Contraction of rat thoracic aorta strips by endothelin-1 in the absence of extracellular Ca^{2+}

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1 Endothelin-1 (ET-1) caused a concentration-dependent contraction of helical strips from rat thoracic aorta in the absence of extracellular Ca^{2+} . The Ca^{2+} -depleted muscle strips, prepared by three repeated applications of 10^{-2} M caffeine or 10^{-6} M noradrenaline in Ca^{2+} -free buffer, were contracted by 10^{-8} M ET-1 in the same manner as non-treated strips.

2 In the absence of extracellular Ca^{2+} , 10^{-7} M phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, induced a small but sustained contraction of the rat thoracic aorta strips within 60 min. Preincubation of the strips with 10^{-7} M PMA for 60 min in Ca^{2+} -free buffer, did not affect the 10^{-8} M ET-1-induced contraction, but decreased the 5×10^{-8} M phorbol 12,13-dibutyrate (PDB), or the 10^{-7} M PMA-induced contraction, and potentiated the contraction induced by 10^{-8} M urotensin II. Preincubation with 10^{-8} M ET-1 (which induced maximum contraction) for 25 min in Ca^{2+} -free buffer did not change the subsequent contraction induced by PMA (10^{-7} M) or urotensin II (10^{-8} M) but gave a somewhat lower maximum tension than in non-treated strips.

3 Calyculin-A, a potent inhibitor of phosphatase, also induced a contraction of the Ca^{2+} -depleted muscle strips in Ca^{2+} -free buffer. Preincubation of the strips with ET-1 (10^{-8} M) or PMA (10^{-7} M) decreased the calyculin-A (3×10^{-8} M)-induced contraction.

4 These results suggest that ET-1 may induce phosphorylation of an unknown protein either without an increase in myoplasmic Ca^{2+} concentration or, alternatively, with mobilization of intracellular Ca^{2+} from noradrenaline- and caffeine-insensitive Ca^{2+} sources, through a mechanism different from that of phorbol ester.

Keywords: Endothelin; phorbol 12-myristate 13-acetate; phorbol 12,13-dibutyrate; urotensin II; calyculin-A; intracellular Ca^{2+}

Introduction

Endothelin (ET), a peptide originally isolated from the culture medium of porcine endothelial cells, is one of the most potent vasoconstrictor substances known (Yanagisawa *et al.*, 1988). Several mechanisms have been proposed to explain the potent vasoconstrictor properties of ET. Early studies postulated that ET mediated its vasoconstrictor effect solely through activation of voltage-dependent Ca^{2+} channels (Yanagisawa *et al.*, 1988; Goto *et al.*, 1989). Subsequent studies indicated that the contractile response to ET consists of two components, one independent of Ca^{2+} influx, the other dependent on Ca^{2+} -influx and insensitive to voltage-dependent Ca^{2+} channel blockers (Auguet *et al.*, 1988; Blackburn & Highsmith, 1990; Bolger *et al.*, 1990). In some instances, ET initiates the metabolism of inositol phosphates and releases intracellularly stored Ca^{2+} (Van Renterghem *et al.*, 1988; Marsden *et al.*, 1989; Sugiura *et al.*, 1989; Takuwa *et al.*, 1990). In aortic smooth muscle cells derived from Wistar rats, ET appears to have no effect on phospho-inositide turnover (Hirata *et al.*, 1988). It has also been suggested that ET causes a stimulation of the diacylglycerol/protein kinase C pathway and induces contraction of vascular smooth muscle (Griendling *et al.*, 1989; Lee *et al.*, 1989; Sugiura *et al.*, 1989). Kodama *et al.* (1989) showed that ET induced contractions in Ca^{2+} -depleted muscle strips from porcine coronary artery, pretreated with caffeine and histamine in Ca^{2+} -free buffer, without intracellular Ca^{2+} mobilization and the contraction was inhibited by an inhibitor of protein kinase C.

To elucidate the mechanism responsible for the vasoconstrictor effects of ET in Ca^{2+} -free buffer, we examined the interactions between the contractile responses of helical strips

of rat thoracic aorta induced by ET-1 and those induced by other agents, such as noradrenaline, caffeine, phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDB), urotensin II and calyculin-A, all of which cause contractions, through different mechanisms, in the absence of extracellular Ca^{2+} .

Methods

Recording of contractile activity

Male Wistar rats (150–250 g) were anaesthetized with sodium pentobarbitone (50 mg kg^{-1} , i.p.; Abbott Laboratories, North Chicago, U.S.A.) and decapitated to obtain the descending thoracic aorta. Helically cut strips, 1.2 mm in width and 12 mm in length, were prepared from the aorta after removal of connective tissues and were suspended vertically in a 5 ml organ bath under 1.2 g tension in Krebs Henseleit solution of the following composition (in mM): NaCl 115, KCl 4.7, CaCl_2 2.5, MgCl_2 1.2, NaHCO_3 25.0, KH_2PO_4 1.2 and dextrose 10.0. The bath medium was maintained at 37°C and bubbled with a mixture of 95% O_2 and 5% CO_2 . The vascular strips were allowed to equilibrate for 90 min before starting experiments. During the equilibration period the bath solution was replaced every 20 min. Changes in muscle tension were recorded isometrically through force displacement transducer (Nihon Kohden, TB-651T). Ca^{2+} -free Krebs Henseleit solution was made by adding 1 mM ethylene glycol-bis (beta-aminoethylether) N,N'-tetraacetic acid (EGTA) instead of 2.5 mM CaCl_2 . Endothelial cells were removed by repeatedly rubbing the aortic strips with intimal surface down over a sheet of filter paper moistened with Krebs Henseleit solution (Furchgott & Zawadzki, 1980). Each strip was subjected only to one ET-1 treatment. All responses are expressed as

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Figure 1 Chart records showing typical effects of endothelin-1 (ET-1) on rat thoracic aorta in the absence of extracellular Ca^{2+} . Aortic strips were preincubated with Ca^{2+} -free buffer containing 1 mM EGTA for 15 min and then ET-1 was added. (a) Contraction induced by cumulative additions of ET-1 in Ca^{2+} -free buffer. (b) ET-1 10^{-8} M-induced contraction of aortic strips in Ca^{2+} -free buffer; ($n = 6$).

means $\pm$ s.e. The data were analysed by use of unpaired Student's *t* test for two means; a value of $P < 0.05$ was considered statistically significant.

Reagents

Endothelin-1 (ET-1) was purchased from Peptide Institute, Osaka, Japan. Urotensin II was generously provided by Dr J. Rivier (Salk Institute, La Jolla, Ca, U.S.A.). Indomethacin, ibuprofen, tetrodotoxin, nitroprusside, prazosin, phorbol 12-myristate 13-acetate (PMA) and EGTA were purchased from Sigma Chemical Co. Noradrenaline (free base), atropine sulphate monohydrate, acetylcholine chloride, papaverine hydrochloride, caffeine, phorbol 12,13-dibutyrate (PDB) and calyculin-A were purchased from Wako Pure Chemical Industries, Ltd. O-2'-dibutyladenosine 3',5'-cyclic monophosphate (dibutyl cyclic AMP) was donated by Daiichi Pharmaceutical Corp. Tokyo, Japan. Ketanserin tartrate was donated by Kyowa Hakko Kogyo Co. Ltd Tokyo, Japan. All other chemicals were of reagent grade or better. The solvent solutions were as follows: 0.25% acetic acid for ET-1 and urotensin II; 100% dimethyl sulphoxide (DMSO) for PMA (10^{-3} M), indomethacin (5×10^{-1} M), ibuprofen (1 M) and calyculin-A (10^{-3} M). The final concentration of DMSO in the muscle bath was never $>0.1\%$ at which concentration it caused no significant effect on the tension.

Results

Endothelin-1-induced contractile response in the absence of extracellular Ca^{2+} and effect of endothelial cells on the contraction

The cumulative contractile effects of ET-1 on an isolated strip of rat descending thoracic aorta in Ca^{2+} -free buffer containing 1 mM EGTA are illustrated in Figure 1a. The minimum effective concentration of ET-1 was 10^{-10} M and the maximum contractile responses was attained at a concentration of 10^{-8} M. In the absence of extracellular Ca^{2+} , 10^{-8} M ET-1 induced a slowly developing contraction and reached

maximum tension (170.8 ± 17.8 mg, $n = 7$) within 25 min, but the tension gradually declined. In the presence of extracellular Ca^{2+} , 10^{-8} M ET-1 induced a sustained contraction (636.0 ± 22.6 mg, $n = 7$, data not shown). Table 1 summarizes the effects of acetylcholine on noradrenaline-precontracted strips and the ET-1-induced contractions in the absence and presence of endothelial cells. The effects of ET-1 on rubbed or unrubbed aortic strips were not significantly different.

Effects of various agents on endothelin-1-induced contractions

Table 2 summarizes the effects of preincubation for 5 min with various agents on the contraction induced by 10^{-8} M ET-1 in Ca^{2+} -free buffer. In each experiment, we used two strips which were contracted by 10^{-7} M noradrenaline in normal Krebs-Henseleit solution. One strip was then preincubated with one of the agents for 5 min in Ca^{2+} -free buffer and the other strip was used as a control. The two strips were equally reactive to noradrenaline. Nitroprusside, papaverine and dibutyl cyclic AMP (which modify the level of cyclic nucleotides in smooth muscle cells) decreased the contractile response to ET-1. High concentrations of indomethacin (5×10^{-4} M), a non-steroidal anti-inflammatory agent, also inhibited the ET-1-induced contraction. However, another non-steroidal anti-inflammatory drug, ibuprofen (even at the high concentration of 10^{-3} M) had no effects on the contraction. Atropine (10^{-6} M), tetrodotoxin (2×10^{-6} M), ketanserin (10^{-6} M), prazosin (10^{-6} M) and verapamil (10^{-5} M) did not change the contractile response to 10^{-8} M ET-1 in Ca^{2+} -free buffer (data not shown).

Effects of endothelin-1 on Ca^{2+} -depleted aortic strips

After 15 min incubation in Ca^{2+} -free buffer, 10^{-2} M caffeine induced a biphasic contraction, which consisted of a small transient contraction followed by relaxation (Figure 2a). Washing with Ca^{2+} -free buffer caused recovery of the strip to the resting tension. The second and third applications of caffeine to the strip in Ca^{2+} -free buffer produced only relaxations. Noradrenaline (10^{-6} M) induced a typical phasic contraction and the second and third application of nor-

Table 2 Inhibition by various agents of 10^{-8} M endothelin-1 (ET-1)-induced contraction in Ca^{2+} -free buffer

Agents	n	Inhibition (%)
Nitroprusside (10^{-7} M)	5	$75.6 \pm 3.5^{***}$
Papaverine (3×10^{-5} M)	4	$63.7 \pm 2.6^{***}$
Dibutyl cyclic AMP (10^{-4} M)	4	$54.7 \pm 3.6^{***}$
Indomethacin (5×10^{-4} M)	4	$35.3 \pm 6.9^*$
Ibuprofen (10^{-3} M)	4	5.1 ± 3.2 NS

Values are means $\pm$ s.e. NS, no significant difference.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (compared with the responses in the absence of agents). The 10^{-8} M ET-1-induced maximum contractile tension in the absence of the agents was 172.0 ± 13.3 mg.

Table 1 Effects of removal of endothelial cells on endothelin-1 (ET-1)-induced contraction in helical strips of rat thoracic aorta in the absence of extracellular Ca^{2+}

Endothelial cells	n	Effects of acetylcholine (10^{-6} M) on noradrenaline (10^{-7} M)-precontracted strips	ET-1-induced contraction	
			EC_{50} (nM)	Maximum tension (mg)
Present	6	% relaxation (74.7 ± 5.5)	2.7 ± 0.6	145.5 ± 13.2
Absent	6	No relaxation	3.3 ± 0.1	160.0 ± 17.3

n, number of determinations. EC_{50} , concentration of ET-1 producing half maximum contraction. Values are means $\pm$ s.e. Noradrenaline (10^{-7} M)-induced responses in the presence and absence of endothelial cells were 480.8 ± 34.1 and 496.1 ± 24.2 mg, respectively. Endothelial cells were removed by rubbing aortic strips with intimal surface down, over a sheet of filter paper moistened with Krebs-Henseleit solution.

Figure 2 Typical chart recordings of endothelin-1 (ET-1, 10^{-8} M)-induced contraction in Ca^{2+} -depleted strips after three repeated applications of 10^{-2} M caffeine (CF) (a) or 10^{-6} M noradrenaline (NA) (b) in Ca^{2+} -free buffer; (▼) washed with Ca^{2+} -free buffer.

adrenaline in Ca^{2+} -free buffer produced only small and tonic contractions (Figure 2b). Such Ca^{2+} -depleted strips were also contracted by 10^{-8} M ET-1 in the same manner as untreated strips in the absence of extracellular Ca^{2+} (Figure 2 and Table 3).

Interactions between the agonists which induce contraction in the absence of extracellular Ca^{2+}

In the absence of extracellular Ca^{2+} , 10^{-7} M PMA induced a small but sustained contraction within 60 min. Precontraction induced by 10^{-7} M PMA did not change the 10^{-8} M ET-1-induced contraction, but decreased the contractile tension induced by 5×10^{-8} M PDB or 10^{-7} M PMA. Furthermore, pretreatment with 10^{-7} M PMA potentiated 10^{-8} M urotensin II-induced contraction. The results of these experiments are shown in Figure 3 and Table 4. The maximum contractions induced by 10^{-7} M PMA or 10^{-8} M urotensin II in the 10^{-8} M ET-1-precontracted strips were not significantly different from the response of untreated strips, but the maximum contractions produced by PMA or urotensin II in the ET-1-treated strips were somewhat lower than those in the untreated strips (Figure 4).

Calyculin-A a potent inhibitor of phosphatase, induced contraction in the absence of extracellular Ca^{2+} . Contraction induced by 10^{-7} M and 3×10^{-8} M calyculin-A required about 30 min and 110 min, respectively, to reach maximum tension (Figure 5a,b). The 3×10^{-8} M calyculin-A-induced increase in tension of aortic strips precontracted with 10^{-7} M PMA or 10^{-8} M ET-1 in Ca^{2+} -free buffer was lower than that developed in untreated strips (Figure 5 and Table 5). Conversely, preincubation with 3×10^{-8} M calyculin-A decreased the 10^{-8} M ET-1- or 10^{-7} M PMA-induced contraction (Table 6). Table 7 summarizes the effects of 10^{-7} M calyculin-A on Ca^{2+} -depleted muscle strips, prepared by three applications of 10^{-2} M caffeine or 10^{-6} M noradrenaline in Ca^{2+} -free buffer. The maximum contractile tensions and the times to reach the maximum contractions obtained with 10^{-7} M calyculin-A in the caffeine or noradrenaline pretreated strips were not significantly different from those in the untreated strips.

Figure 3 Chart records showing the effects of cumulative applications of endothelin-1 (ET-1, 10^{-8} M, $n = 6$) (a); phorbol 12,13-dibutyrate (PDB, 5×10^{-8} M, $n = 6$) (b); phorbol 12-myristate 13-acetate (PMA, 10^{-7} M, $n = 4$) (c); or urotensin II (UII, 10^{-8} M, $n = 5$) (d) on aortic strips precontracted with 10^{-7} M PMA. DMSO, 0.01% dimethyl sulphoxide. W (▼), washed with Ca^{2+} -free buffer.

Figure 4 Typical chart records of the effects of the cumulative addition of phorbol 12-myristate 13-acetate (PMA 10^{-7} M) (a) or urotensin II (UII 10^{-8} M) (b) on aortic strips precontracted with endothelin-1 (ET-1 10^{-8} M). PMA 10^{-7} M-induced contractile tension in the non-treated strips and in the ET-1-precontracted strips were 165 ± 20.6 mg ($n = 5$) and 122.4 ± 26.2 mg ($n = 6$), respectively. 10^{-8} M Urotensin II-induced contractile tension in the untreated strips and in the ET-1-precontracted strips were 36.8 ± 5.3 mg ($n = 6$) and 26.0 ± 3.0 mg ($n = 6$), respectively. W (▼), washed with Ca^{2+} -free buffer.

Table 3 Endothelin-1 (ET-1 10^{-8} M)-induced contraction of Ca^{2+} -depleted muscle strips

Treatments	n	Time to reach maximum contraction (min)	ET-1 (10^{-8} M)-induced maximum contractile tension (mg)
Preincubation for 15 min in Ca^{2+} -free buffer	6	21.1 ± 1.3	185.0 ± 12.8
Repeated applications of 10^{-2} M caffeine in Ca^{2+} -free buffer	7	24.1 ± 0.7	172.0 ± 15.8
Repeated applications of 10^{-6} M noradrenaline in Ca^{2+} -free buffer	6	21.6 ± 1.0	172.8 ± 18.0

n , number of experiments. Values are means $\pm$ s.e. NS, no significant difference.

Table 4 Effects of endothelin-1 (ET-1), phorbol 12,13-dibutyrate (PDB), phorbol 12-myristate 13-acetate (PMA) or urotensin II on aortic strips precontracted with 10^{-7} M PMA

Treatments	Tension (mg)			
	ET-1 (10^{-8} M)	PDB (5×10^{-8} M)	PMA (10^{-7} M)	Urotensin II (10^{-8} M)
Control	170.8 ± 17.8 (6)	190.8 ± 13.0 (6)	146.1 ± 20.1 (4)	29.1 ± 2.0 (6)
10^{-7} M PMA	193.1 ± 8.8 (6) NS	111.6 ± 11.0 (6)	28.7 ± 7.1 (4)	71.5 ± 9.8 (6)

Aortic strips were preincubated with Ca^{2+} -free buffer containing 1 mM EGTA and then 0.01% DMSO or 10^{-7} M PMA was added to the bath. DMSO 0.01% had no effect on the strips and 10^{-7} M PMA induced a sustained contraction (161.3 ± 12.5 mg, $n = 22$) within 60 min. After 60 min of the pretreatment, 10^{-8} M ET-1, 5×10^{-8} M PDB, 10^{-7} M PMA or 10^{-8} M urotensin II were added cumulatively. NS, no significant difference. ** $P < 0.01$; *** $P < 0.001$, compared with control strips. Values are means $\pm$ s.e. Number of determinations in parentheses.

Figure 5 Typical chart records of the effects of calyculin-A (CL-A) on untreated aortic strips (a, $n = 5$; b, $n = 6$); on phorbol 12-myristate 13-acetate (PMA, 10^{-7} M) precontracted strips (d, $n = 5$) or on endothelin-1 (ET-1, 10^{-8} M)-precontracted strips (c, $n = 4$) in the absence of extracellular Ca^{2+} . W (▼), washed with Ca^{2+} -free buffer.**Table 5** Effects of calyculin-A (3×10^{-8} M) on aortic strips precontracted with phorbol 12-myristate 13-acetate (PMA) (10^{-7} M) or endothelin-1 (ET-1) (10^{-8} M)

Treatments	n	Calyculin-A (3×10^{-8} M)-induced contraction (mg)
Control	6	198.3 ± 23.0
PMA (10^{-7} M)	4	10.0 ± 6.3
ET-1 (10^{-8} M)	5	58.0 ± 4.8

After 10^{-8} M ET-1- or 10^{-7} M PMA-induced contraction reached maximum tension, 3×10^{-8} M calyculin-A was added. Values are means $\pm$ s.e. *** $P < 0.001$, compared with the response of untreated strips.

Table 6 Effects of endothelin-1 (ET-1, 10^{-8} M) or phorbol 12-myristate 13-acetate (PMA, 10^{-7} M) on aortic strips precontracted with calyculin-A (3×10^{-8} M)

Treatments	n	Tension (mg)	
		ET-1 (10^{-8} M)	PMA (10^{-7} M)
Control	5	190.0 ± 14.8 **	19.0 ± 9.2 **
Calyculin-A	4	75.0 ± 19.3 **	77.0 ± 18.7 **

Aortic strips were preincubated with Ca^{2+} -free buffer containing 1 mM EGTA for 15 min and then 0.03% DMSO (control) or 3×10^{-8} M calyculin-A was added to the bath. DMSO 0.03% had no effect on the strips and 3×10^{-8} M calyculin-A induced a maximum contraction within 110 min. After 110 min of the pretreatment, 10^{-8} M ET-1 or 10^{-7} M PMA was added cumulatively. ** $P < 0.01$, compared with control strips. Values are means $\pm$ s.e. n, number of determinations.

Discussion

In the present study, we examined the effects of ET-1 on rat thoracic aorta strips, focusing on the mechanisms of ET-1-induced contraction in the absence of extracellular Ca^{2+} . It

has been reported that caffeine and noradrenaline release Ca^{2+} from intracellular stores of vascular smooth muscle through different mechanisms, and thus induce contractions in the absence of extracellular Ca^{2+} (Itoh *et al.*, 1983). Moreover, an overlap between an ET-sensitive intracellular Ca^{2+} store and caffeine-sensitive pool in vascular smooth muscle has been shown (Kai *et al.*, 1989). It has also been shown that ET, like noradrenaline, stimulates phospholipase C to produce inositol triphosphate and to mobilize intracellular Ca^{2+} in vascular smooth muscle (Marsden *et al.*, 1988; Van Renterghem *et al.*, 1988; Sugiura *et al.*, 1989; Takuwa *et al.*, 1990). However, our findings have shown that the Ca^{2+} -depleted muscle strips, after three repeated applications of caffeine or noradrenaline in Ca^{2+} -free buffer, were contracted by 10^{-8} M ET-1 in the same manner as untreated strips (Table 3 and Figure 2). These results suggest that the ET-1-induced contraction in Ca^{2+} -free buffer may not require an increase in intracellular Ca^{2+} concentration or, alternatively, it may be induced by intracellular Ca^{2+} from noradrenaline- and caffeine-insensitive Ca^{2+} sources. Kodama *et al.* (1989) reported that ET evoked a contraction of porcine coronary artery strips in Ca^{2+} -free buffer not only with intracellular Ca^{2+} mobilization but also without an increase in intracellular Ca^{2+} concentration, as determined by front surface fluorometry in arterial strips loaded with fura-2. Therefore, the ET-1-induced contraction of the Ca^{2+} -depleted strips from rat thoracic aorta may not be due to an intracellular Ca^{2+} mobilization.

Griendling *et al.* (1989) and Lee *et al.* (1989) reported that ET caused a pronounced stimulation of diacylglycerol and activation of protein kinase C in cultured vascular smooth muscle cells. Furthermore, ET-induced contraction of vascular smooth muscle in Ca^{2+} -free buffer was inhibited by H-7, a protein kinase C inhibitor (Kodama *et al.*, 1989; Sugiura *et al.*, 1989). Phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, was shown to cause small and sustained contractions of rat thoracic aorta strips in Ca^{2+} -free buffer (Itoh & Lederis, 1987). These findings suggest that an activation of protein kinase C is involved in the ET-induced contraction in Ca^{2+} -free buffer. In the course of our study, however, interesting differences became apparent between ET-1 and PMA. The preincubation with 10^{-7} M PMA for 60 min in Ca^{2+} -free buffer did not affect the 10^{-8} M ET-1-induced contraction, but decreased the 5×10^{-8} M phorbol 12,13-dibutyrate (PDB) or another 10^{-7} M PMA-induced contraction (Table 4 and Figure 3a,b,c). Moreover, preincubation with 10^{-8} M ET-1, which causes maximum contraction in Ca^{2+} -free buffer, did not change the contraction induced by 10^{-8} M urotensin II, whereas preincubation with 10^{-7} M PMA potentiated the urotensin II-induced contraction (Table 4, Figure 3d, and Figure 4b). It is likely that the contractions induced by cumulative applications of two agents with different mechanisms may be additive, whereas in the case of contractions induced by cumulative applications of two agents with similar mechanisms, the first agent-induced precontraction may influence the second agent-induced contraction. Therefore, ET-1 and active phorbol esters may induce contraction of vascular strips in Ca^{2+} -free buffer through differ-

Table 7 Calyculin-A-induced contraction of Ca^{2+} -depleted muscle strips

Treatments	n	Time to reach maximum contraction (min)	Calyculin-A (10^{-7} M) induced maximum contractile tension (mg)
Preincubation for 15 min in Ca^{2+} -free buffer	5	28.2 ± 1.1	399.2 ± 38.7
Repeated applications of 10^{-2} M caffeine in Ca^{2+} -free buffer	4	30.5 ± 3.2	399.7 ± 55.8
Repeated applications of 10^{-6} M noradrenaline in Ca^{2+} -free buffer	4	31.7 ± 2.6	423.5 ± 48.6

Ca^{2+} -depleted muscle strips were pretreated with three repeated applications of 10^{-2} M caffeine or 10^{-6} M noradrenaline in Ca^{2+} -free buffer. n, number of experiments. Values are means $\pm$ s.e. NS, no significant difference.

ent mechanisms. Since H-7 inhibits not only protein kinase C but also other protein kinases, such as cyclic GMP-dependent protein kinase, cyclic AMP-dependent protein kinase and myosin light chain kinase (Hidaka *et al.*, 1984), attention should be paid to the interpretation of the effects induced by H-7. The urotensin II-induced contraction in the absence of extracellular Ca^{2+} consisted of two components: a relatively rapid contraction through intracellular Ca^{2+} mobilization from noradrenaline-sensitive Ca^{2+} store sites and a slow tonic contractile phase after the treatment with noradrenaline (Itoh *et al.*, 1987). The mechanism of the PMA-induced potentiation of the slow tonic contraction induced by urotensin II remains to be determined.

Calyculin-A, a potent phosphatase inhibitor, induces contraction of rat aortic strips in the absence of extracellular Ca^{2+} without an increase in cytoplasmic Ca^{2+} concentration, suggesting that the contraction is due to a Ca^{2+} -independent phosphorylation of myosin light chain and other proteins, by an inhibition of phosphatase activity (Ishihara *et al.*, 1989). In our experiments, the Ca^{2+} -depleted muscle strips were also contracted by calyculin-A. In the absence of extracellular Ca^{2+} , the pretreatments with 10^{-8} M ET-1 and 10^{-7} M PMA decreased contraction induced by 3×10^{-8} M calyculin-A (Figure 5, Table 5). The preincubation of the strips with 3×10^{-8} M calyculin-A also decreased the contractions induced by 10^{-8} M ET-1 and 10^{-7} M PMA (Table 6). The interactions between calyculin-A and ET-1 were similar to the interactions between calyculin-A and PMA, which activates protein kinase C and induces physiological actions through phosphorylation of some proteins by the kinase (Nishizuka, 1984). Therefore, it is possible that the ET-1-induced contraction in Ca^{2+} -free buffer may also involve the phosphorylation of unknown proteins.

Papaverine, dibutyryl cyclic AMP and nitroprusside, all of which influence postreceptor events mainly by modulating the levels or actions of cyclic nucleotides in vascular smooth muscle, inhibited the contractile response to ET-1. A high concentration of indomethacin (5×10^{-4} M), a cyclooxygenase inhibitor, decreased the contraction induced by ET-1 in Ca^{2+} -free buffer, whereas ibuprofen, another cyclooxygenase inhibitor, did not. Because indomethacin has also been shown to inhibit phosphodiesterase like papaverine (Flores *et al.*, 1972), the inhibitory effect of indomethacin on the ET-1-induced contraction may be due to its inhibitory action of phosphodiesterase, but not to that of cyclooxygenase.

Recent reports indicate that ET stimulates the release of

endothelium derived relaxant factor (EDRF) and cyclooxygenase products from endothelial cells (Warner *et al.*, 1987; Folta *et al.*, 1989). However, in the present experiments the presence or absence of endothelial cells did not change the response to ET-1 in Ca^{2+} -free buffer (Table 1). Since the production of EDRF is dependent on extracellular Ca^{2+} (Griffith *et al.*, 1986), the ET-1-induced contraction in the absence of extracellular Ca^{2+} might not be affected by endothelial cells.

The phosphorylation of 20 kDa myosin light chain (MLC) is considered to lead to an interaction of actin with myosin which underlies the initiation of the contractile response (Adelstein & Eisenberg, 1980; Hartshorne & Siemankowski, 1981). In many forms of smooth muscle, however, both the agonist-induced rise in intracellular Ca^{2+} concentration and the increase in the content of phosphorylated MLC are transient (Hartshorne & Siemankowski, 1981; Silver & Stull, 1984). Murphy and coworkers (Dillon *et al.*, 1981) have proposed that stress maintenance depends upon the formation of a so-called latch bridge between actin and myosin, being independent of an increase in myoplasmic Ca^{2+} concentration and MLC phosphorylation. Therefore, the Ca^{2+} -independent contractile responses induced by several agents such as ET-1, phorbol esters, urotensin II, calyculin-A may be appropriate models to analyze the latch bridge state of smooth muscle.

Recently, two independent groups (Arai *et al.*, 1990; Sakurai *et al.*, 1990) reported the structure of two different receptors for endothelins. Each is a rhodopsin-like receptor coupled to a G protein. One shows high specificity for ET-1 and is widely distributed in the central nervous system, the heart and the lung. The other is a non-selective ET receptor for the three ET isopeptides which is coupled through a G protein to phospholipase C, leading to transient increases in intracellular Ca^{2+} , but its messenger RNA is not detected in rat aorta. Therefore, the selective ET-1 receptor may be responsible for the contraction of rat thoracic aorta in Ca^{2+} -free buffer.

In summary, the present results indicate that ET-1 can cause phosphorylation of an unknown protein without an increase in myoplasmic Ca^{2+} through a different mechanism from phorbol esters, activators of protein kinase C, and induce a contractile response of rat thoracic aorta in Ca^{2+} -free buffer. Further work will be required to establish Ca^{2+} -independent phosphorylation of smooth muscle.

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