for [b]. [d] Significantly different from respective control with $P < 0.05$. [e] Significantly different from the muscle pretreated with sarafotoxin S6c with $P < 0.05$. [f] Significantly different from the vehicle control with $P < 0.05$. n: number of experiments.

M^{-1}. Force of contraction was measured with an isometric transducer under a resting force of 5 mN. Intracellular Ca^{2+} stores were depleted by treating the muscle with 10 μM ionomycin for 20 min (Nishimura et al., 1988). In a preliminary experiment, it was found that endothelin-1, endothelin-3 and sarafotoxin S6c augmented the Ca^{2+}-induced contraction only in the presence of guanosine triphosphate. Based on this result, all the experiments were done in the presence of 10 μM guanosine triphosphate.

2.6. Chemicals

Endothelin-1, endothelin-3 and sarafotoxin S6c were purchased from the Peptide Institute (Osaka, Japan). IRL 1620 (succinyl-[Glu^9,$Ala^{11,15}$]endothelin-1-(8–21)) was a gift from Dr. T. Okada, Ciba-Geigy Japan and *Staphylococcus aureus* α-toxin was a gift from Drs. I. Kato and K. Noda, Chiba University. Other chemicals used were calphostin C (2-[12-[2-(benzyloxy)propyl]-3,10-dihydro-4,9-dihydroxy-2,6,7,11-tetramethoxy-3,10-dioxo-1-perylenyl]-1-methylethyl carbonic acid 4-hydroxyphenyl ester) (Sigma Chemicals, St. Louis, MO, USA), phorbol 12-myristate 13-acetate, 12-deoxyphorbol 13-isobutyrate (Funakoshi, Tokyo, Japan) and guanosine triphosphate (Boehringer Mannheim Corp., New York, NY, USA).

2.7. Statistics

The results of the experiments are expressed as means ± S.E.M. The statistical significance of differences between points was determined with an unpaired Student's *t*-test and for those between concentration-response curves, by two-way analysis of variance. In some experiments, concentration-response curves were analyzed as reported by Tajimi et al. (1991) to find if the curve was composed of more than one component. $P < 0.05$ was taken as significant.

3. Results

3.1. Effects of calphostin C

Fig. 1A shows the concentration-response curve for endothelin-1 in the rabbit saphenous vein. Analysis of the curve suggested that it is composed of two phases; the first a high-affinity component with an EC_{50} of 13.14 ± 0.12 ($n = 8$) and the second, a low-affinity component with an EC_{50} of 9.45 ± 0.30 ($n = 8$). In the presence of 300 nM and 500 nM calphostin C, the concentration-response curve for endothelin-1 was shifted to the right, increasing the EC_{50} 9.1- and 28.1-fold, respectively (Table 1), whereas 100 nM calphostin C did not show a significant effect. In addition, calphostin C more strongly inhibited the effects of lower concentrations of endothelin-1 and changed a two-component curve to a single-component curve.

To examine the selectivity of calphostin C, effects of 500 nM calphostin C on the contractions induced by high K^+ were examined. Result indicated that calphostin C did not change the cumulative concentration-response curve for KCl (11.5–51.5 mM)($n = 4$, data not shown).

Fig. 2A shows the effects of cumulative addition of endothelin-3. Calphostin C (300 and 500 nM) shifted the concentration-response curves to the right, increasing the EC_{50} 4.0- and 7.4-fold, respectively, although 100 nM calphostin C was almost ineffective (Table 1). Analysis of the curve suggested that it was composed of two components with EC_{50} of 12.01 ± 0.20 ($n = 8$) and 9.08 ± 0.30 ($n = 8$), respectively. Calphostin C changed this to a single component by preferentially inhibiting the effects of lower concentrations of endothelin-3.

Fig. 2B shows the effects of cumulative addition of sarafotoxin S6c. At the concentrations of 1–100 pM, sarafotoxin S6c induced a much greater contraction than endothelin-1 or endothelin-3. At these concentra-

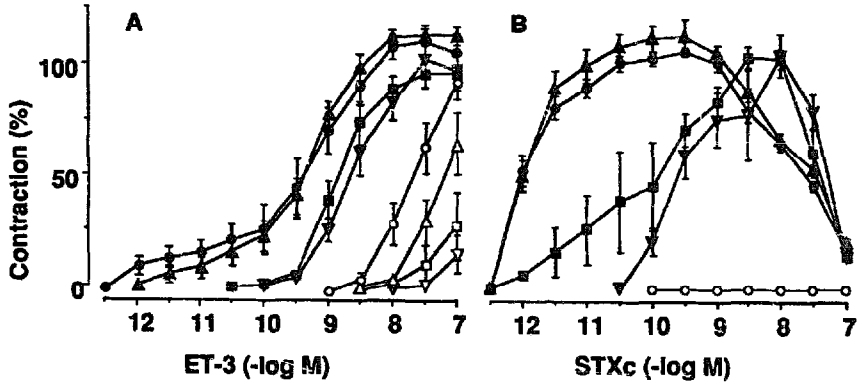

Fig. 2. Effects of calphostin C on the concentration-response curves for endothelin-3 (A) and sarafotoxin S6c (B) in the rabbit saphenous vein. Closed symbols: before desensitization of the endothelin ET_B receptor. ●: Control. ▲, ■ and ▼: Calphostin C 100, 300 and 500 nM, respectively. Open symbols: after endothelin ET_B receptor desensitization. ○: After receptor desensitization. △, □ and ▽: Calphostin C 100, 300 and 500 nM, respectively. For further explanation see Fig. 1.

tions, endothelin-1 and endothelin-3 activated the high affinity component and induced only small contractions. Higher concentrations of sarafotoxin S6c (> 100 pM) showed an inhibitory effect, yielding a bell-shaped concentration-response curve, as reported previously (Sudjarwo et al., 1994). Calphostin C (300 nM) shifted the concentration-response curve to the right and increased the EC_{50} 62-fold. The resulting concentration-response curve was quite similar to those for endothelin-1 or endothelin-3 except that higher concentrations of sarafotoxin S6c still showed an inhibitory effect (Fig. 2B). A higher concentration of calphostin C (500 nM) showed an additional inhibitory effect, increasing the EC_{50} 109-fold. However, 100 nM calphostin C was almost ineffective (Table 1).

Cumulative addition of IRL 1620 resulted in a graded contraction. The concentration-response curve for IRL 1620 was similar to that for sarafotoxin S6c in the presence of 300 nM calphostin C. The threshold concentration was 3 pM and the maximum was reached at 30 nM. However, 100 nM IRL 1620 showed an inhibitory effect, yielding a bell-shaped concentration-response curve, as reported previously (Sudjarwo et al., 1994). Calphostin C (100 and 300 nM) shifted the concentration-response curve to the right, increasing the EC_{50} 3.9- and 8.7-fold, respectively, although 500 nM calphostin C did not induce additional inhibition (Table 1).

3.2. Effects of calphostin C on the endothelin ET_B receptor-desensitized muscle

In the preparations in which the endothelin ET_B receptor was desensitized, the selective agonists of the endothelin ET_B receptor, sarafotoxin S6c (Fig. 2B) and IRL 1620 (data not shown), were not effective. In the receptor-desensitized preparations, the effects of low concentrations of endothelin-1 were inhibited without changes in the effects of higher concentrations and thus a two-component curve became a single-component curve (Fig. 1B). Calphostin C (100, 300 and 500 nM) shifted the concentration-response curve to the right, increasing the EC_{50} 43-, 88- and 163-fold, respectively (Table 1).

As shown in Fig. 2A and Table 1, the EC_{50} for endothelin-3 was increased 64-fold by desensitization of the endothelin ET_B receptor. Calphostin C (100, 300 and 500 nM) further shifted the concentration-response curves to the right.

3.3. Effect of down-regulation of protein kinase C

In the preparations pretreated with 1 μM phorbol 12-myristate 13-acetate for 20 h, the effect of 12-deoxyphorbol 13-isobutyrate to induce contraction was completely inhibited ($n = 24$, data not shown), suggesting that protein kinase C was down-regulated. In the protein kinase C-down-regulated preparations, the effects of lower concentrations of endothelin-1 were inhibited (Fig. 3A), increasing the EC_{50} 4.3-fold (Table 1) and changing a two-component curve into a single component curve. In the preparations pretreated with vehicle, in contrast, the contractile effect of endothelin-1 was not significantly affected (Table 1). Combination of the protein kinase C down-regulation and the endothelin ET_B receptor desensitization showed a stronger inhibitory effect than protein kinase C down-regulation alone (Fig. 3A), increasing the EC_{50} 12.5-fold (Table 1). However, the shift of the concentration-response curve was not parallel; the effects of lower concentrations of endothelin-1 were more strongly inhibited than the effects of higher concentrations.

Vehicle treatment increased the EC_{50} for endothelin-3 5.3-fold (Table 1). Down-regulation of protein kinase C had a greater inhibitory effect than vehicle, increasing the EC_{50} 26-fold compared to the control or 4.9-fold compared to the vehicle control (Fig. 3B and Table 1).

Vehicle treatment also increased the EC_{50} for sarafotoxin S6c and IRL 1620 3.8-fold and 3.5-fold,

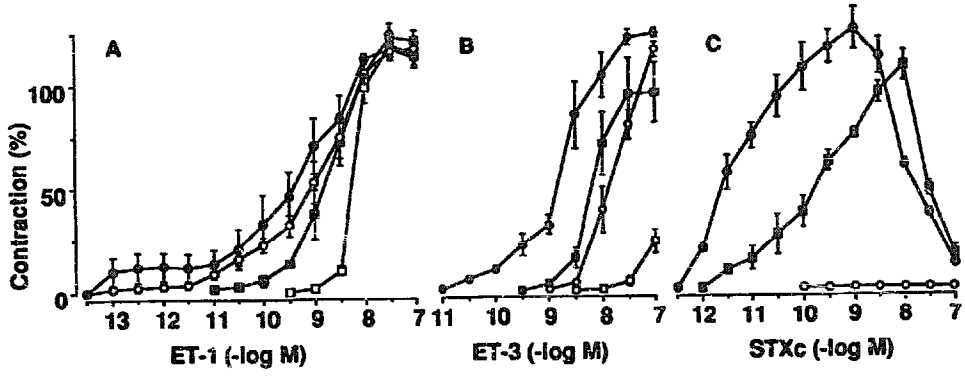

Fig. 3. Effects of down-regulation of protein kinase C (by pretreatment with phorbol ester) on the concentration-response curves for endothelin-1 (A), endothelin-3 (B) and sarafotoxin S6c (C) in the rabbit saphenous vein before and after the desensitization of the endothelin ET_B receptor. ●: Vehicle control. ○: After receptor desensitization. ■: After treatment with phorbol ester. □: Combination of receptor desensitization and phorbol ester pretreatment. For further explanation see Fig. 1.

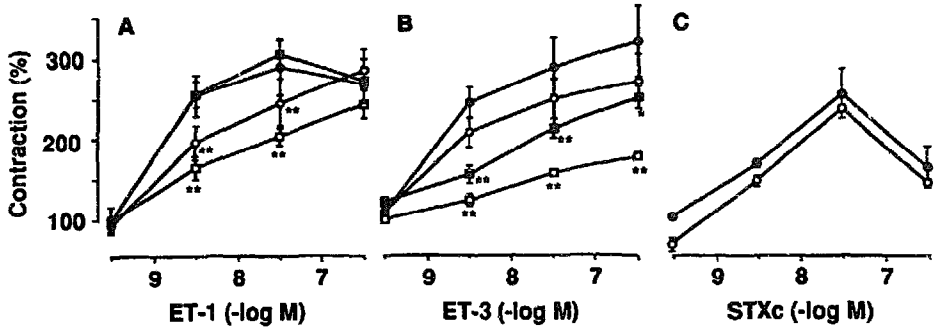

Fig. 4. Effects of calphostin C on the augmentation of Ca^{2+}-induced contraction induced by endothelin-1 (A), endothelin-3 (B) or sarafotoxin S6c (C) in the permeabilized rabbit saphenous vein. After the contraction induced by 300 nM Ca^{2+} reached a steady level, agonist was added cumulatively. ●: Control. ○: Calphostin C 500 nM. ■: After desensitization of the endothelin ET_B receptor. □: Combination of receptor desensitization and 500 nM calphostin C. 100% represents the magnitude of the contraction induced by 300 nM Ca^{2+}. Each point represents the mean of four to eight experiments and S.E.M. is shown by a vertical bar. * and ** Significantly different from control with $P < 0.05$ and 0.01, respectively.

respectively (Table 1). Down-regulation of protein kinase C had a greater inhibitory effect than vehicle, increasing the EC_{50} for sarafotoxin S6c 59-fold (Fig. 3C and Table 1) and IRL 1620 34-fold compared to the vehicle control (Table 1).

3.4. Permeabilized muscle

Application of 300 nM Ca^{2+}-induced contraction in the vein permeabilized with bacterial α-toxin. Endothelin-1, at approximately 30000 times higher concentrations (> 3 nM) than needed to induce contraction in intact muscle (> 0.1 pM), augmented the contraction induced by Ca^{2+}. Calphostin C (500 nM) inhibited the effect of lower concentrations of endothelin-1 to augment the Ca^{2+}-induced contraction (Fig. 4A). Endothelin ET_B receptor desensitization did not change the effect of endothelin-1. Calphostin C showed a similar inhibitory effect on the endothelin ET_B receptor-desensitized muscle to that on muscle without receptor desensitization.

Fig. 4B shows that endothelin-3 (> 3 nM) has an effect similar to that of endothelin-1 to augment the Ca^{2+}-induced contraction. This effect was not significantly inhibited by 500 nM calphostin C. Desensitization of the endothelin ET_B receptor inhibited the effect of endothelin-3 whereas combination of receptor desensitization and 500 nM calphostin C showed an additional inhibitory effect.

Fig. 4C shows that sarafotoxin S6c (> 3 nM) augmented the Ca^{2+}-induced contraction. Calphostin C (500 nM) did not change the effect of sarafotoxin S6c. Desensitization of the endothelin ET_B receptor completely abolished the effect of sarafotoxin S6c ($n = 4$, data not shown).

Fig. 5A shows that down-regulation of protein kinase C inhibited the effect of lower concentrations of endothelin-1, as was the case with calphostin C. Combination of desensitization of the endothelin ET_B receptor and down-regulation of protein kinase C had an effect similar to that of down-regulation of protein kinase C alone.

Fig. 5B shows that down-regulation of protein kinase C inhibited the effect of endothelin-3. Combina-

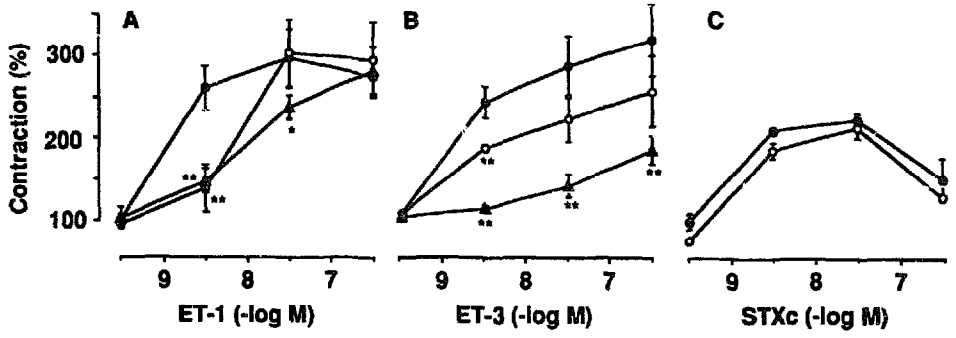

Fig. 5. Effects of down-regulation of protein kinase C on the augmentation of Ca^{2+}-induced contraction induced by endothelin-1 (A), endothelin-3 (B) or sarafotoxin S6c (C) in the permeabilized rabbit saphenous vein. After the contraction induced by 300 nM Ca^{2+} reached a steady level, agonist was added cumulatively. ●: Control. ○: Protein kinase C down-regulated. ▲: Combination of receptor desensitization and protein kinase C down-regulation. For further explanation see Fig. 4.

tion of desensitization of the endothelin ET_B receptor and down-regulation of protein kinase C showed an additional inhibitory effect.

Fig. 5C shows that down-regulation of protein kinase C did not inhibit the effect of sarafotoxin S6c.

4. Discussion

In the present experiments, we confirmed our previous finding in the rabbit saphenous vein that the concentration-response curve for endothelin-1 was composed of high- and low-affinity components (Karaki et al., 1994). Analyzing the ^{125}I-endothelin-3 binding in the rabbit saphenous vein, Gray et al. (1994) have also shown the presence of high- and low-affinity endothelin binding sites. Desensitization of the endothelin ET_B receptor selectively inhibited the high-affinity component of the concentration-response curve. These results support the suggestion that the high-affinity component for endothelin-1 is mediated by the endothelin ET_B receptor whereas the low affinity component is mediated by both the endothelin ET_A and ET_B receptors (Karaki et al., 1994; Sudjarwo et al., 1994).

An inhibitor of protein kinase C, calphostin C, shifted the concentration-response curve for endothelin-1 to the right in a concentration-dependent manner. Furthermore, calphostin C abolished the high-affinity component. Down-regulation of protein kinase C had an effect similar to that of calphostin C. These results suggest that lower concentrations of endothelin-1 activate protein kinase C through the endothelin ET_B receptor and induce contraction. After desensitization of the endothelin ET_B receptor, endothelin-1-induced contraction by activating the endothelin ET_A receptor. Calphostin C and down-regulation of protein kinase C shifted the concentration-response curves for endothelin-1 to the right. These results suggest that the contraction mediated by the endothelin ET_A receptor is also due to activation of protein kinase C.

The concentration-response curve for endothelin-3 also had two components. Desensitization of the endothelin ET_B receptor shifted the concentration-response curve for endothelin-3 to the right, suggesting that lower concentrations (> 1 pM) selectively activate the endothelin ET_B receptor whereas higher concentrations (> 3 nM) have an additional effect to activate the endothelin ET_A receptor. Calphostin C and down-regulation of protein kinase C showed an inhibitory effect similar to that of endothelin ET_B receptor desensitization, suggesting that the contraction mediated by the endothelin ET_B receptor is due to activation of protein kinase C. After desensitization of the endothelin ET_B receptor, calphostin C and down-regulation of protein kinase C still inhibited the contraction induced by endothelin-3. These results support the suggestion that the endothelin ET_A receptor is coupled to the activation of protein kinase C.

A selective agonist of the endothelin ET_B receptor, sarafotoxin S6c, induced greater contractions than endothelin-1, endothelin-3 or IRL 1620 at concentrations that stimulated the high-affinity component of the ET_B receptor. Calphostin C and down-regulation of protein kinase inhibited the contraction induced by sarafotoxin S6c and changed the concentration-response curves as they did to those for endothelin-1, endothelin-3 or IRL 1620. These results suggest that lower concentrations of sarafotoxin S6c (1–100 pM) activate more strongly the high-affinity endothelin ET_B receptor, inducing a greater contraction than endothelin-1, endothelin-3 or IRL 1620 and that this contraction is mediated by protein kinase C.

The concentration-response curve for another selective agonist of the endothelin ET_B receptor, IRL 1620, was similar to that for sarafotoxin S6c in the presence of 300 nM calphostin C. This suggests that IRL 1620 has weaker effect than sarafotoxin S6c to activate the high-affinity endothelin ET_B receptor. The effects of IRL 1620 were inhibited by calphostin C and desensitization of protein kinase C, again suggesting that the contractile endothelin ET_B receptor is coupled to protein kinase C.

From these results, we concluded that both the endothelin ET_A and ET_B receptors are coupled to activation of protein kinase C, possibly by generation of diacylglycerol. This conclusion agrees with the finding that the endothelin ET_A receptor is linked to phospholipase C (see Introduction). In contrast, the endothelin ET_B receptor in smooth muscle does not seem to activate the inositol trisphosphate/Ca^{2+} release pathway (see Introduction). One of the possible explanations is that this receptor is coupled to phospholipase D and, thus, generates diacylglycerol without generating inositol trisphosphate. Further experiments are necessary to examine this possibility.

It has been shown that both the endothelin ET_A and ET_B receptors are coupled to Ca^{2+} sensitization in intact arterial and venous smooth muscle and induce greater contraction at a given Ca^{2+} level (Sudjarwo et al., 1995). In the permeabilized mesenteric artery, furthermore, endothelin-1 increases Ca^{2+} sensitivity through activation of both protein kinase C-dependent and -independent mechanisms (Hori et al., 1993; Yoshida et al., 1994). To find if protein kinase C plays a role in Ca^{2+} sensitization, we examined the effects of endothelins and sarafotoxin S6c on the permeabilized preparation. It was found that endothelin-1, endothelin-3 and sarafotoxin S6c augment the Ca^{2+}-induced contraction. However, Ca^{2+} sensitivity was increased by concentrations of the stimulants more than 3000-fold higher (threshold concentration of approximately 3 nM) than needed to induce contraction (threshold concen-

tration of 0.1–1 pM). Desensitization of the endothelin ET_B receptor did not change the effect of endothelin-1, partially inhibited the effect of endothelin-3 and completely inhibited the effect of sarafotoxin S6c. These results support the suggestion that both the endothelin ET_A and ET_B receptors mediate Ca^{2+} sensitization (Sudjarwo et al., 1995).

Calphostin C and down-regulation of protein kinase C inhibited the effects of endothelin-1 and endothelin-3 but not of sarafotoxin S6c. In the endothelin ET_B receptor-desensitized muscle, calphostin C and down-regulation of protein kinase C still inhibited the effects of endothelin-1 and endothelin-3. These results suggest that protein kinase C is involved in the endothelin ET_A receptor-mediated Ca^{2+} sensitization but not in the endothelin ET_B receptor-mediated Ca^{2+} sensitization. This result is surprising because stimulation of the endothelin ET_B receptor activated protein kinase C and induced contraction in intact smooth muscle. Since it has been shown that Ca^{2+} sensitization in smooth muscle is mediated by protein kinase C-dependent and -independent mechanisms (Hori et al., 1993), it is suggested that the endothelin ET_B receptor is coupled to both these pathways and, thus, inhibition of only the protein kinase C pathway did not inhibit Ca^{2+} sensitization. Further experiments are necessary to examine this possibility.

From these results, it is concluded that protein kinase C is involved in the contraction and the Ca^{2+} sensitization induced by endothelins in the rabbit saphenous vein.

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References

Arai, H., S. Hori, I. Aramori, H. Ohkubo and S. Nakanishi, 1990, Cloning and expression of a cDNA encoding an endothelin receptor, Nature 348, 730.

Danthuluri, N.R. and T.A. Brock, 1990, Endothelin receptor coupling mechanisms in vascular smooth muscle: a role for protein kinase C, J. Pharmacol. Exp. Ther. 254, 393.

Emori, T., Y. Hirata and F. Marumo, 1990, Specific receptors for endothelin-3 in cultured bovine endothelial cells and its cellular mechanism of action, FEBS Lett. 263, 261.

Gray, G.A., B.-M. Loffler and M. Clozel, 1994, Characterization of endothelin receptors mediating contraction of rabbit saphenous vein, Am. J. Physiol. 266, H959.

Griendling, K.K., T. Tsuda and R.W. Alexander, 1989, Endothelin stimulates diacylglycerol accumulation and activates protein kinase C in cultured vascular smooth muscle, J. Biol. Chem. 264, 8237.

Hori, M., K. Sato, S. Miyamoto, H. Ozaki and H. Karaki, 1993, Different pathways of calcium sensitization activated by receptor agonists and phorbol esters in vascular smooth muscle, Br. J. Pharmacol. 110, 1527.

Ihara, M., T. Fukuroda, T. Saeki, M. Nishikibe, K. Kojiri, H. Suda and M. Yano, 1991, An endothelin receptor (ET_A) antagonist isolated from Streptomyces misakiensis, Biochem. Biophys. Res. Commun. 178, 132.

Karaki, H., S.A. Sudjarwo and M. Hori, 1994, Novel antagonist of endothelin ETB1 and ETB2 receptors, BQ-788: effects on blood vessel and small intestine, Biochem. Biophys. Res. Commun. 205, 168.

Kasuya, Y., Y. Takuwa, M. Yanagisawa, T. Masaki and K. Goto, 1992, A pertussis toxin sensitive mechanism of endothelin action in porcine coronary artery smooth muscle, Br. J. Pharmacol. 107, 456.

Kobayashi, E., H. Nakano, M. Morimoto and T. Tamaoki, 1989, Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C, Biochem. Biophys. Res. Commun. 159, 548.

Masaki, T., J.R. Vane and P.M. Vanhoutte, 1994, International Union of Pharmacology nomenclature of endothelin receptors, Pharmacol. Rev. 46, 137.

Nishimura, J., M. Kolber and C. Van Breemen, 1988, Norepinephrine and GTPgS increase myofilament Ca^{2+} sensitivity in α-toxin permeabilized arterial smooth muscle, Biochem. Biophys. Res. Commun. 157, 677

Nishimura, J., S. Moreland, H.Y. Ahn, T. Kawase, R.S. Moreland and C. Van Breemen, 1992, Endothelin increases myofilament Ca^{2+} sensitivity in α-toxin-permeabilized rabbit mesenteric artery, Circulation Res. 71, 951.

Ohlstein, E.H., S. Horohonich and D.W.P. Hay, 1989, Cellular mechanism of endothelin in rabbit aorta, J. Pharmacol. Exp. Ther. 250, 548.

Resink, T.J., T. Scott-Burden, C. Boulanger, E. Weber and F. Buhler, 1990, Internalization of endothelin by cultured human vascular smooth muscle cells: characterization and physiological significance, Mol. Pharmacol. 38, 244.

Rubanyi, G.M. and M.A. Polokoff, 1994, Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology, Pharmacol. Rev. 46, 325.

Sakurai, T., M. Yanagisawa, Y. Takuwa, H. Miyazaki, S. Kimura, K. Goto and T. Masaki, 1990, Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor, Nature 348, 732.

Shimamoto, H., Y. Shimamoto, C. Kwan and E.E. Daniel, 1992, Participation of protein kinase C in endothelin-1-induced contraction in rat aorta: studies with new tool, calphostin C, Br. J. Pharmacol. 107, 282.

Sudjarwo, S.A., M. Hori and H. Karaki, 1992, Effects of endothelin-3 on cytosolic calcium level in vascular endothelium and on smooth muscle contraction, Eur. J. Pharmacol. 229, 137.

Sudjarwo, S.A., M. Hori, T. Tanaka, Y. Matsuda, T. Okada and H. Karaki, 1994, Subtypes of endothelin ET_A and ET_B receptors mediating venous smooth muscle contraction, Biochem. Biophys. Res. Commun. 200,627.

Sudjarwo, S.A., M. Hori, T. Tanaka, Y. Matsuda and H. Karaki, 1995, Coupling of the endothelin ET_A and ET_B receptors to calcium mobilization and calcium sensitization in the artery and vein, Eur. J. Pharmacol. Mol. Pharmacol. Sect. 289, 197.

Tajimi, M., H. Ozaki, K. Sato and H. Karaki, 1991, Effect of a novel inhibitor of cyclic AMP phosphodiesterase, E-1020, on cytosolic Ca^{2+} level and contraction in vascular smooth muscle, Naunyn-Schmied. Arch Pharmacol. 344, 602.

Yanagisawa, M., H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, K. Goto and T. Masaki, 1988, A novel potent vasoconstrictor peptide produced by vascular endothelial cells, Nature 332, 411.

Yoneyama, T., M. Hori, M. Makatani, T. Yamamura, T. Tanaka, Y. Matsuda and H. Karaki, 1995, Subtypes of endothelin ET_A and ET_B receptors mediating tracheal smooth muscle contraction, Biochem. Biophys. Res. Commun. 207, 668.

Yoshida, M., A. Suzuki and T. Itoh, 1994, Mechanisms of vasoconstriction induced by endothelin-1 in smooth muscle of rabbit mesenteric artery, J. Physiol. 477, 253.