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Potentiation by endothelin-1 of 5-hydroxytryptamine-induced contraction in coronary artery of the pig

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1 In order to elucidate the physiological and potential pathological roles of endothelin-1 (ET-1) in coronary artery contraction and relaxation, we undertook the present study to examine the action of ET-1 itself, and the combined effects of ET-1 with vasoconstrictor agonists such as acetylcholine (ACh), histamine, and 5-hydroxytryptamine (5-HT), all of which have been implicated in the genesis of coronary spasm.

2 Isometric tension and cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in a ring segment of porcine coronary artery loaded with fura-2 were measured simultaneously.

3 ET-1 contracted the artery in a concentration-dependent manner; and nisoldipine, a Ca^{2+} channel blocking drug of the 1,4-dihydropyridine type, antagonized the ET-1 action non-competitively. A radioreceptor binding assay also indicated the mutually exclusive binding of ET-1 and (+)-[^{3}H]-PN200-110, a Ca^{2+} channel ligand, to the membrane fraction of porcine coronary artery.

4 ET-1 (10–100 pM) increased tension and $[Ca^{2+}]_i$ in a parallel manner, while at higher concentrations (1–10 nM) it produced further contraction with a small increase in $[Ca^{2+}]_i$.

5 ET-1 (30–100 pM) selectively potentiated the 5-HT-induced contraction 1.5 to 2 times over the control without causing a significant increase in $[Ca^{2+}]_i$, which seems to be qualitatively similar to a tumour promoting phorbol ester, 12-deoxyphorbol 13-isobutylate (DPB). Bay K 8644 (10 nM), on the other hand, potentiated the contraction in response to practically all agonists used and affected a concomitant increase in $[Ca^{2+}]_i$.

6 A Ca^{2+} channel blocking drug such as diltiazem abolished the increase in $[Ca^{2+}]_i$ and partially attenuated the mechanical potentiation produced by a small amount of ET-1 in combination with 5-HT.

7 The results suggest that ET-1 and 5-HT interact functionally at the cellular or subcellular level and modulate the Ca^{2+} sensitivity of the contractile elements through the possible activation of protein kinase C.

Keywords: Endothelin; coronary artery; 5-hydroxytryptamine; contraction; cytosolic Ca^{2+} concentration; fura-2

Introduction

Endothelin-1 (ET-1), a 21-residue peptide originally isolated from vascular endothelial cells, exhibits potent vasoconstrictor activity (Yanagisawa *et al.*, 1988). In pig coronary artery, ET-1-induced contraction was shown to be greatly attenuated by Ca^{2+} antagonists of the 1,4-dihydropyridine type (Yanagisawa *et al.*, 1988; Goto *et al.*, 1989). Moreover, ET-1 increased the voltage-dependent Ca^{2+} channel current (Goto *et al.*, 1989) and phosphoinositide (PI) hydrolysis (Kasuya *et al.*, 1989a; Lee *et al.*, 1989; Danthuluri & Brock, 1990). It has also been reported that ET-1 produces endothelium-dependent vascular relaxation (De Nucci *et al.*, 1988; Sakata *et al.*, 1989).

Although ET-1 immunoreactivity is widely detected in various tissues including those of the central nervous system and kidney, the observations on vascular reactivity have led to the idea that ET-1 may act as an endogenous modulator of vascular tone. Most studies on the action of ET-1 in biological systems have employed relatively large concentrations of the peptide (nM to μM). If ET-1 is released locally and plays an important role in the cardiovascular system under physiological and pathological conditions, it is of particular significance to know how ET-1 at lower concentrations (pM to nM) can act *per se*, or if, at these lower levels, it modulates the actions of other vasoconstrictor stimuli on cardiovascular functions. It has been reported that a low dose of ET-1 sensitized vascular smooth muscle to various agents including biogenic amines such as noradrenaline, histamine and 5-hydroxytryptamine (5-HT) as well as adenosine 5'-triphosphate (ATP), vasopressin (La *et al.*, 1990; Aitkenhead *et al.*, 1990), neuropeptide Y (MacLean & McGrath, 1990), clonidine, and Bay K 8644, a Ca^{2+} agonist of the 1,4-dihydropyridine type responsible for the opening of Ca^{2+} channels (Godfraind *et al.*, 1989). Of various endogenous substances, 5-HT (Ashton *et al.*, 1986), histamine (Shimokawa *et al.*, 1983), and acetylcholine (ACh) (Yasue *et al.*, 1976) have been particularly implicated in the genesis of the coronary vasospasm of Prinzmetal's (variant) angina pectoris (Fleckenstein, 1983).

The present study was thus undertaken to improve our understanding of the effects of ET-1 on coronary artery contraction and relaxation. In this study, the mechanical activity and cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured simultaneously (Nakayama & Tanaka, 1988; 1989). Since tumour promoting phorbol esters mimic 1,2-diacylglycerol (DAG)-like action and activate protein kinase C (PKC) directly (Castagna *et al.*, 1982), we compared the actions of ET-1 with those of 12-deoxyphorbol 13-isobutylate (DPB), a phorbol ester, and Bay K 8644, on the tension-$[Ca^{2+}]_i$ relationship in isolated porcine coronary artery.

Methods

Isolation of arteries

A main branch of the anterior descending coronary artery, (outer diameter of about 3 mm), was carefully isolated from healthy pig hearts dissected within 15 min after slaughter of the animals. The pigs were of Yorkshire strain, either sex, 6–8 months old and weighed about 100 kg. The coronary arteries were immersed in Tyrode solution at 4°C, oxygenated with

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95% O_2 and 5% CO_2, and transferred to the laboratory. The arteries were cleared of connective tissue and adventitia under a dissection microscope and cut into ring segments about 2 mm wide. In experiments designed to examine the effect of removal of endothelium, the intimal layer of the artery was rubbed with a moist cotton pledget. Repeated histological examination of the intimal surface of such arteries by scanning electron microscopy has indicated the absence of endothelial cells (Nakayama, 1988). The effectiveness of the endothelial removal was established by the absence of relaxation of the preconstricted artery to the endothelium-dependent relaxant, substance P.

Recording of isometric tension

Two L-shaped tungsten wires (150 μm diameter) were inserted through the lumen of the rings. The lower wire was attached to a supporting hook and the upper one was connected to the lever of a mechanoelectric transducer made in our laboratory. Each artery was mounted in an organ bath containing 10 ml of Tyrode solution (mM: NaCl 158.3, KCl 4.0, $NaHCO_3$ 10.0, NaH_2PO_4 0.42, $CaCl_2$ 2.0, $MgCl_2$ 1.05 and glucose 5.6). Isotonic high-K^+ Tyrode solution was prepared by replacing the NaCl by an equimolar amount of KCl. Ca^{2+}-free Tyrode solution was prepared by omitting $CaCl_2$ and adding 0.2 mM EGTA. The solution was bubbled with 95% O_2 and 5% CO_2 and maintained at a pH of 7.35 at 35°C. The coronary artery in the organ bath was allowed to equilibrate for 1.5 h under an optimal basal tension of 3.0 g before the actual experiments were started. Isometric tension measured with a force transducer was displayed on a penwriting recorder (R-52, Rikadenki, Tokyo, Japan).

Effects of antagonists on contractions produced by agonistic stimuli such as 5-HT, histamine, ACh, and Bay K 8644 were examined. The cumulative increase in concentrations of each agonist produced a concentration-dependent contraction. The pD_2 is the negative logarithm of the agonist concentration required to produce half the maximum response. The maximum contraction was taken as 100% in each preparation. After an incubation period of 30 min for each antagonist, a concentration-response curve for each agonistic stimulus was again obtained. The values of pA_2 and slope of Schild's plot were obtained according to the definition of Arunlakshana & Schild (1959).

The amplification phenomena described here comprises two elements; (1) augmentation, an increase in response amplitude at a given agonist concentration resulting in an increase in the agonist curve maximum with only a small effect of pD_2; (2) sensitization, an apparent increase in agonist potency which is manifest as an increase in pD_2, in this case accompanied by a change in curve maximum (Poech & Holzman, 1980; Leff & Morse, 1987).

Cytosolic Ca^{2+} level measured simultaneously with mechanical activity

Artery segments cleared from both adventitia and endothelium were loaded with 5 μM fura-2-AM for 4 to 5 h at room temperature in Tyrode solution containing 5 mg ml^{-1} albumin and a noncytotoxic detergent, Cremophor EL (0.5%). After the fura-2 loading, each segment was rinsed with normal Tyrode solution for 15 min, mounted in a temperature-controlled quartz glass chamber (7 ml), and perfused with normal Tyrode solution. $[Ca^{2+}]_i$ and isometric tension development were recorded simultaneously, the former with a fluorometer (CAF-100, Japan Spectrophotometric, Tokyo, Japan). U.v. excitation light obtained from a xenon high-pressure lamp (340 nm, F_{340}; and 380 nm, F_{380}; each within ± 5 nm) was focused on the artery and the corresponding emission signals (500 ± 10 nm) as well as the ratio signal (F_{340}/F_{380}), referred to as R, a measure of $[Ca^{2+}]_i$, were monitored. The emitted signals from the artery were collected into a photomultiplier through a 500 ± 10 nm filter. The time constant of the optical system was 0.25 ms. Two L-shaped tungsten wires (150 μm) were inserted through the lumen of the ring segment that was horizontally mounted in the organ bath. One end of each wire was anchored and the other end was connected to a force displacement transducer. All recordings were displayed on a multi-pen recorder.

Absolute $[Ca^{2+}]_i$ was calculated by the ratio method originally described by Grynkiewicz *et al.* (1985) and modified by Himpens *et al.* (1989). The following equation was used: Cytosolic Ca^{2+} concentration $= K_D \times B \times [(R - R_{min})/(R_{max} - R)]$, where K_D is the dissociation constant of fura-2 for Ca^{2+}, assumed to be 224 nM *in vivo* (Grynkiewicz *et al.*, 1985), and B is the ratio of F_{380} in Ca^{2+}-free solution to that in Ca^{2+}-containing solution. R is the fluorescence ratio at F_{340}/F_{380}. The minimum fluorescence (R_{min}) was obtained by superfusion of the artery with a 140 mM K^+, Ca^{2+}-free solution containing 2 mM EGTA. Twenty minutes after superfusion with this solution, 10 μM ionomycin was added, and R_{min} was determined. After determination of R_{min}, the artery was superfused with an excess of Ca^{2+} (10 mM), which gave the maximal signal ratio, R_{max}, $MnCl_2$ (20 mM) added thereafter totally quenched the fluorescence. The procedure for the calibration of $[Ca^{2+}]_i$ is depicted in Figure 1.

Binding assay

The coronary arteries (main branches of left anterior descending artery and left circumflex artery) of pigs were isolated. The arteries were cleared and minced with scissors and then homogenized by use of Potter and Polytron homogenizers in 10 volumes of 50 mM Tris-HCl buffer containing 0.25 M sucrose and 10 μM $MgCl_2$ at pH 7.5. The membrane fraction was prepared according to the method of De Pover *et al.* (1982) and modified by us (Yamada *et al.*, 1990). Briefly, the arterial homogenates were centrifuged at 500 *g* for 10 min and the supernatant fraction, after filtration through four layers of cheesecloth was centrifuged at 9,000 *g* for 10 min. The resulting supernatant fraction was further centrifuged at 120,000 *g* for 30 min and the pellet was resuspended in the buffer to a concentration of 0.5 to 1.5 mg ml^{-1} of protein and used in the binding assays. All steps were performed at 4°C. Freshly prepared membranes from pig coronary artery were incubated with 20 pmol–2 nmol (+)-[^{3}H]-PN200–110 in 50 mM Tris-HCl buffer (pH 7.5) for 60 min at 25°C. The reaction was terminated by rapid vacuum filtration through Whatman GF/B glass fibre filters by use of a cell harvester (Brandel, M-24R, Gaithersburg, U.S.A., M.D.), and the filters were immediately washed 3 times with 3 ml of ice-cold buffer. Tissue-bound radioactivity was extracted from the filters overnight in scintillation fluid, and the radioactivity was determined by liquid scintillation counting. Specific binding of (+)-[^{3}H]-PN200-110 was defined as the difference in binding

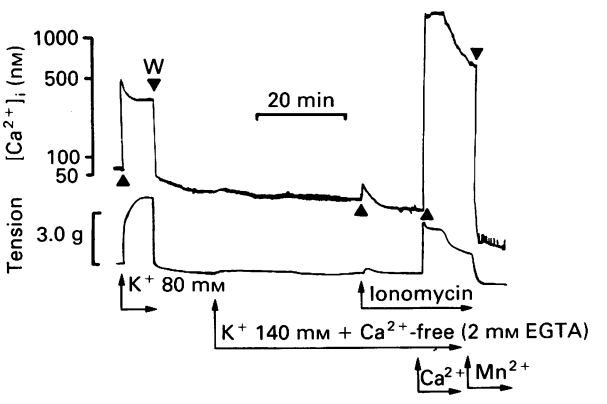

Figure 1 A tracing showing the tension-$[Ca^{2+}]_i$ relation in a coronary ring segment during 80 mM K^+-induced contracture and the actual procedure to obtain R_{min} and R_{max}. Ionomycin, 10 μM; Ca^{2+}, 10 mM; and $MnCl_2$, 20 mM. W: Washout. Muscle wet weight: 6.4 mg.

determined in the absence and presence of nifedipine (1 μM). The ability of drugs to inhibit specific (+)-[^{3}H]-PN200-110 binding was estimated by IC_{50} values which are the molar concentrations of unlabelled drugs necessary to displace 50% of the specific binding determined by log probit analysis. All experiments were carried out in duplicate.

Drugs

The drugs used in the present study were as follows: human endothelin-1 (Peptide Research Institute, Osaka, Japan), acetylcholine chloride (Daiichi, Tokyo, Japan), ionomycin calcium (Hoechst Japan, Tokyo, Japan), and Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) (Bayer Yakuhin, Osaka, Japan). Fura-2/AM, ethyleneglycol-*bis*-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), and tris(hydroxymethyl)-aminomethane (Tris) were obtained from Dojindo Laboratories (Kumamoto, Japan). 12-Deoxyphorbol 13-isobutylate (DPB) and 4α-phorbol didecanoate (4α-PDD) were obtained from Funakoshi (Tokyo, Japan), and dissolved in dimethyl sulphoxide (DMSO) at a concentration of 1 mM and diluted to the desired concentrations with normal Tyrode solution. Ketanserin tartrate was supplied by Kyowahakko (Tokyo). Histamine dihydrochloride, 5-hydroxytryptamine creatinine sulphate and other drugs of reagent grade were obtained from Sigma (St. Louis, M.O., U.S.A.).

Statistical analysis

Results are expressed as means ± s.e. Statistical analysis was made by use of Student's paired or unpaired *t* test. *P* values less than 0.05 were considered significant.

Results

Recording of isometric tension in response to endothelin-1 and Bay K 8644

ET-1 and Bay K 8644 produced a concentration-dependent increase in tension of porcine coronary artery with and without endothelium. The sensitivities (pD_2) to ET-1 and Bay K 8644 of the coronary artery without endothelium were significantly increased in comparison with those of the arteries with endothelium (Table 1). The maximum tensions developed by ET-1 and Bay K 8644 in the endothelium-free artery were also increased, but not significantly in the case of ET-1, when compared with those of endothelium-intact artery. The amplitude of tonic contractions produced by 40 mM K^+ was about the same in the arteries with (6.4 ± 0.6 g, $n = 7$) and without (7.1 ± 0.5 g, $n = 7$) endothelium. Thus, the following experiments were carried out on arteries without endothelium in order to avoid the influence of endothelium on the contraction.

Table 1 Comparison of parameters of cumulative concentration-effect curves with and without endothelium elicited by ET-1 and Bay K 8644[a]

	pD_2	*Maximum response*[b]	n
ET-1			
E(+)	8.57 ± 0.06	105.6 ± 6.9	7
E(−)	9.02 ± 0.06**	122.6 ± 6.6	7
Bay K 8644			
E(+)	7.20 ± 0.08	37.0 ± 14.1	5
E(−)	7.40 ± 0.15**	69.6 ± 11.7*	5

[a] Rings of porcine coronary arteries with [E(+)] or without [E(−)] endothelium were studied in parallel by exposure to increasing concentration of ET-1 or Bay K 8644.
[b] The maximum contractile responses to ET-1 or Bay K 8644 were expressed as a percentage of the amplitude of tonic contraction produced by 40 mM K^+ (= 100%).
Each value represents means ± s.e. of number of preparations (*n*). The difference from the corresponding control response is statistically significant (* $P < 0.05$; ** $P < 0.01$).

Figure 2a shows that increasing concentrations of nisoldipine, a potent Ca^{2+} antagonist of the 1,4-dihydropyridine type, attenuated the slope as well as the maximum response of concentration-response curves for ET-1. Cumulative results are plotted in double reciprocal fasion in the inset of Figure 2a. Firstly, it can be seen that these two lines pass through different points on the Y axis, showing again the non-competitive nature of the interactions of nisoldipine and ET-1 with their binding site in the Ca^{2+} channel. Secondly, the K_D values can be calculated, and are (2.0 ± 0.4) × 10^{-9} M ($n = 4$) for the ET-1 control and (6.9 ± 4.2) × 10^{-9} M ($n = 4$) for nisoldipine (10 nM). These K_D values are not significantly different from each other. In contrast, nisoldipine caused a parallel shift in the concentration-response curves for Bay K 8644 (data not shown) and the pA_2 value was 9.0 ± 0.1 ($n = 6$), indicating an apparently competitive antagonism between Bay K 8644 and nisoldipine.

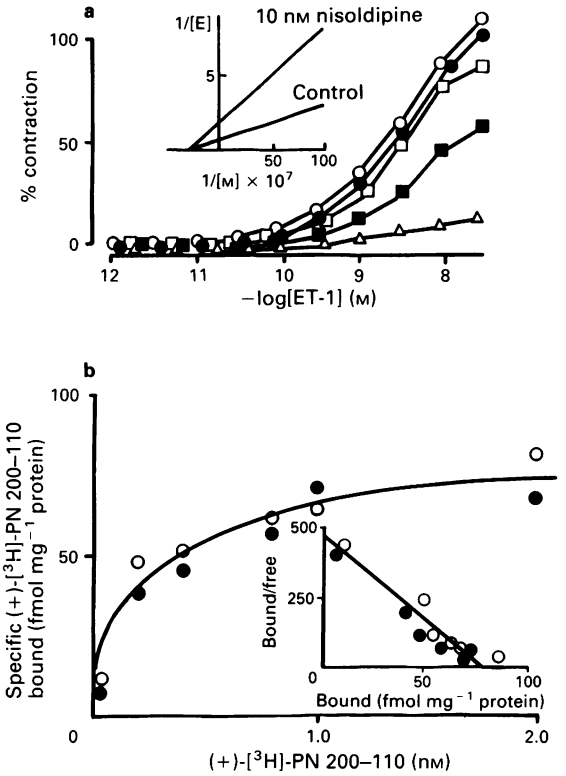

Figure 2 (a) Concentration-response curves for the effect of endothelin-1 (ET-1) on the basal tension of pig coronary artery. The increase in basal tension was normalized as % of amplitude of the tonic component of the 40 mM K^+-induced contracture (= 100%) in the corresponding coronary artery. Each curve shows mean value after the incubation with various concentrations of nisoldipine for 40 min: 0.1 nM (●), 1 nM (□), and 10 nM (■). Control concentration-response curves of the tonic contractions produced by ET-1 in the medium containing 2 mM Ca^{2+} (○) and in the Ca^{2+}-free medium containing 0.2 mM EGTA (△) are also given. Each point represents the mean of 6 to 8 experiments. Standard error at each point is smaller than symbols. Double reciprocal plots of either control response in the presence of 2 mM Ca^{2+} or the response after incubation of the coronary artery with nisoldipine (10 μM) are depicted in the inset. [E]: Effect, [M]: Molar concentration of ET-1. Calculated K_D value, i.e., dissociation constant, is (2.0–9.6) × 10^{-9} M. (b) Specific binding of (+)-[^{3}H]-PN200-110 in absence (○) and presence (●) of ET-1 (30 nM). Inset: Scatchard plot analysis in absence (○) and presence (●) of ET-1 (30 nM).

Binding studies

The effect of ET-1 on the specific binding of (+)-[^{3}H]-PN200-110, a Ca^{2+} antagonist of the 1,4-dihydropyridine type was studied. ET-1 (30 nM) had no apparent effect on the (+)-[^{3}H]-PN200-110-binding (Figure 2b). The Scatchard plot showed that the dissociation constant (K_D) and the maximum number of binding sites (B_{max}) of (+)-[^{3}H]-PN200-110 were 0.172 nM and 79.1 fmol mg^{-1} protein, respectively. In the presence of ET-1 (30 nM) the value was 0.17 nM for K_D, and 74.8 fmol mg^{-1} protein for B_{max}, values which were not significantly different from their controls.

Effects of endothelin-1, 12-deoxyphorbol 13-isobutylate and Bay K 8644 on the agonist-induced contractions

ET-1 (100 pM) potentiated 5-HT-induced contraction of the coronary segment (Figure 3a). Cumulative addition of 5-HT produced a concentration-dependent increase in tension. Low concentrations of ET-1 (10–100 pM) potentiated the contractile response to 5-HT in a concentration-dependent manner, whereas ET-1 in concentrations above 1 nM rather depressed or potentiated less reproducibly the 5-HT-induced contraction. In order to obtain a stable contractile potentiation for quantitative analysis the effects of low concentrations of ET-1 (30–100 pM) were examined. These amounts of ET-1 increased the basal tension to only about 10–15% of the contraction produced by 40 mM K^+ (see Figure 3a). The potentiation of the 5-HT-induced contraction by ET-1 was calculated from the following formula; (ii − i)/i × 100 (see Figure 3a), and normalized as % of amplitude of the tonic component of the 40 mM K^+-induced contraction in the corresponding coronary arteries. Pretreatment with ET-1 (100 pM) for 40 min significantly augmented the contraction amplitude in response to 5-HT (Figure 3b). The sensitivity to 5-HT was not significantly changed before ($pD_2 = 6.4 \pm 0.04$, $n = 6$) and after ($pD_2 = 6.5 \pm 0.04$, $n = 6$) application of ET-1. ET-1 (10 nM), on the other hand, depressed the 5-HT-induced contraction. The contractile response to 5-HT itself was not inhibited by prazosin, phentolamine (10 μM) or chlorpheniramine (1 μM), but was attenuated by ketanserin (0.1 μM). With a concentration range of ketanserin between 3 nM–0.1 μM, the concentration-response curves for 5-HT in the coronary artery showed a parallel shift to the right (data not shown). The pA_2 value for ketanserin was 8.77 ± 0.39 ($n = 5$), and the slope of Schild plot was 1.06. The value of pA_2 and slope of the Schild plot were not significantly affected by pretreatment with 1 μM prazosin; pA_2, 9.14 ± 0.25 ($n = 5$); the slope of the Schild plot, 0.86. Furthermore, the contractile response to 5-HT augmented by ET-1 was not inhibited by prazosin, phentolamine (10 μM) or chlorpheniramine (1 μM), but was attenuated by ketanserin (0.1 μM) (Figure 3a).

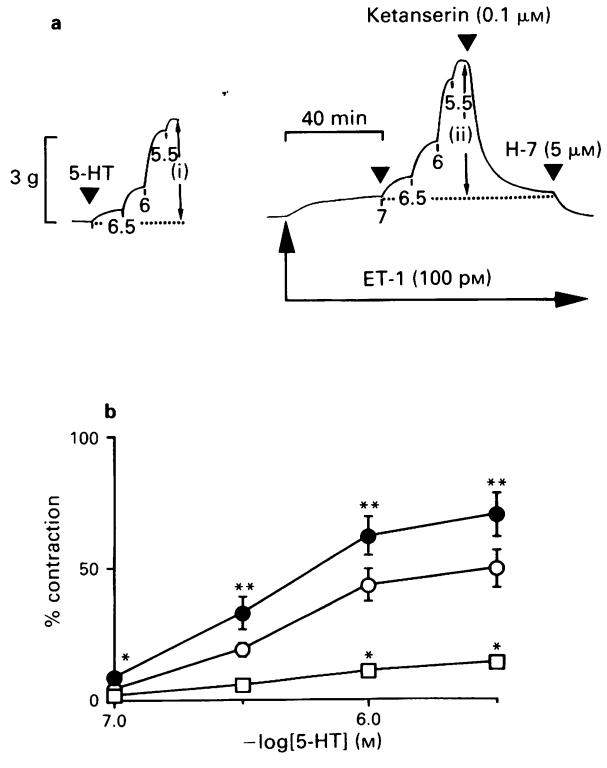

Figure 3 Effect of endothelin-1 (ET-1) on 5-hydroxytryptamine (5-HT)-induced contraction. (a) Representative tracings showing concentration-dependent increase in basal tension produced by 5-HT before (left panel) and after (right panel) administration of ET-1 (100 pM) to the same coronary ring. The numbers on the curves indicate the negative log of molar concentration of 5-HT. (i) and (ii) refer to the 5-HT-induced maximal contraction before and after application of ET-1 (100 pM), respectively. (b) Concentration-response curves for the effect of 5-HT on the basal tension of coronary arteries before (○) and after administration of ET-1 at 100 pM (●) or 10 nM (□). Points and bars indicate mean ± s.e. of 6 to 8 preparations. * $P < 0.05$; ** $P < 0.01$ vs corresponding control values of arteries.

When a single dose of DPB (0.1 nM–1 μM) was applied, the tension increased slowly in a concentration-dependent fashion (data not shown). The tension developed to DPB (3 nM) was less than 10% of that produced by the 40 mM K^+ medium, and the maximum tension developed in response to DPB (1 μM) was 147.9 ± 20.8% ($n = 6$) of the 40 mM K^+-induced contraction. The pD_2 value was 7.2 ± 0.3 ($n = 6$). In contrast, 4α-PDD (1 μM) showed no apparent effect on the tension.

Pretreatment with ET-1 (100 pM) or DPB (3 nM) (Table 2) potentiated the 5-HT-induced contraction, but did not show any apparent potentiating effect on the contractions produced by histamine and ACh. A small amount of Bay K 8644 (10 nM), (producing 10–15% of the increase in tension produced by 40 mM K^+) strongly potentiated the contraction in response not only to 5-HT but also histamine and ACh (Table 2). The contractile response to histamine, ACh, and 5-HT, and their amplification phenomena produced by ET-1 and DPB, or by Bay K 8644 were inhibited by chlorpheniramine and atropine (each 1 μM) (data not shown), or by ketanserin (0.1 μM), respectively. The results indicate that ET-1 selectively potentiated the 5-HT-induced contraction, while Bay K 8644 augmented the contraction amplitude and/or sensitized the coronary artery to the agonistic stimuli used in the present study.

Recording of tension and $[Ca^{2+}]_i$ in coronary artery

Effects of high-K^+, Bay K 8644, endothelin-1 and 12-deoxyphorbol 13-isobutyrate For further elucidation of the potentiating action of ET-1 on the 5-HT-induced contraction, $[Ca^{2+}]_i$ assessed by use of fura-2 and tension development were measured simultaneously. In the fura-2-loaded porcine coronary artery, high-K^+ (20–80 mM) increased $[Ca^{2+}]_i$ which was followed by an increase in basal tone (see Figure 1). The tension development due to high-K^+ was preceded by less than 1 s by the fluorescence changes and the rate of increase in the fluorescence was faster than that of tension development. The mean $[Ca^{2+}]_i$ at basal tone was 69.1 ± 2.3 nM ($n = 42$). The $[Ca^{2+}]_i$ increased during the tonic phase of 40 mM K^+-induced contraction was 228.4 ± 11.0 nM ($n = 42$). A single application of high-K^+ produced a concentration-dependent increase in both tension and $[Ca^{2+}]_i$, indicating the existence of a positive correlation between the two (Figure 4a).

Although Bay K 8644, a Ca^{2+} agonist of the 1,4-dihydropyridine type, was light-sensitive and the time for exposure to u.v. light was limited, a positive tension-$[Ca^{2+}]_i$ relationship could be obtained by single application of Bay K 8644. There is a positive correlation between tension and $[Ca^{2+}]_i$ measured after a 30 min exposure to Bay K 8644 (Figure 4b). Both mechanical activity and $[Ca^{2+}]_i$ augmented by 40 mM K^+ or Bay K 8644 (100 nM) were abolished by diltiazem (10 μM).

Table 2 Effects of endothelin-1 (ET-1, 100 pM), 12-deoxyphorbol 13-isobutylate (DPB, 3 nM), and Bay K 8644 (10 nM) on the contractions of porcine coronary artery produced by cumulative addition of 5-hydroxytryptamine (5-HT), histamine and acetylcholine (ACh)[a]

	Control + ET-1			*Control + DPB*			*Control + Bay K 8644*		
	n	pD_2	*Maximum response*[b]	n	pD_2	*Maximum response*	n	pD_2	*Maximum response*
5-HT	6	6.40 ± 0.03	49.6 ± 7.0	7	6.54 ± 0.09	55.2 ± 6.1	8	6.51 ± 0.07	52.7 ± 6.8
	6	6.46 ± 0.04	70.4 ± 8.2**	7	6.53 ± 0.11	66.3 ± 6.7*	8	6.72 ± 0.09**	84.7 ± 6.5**
Histamine	6	5.47 ± 0.10	135.6 ± 6.3	5	6.21 ± 0.13	121.6 ± 14.3	6	5.16 ± 0.08	141.1 ± 16.3
	6	5.51 ± 0.10	130.3 ± 7.9	5	6.14 ± 0.14	121.0 ± 12.6	6	5.55 ± 0.14**	156.1 ± 22.9
ACh	6	6.23 ± 0.11	61.2 ± 9.3	5	6.59 ± 0.11	101.6 ± 20.9	5	6.01 ± 0.06	24.5 ± 9.7
	6	6.17 ± 0.12	63.1 ± 9.4	5	6.43 ± 0.20	101.0 ± 17.3	5	6.37 ± 0.21	52.5 ± 7.5*

[a] Rings of porcine coronary arteries without endothelium were studied in parallel in the absence (control) or presence of ET-1 (100 pM), DPB (3 nM), or Bay K 8644 (10 nM) and were exposed to increasing concentration of 5-HT, histamine, or ACh.
[b] The maximum contractile response to each agonist was expressed as percentage of amplitude of tonic contraction produced by 40 mM K^+ (= 100%).
Each value represents mean ± s.e. of number of preparations (*n*). The difference from the corresponding control response is statistically significant (* $P < 0.05$; ** $P < 0.01$).

Figure 5 shows the tension-$[Ca^{2+}]_i$ relationship obtained by a single application of ET-1 (30 pM–10 nM). Figure 5a depicts an experiment showing that the $[Ca^{2+}]_i$ was increased from the control level (70.7 ± 2.9 nM, $n = 4$) to the initial peak (170.0 ± 17.1 nM, $n = 4$) within 3 min with a slight effect on the tension when ET-1 at a high concentration of 10 nM was applied. The muscle tension then increased gradually with time and reached a maximum within 20 to 30 min, while $[Ca^{2+}]_i$ slowly declined and reached a steady state level (120.1 ± 14.0 nM, $n = 4$). There was a positive correlation

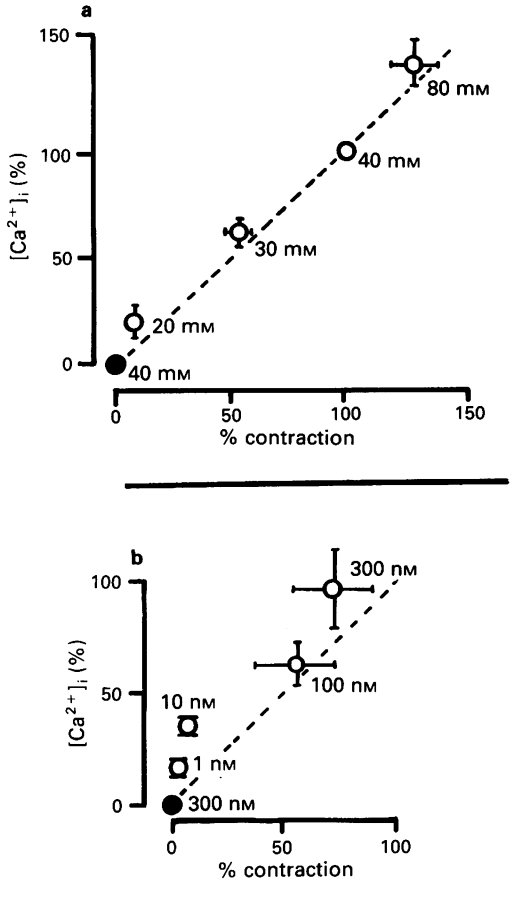

Figure 4 Relationship between tension development and cytosolic Ca^{2+} signal ($[Ca^{2+}]_i$) during contractions produced by high-K^+ (a) or Bay K 8644 (b). A positive correlation between tension and $[Ca^{2+}]_i$ in the amplitude of the tonic phase of high K^+- or Bay K 8644-induced contractions is evident. The responses to a single application of various concentrations of K^+ or Bay K 8644 were plotted, and those to 40 mM K^+ were taken as 100% on the ordinate and abscissa scales. Note that tension and $[Ca^{2+}]_i$ augmented by 40 mM K^+ (a) or Bay K 8644 (b) were abolished by diltiazem (10 μM) (●). Points and bars indicate mean ± s.e. of at least 4 preparations for each concentration. (b) Relationship between tension development and $[Ca^{2+}]_i$ during Bay K 8644-induced contraction. The responses to a single application of various concentrations of Bay K 8644 were plotted, and those to 40 mM K^+ were taken as 100% on the ordinate and abscissa scales. Points and bars indicate mean ± s.e. of at least 4 preparations for each concentration.

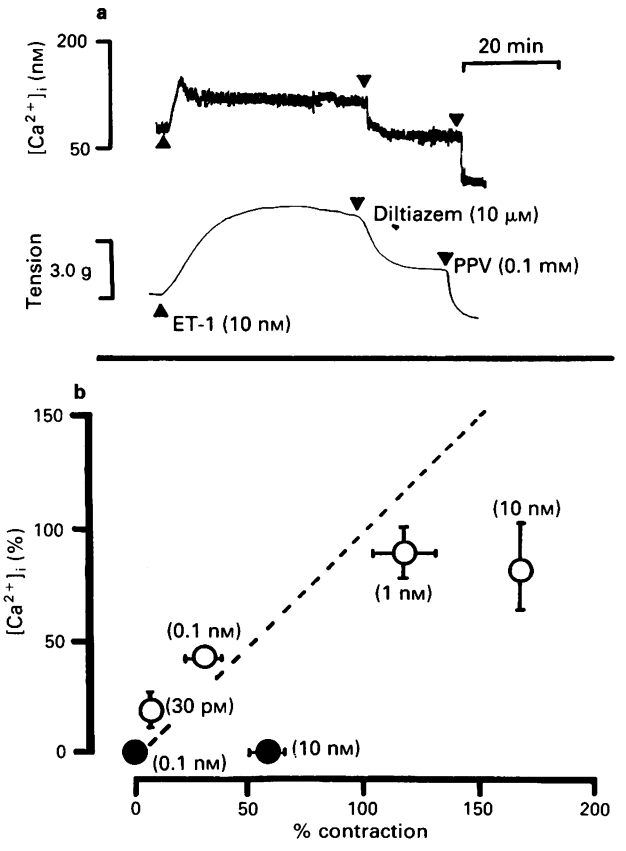

Figure 5 Relationship between tension development and $[Ca^{2+}]_i$ during endothelin-1 (ET-1)-induced contraction. (a) Typical tracings showing the tension-$[Ca^{2+}]_i$ relationship at 10 nM ET-1 in the coronary ring segment. PPV: Papaverine hydrochloride (0.1 mM). Muscle wet weight: 5.3 mg. (b) Relation between tension amplitude and $[Ca^{2+}]_i$ in the tonic phase of ET-1-induced contraction. The responses to a single application of various concentrations of ET-1 were plotted before (○) and after (●) administration of diltiazem (10 μM), and those to 40 mM K^+ were taken as 100% on the ordinate and abscissa scales. Note that tension and $[Ca^{2+}]_i$ were augmented in parallel by ET-1 at relatively lower concentrations but that the tension was augmented without an increase in $[Ca^{2+}]_i$ at the higher concentrations of the peptide. Points and bars, indicate mean ± s.e. of 4–6 preparations.

between tension and $[Ca^{2+}]_i$ measured 40 min after application of ET-1 (30–100 pM) (Figure 5b). ET-1 at higher concentrations, over 1 nM, produced a larger contraction than that due to 40 mM K^+ with still only a small increase in $[Ca^{2+}]_i$. Diltiazem (10 μM) abolished the increase in tension and $[Ca^{2+}]_i$ produced by a low concentration of ET-1 (0.1 nM), while the antagonist only partially inhibited the augmented tension in response to ET-1 (10 nM) in spite of abolition of the increase in $[Ca^{2+}]_i$ (Figure 5a and b). Papaverine (0.1 mM) totally inhibited both tension and the increase in $[Ca^{2+}]_i$.

Figure 6 shows the tension-$[Ca^{2+}]_i$ relationship of DPB, which is qualitatively similar to that of ET-1: a single application of DPB (1 nM–30 nM) slowly increased the basal tension and $[Ca^{2+}]_i$ in a concentration-dependent manner, and there was a positive correlation between these. However, DPB at higher concentrations, over 0.1 μM, produced a larger contraction than that due to 40 mM K^+ with a slight increase in $[Ca^{2+}]_i$. The increased $[Ca^{2+}]_i$ and the tension produced by low concentration of DPB (30 nM) was abolished by diltiazem (10 μM). However, diltiazem (10 μM) only partially inhibited the augmented tension in spite of abolition of the increase in $[Ca^{2+}]_i$ when a high concentration of DPB (1 μM) was given (Figure 6). 4α-PDD (1 μM) had no apparent effect on the tension and $[Ca^{2+}]_i$.

Effects of 5-hydroxytryptamine before and after application of endothelin-1, 12-deoxyphorbol 13-isobutylate and Bay K 8644 Figure 7 shows the tension-$[Ca^{2+}]_i$ relationship for a single application of 5-HT at various concentrations before and after application of ET-1 (a) or DPB (b). The $[Ca^{2+}]_i$-tension relationship of 5-HT alone was similar to that of high-K^+ (see open circles in Figure 4a). ET-1 (30 pM) or DPB (3 nM) strongly augmented the 5-HT-induced tension without a significant change in $[Ca^{2+}]_i$ such that the tension-$[Ca^{2+}]_i$ relation shifted almost parallel to the right (closed circles in Figure 7a and b). 4α-PDD (3 nM) showed no apparent effect on the tension-$[Ca^{2+}]_i$ relation.

When diltiazem (10 μM) was applied, the increase in tension and $[Ca^{2+}]_i$ induced by 5-HT (3 μM) itself was abolished (open squares in Figure 7a and b). The increased $[Ca^{2+}]_i$ produced by 5-HT (3 μM) plus ET-1 (30 pM) or DPB (3 nM) was completely inhibited by diltiazem (10 μM), while the 5-HT (3 μM)-induced contraction which was potentiated by ET-1 (30 pM) or DPB (3 nM) was partially attenuated (closed squares in Figure 7a and b).

Figure 8 shows the tension-$[Ca^{2+}]_i$ relation of 5-HT before and after application of Bay K 8644 (10 nM). Bay K 8644

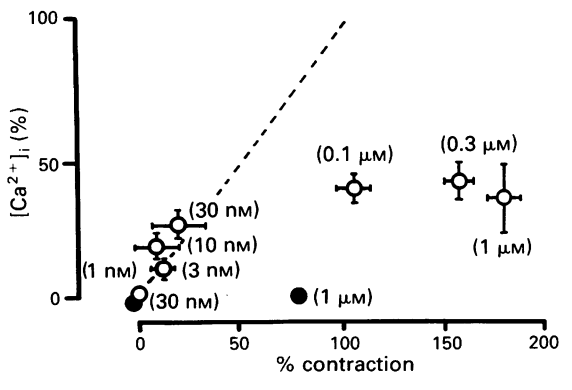

Figure 6 Relationship between tension development and $[Ca^{2+}]_i$ during 12-deoxyphorbol 13-isobutylate (DPB)-induced contraction. The tonic contractile responses to a single application of various concentrations of DPB were plotted (○); after administration of diltiazem (10 μM) (●). The responses to 40 mM K^+ were taken as 100% on the ordinate and abscissa scales. Note that tension and $[Ca^{2+}]_i$ were augmented in parallel by DPB at relatively lower concentrations but that the tension was augmented without an increase in $[Ca^{2+}]_i$ at the higher concentration of DPB. Points and bars, indicate mean ± s.e. of 4–6 preparations.

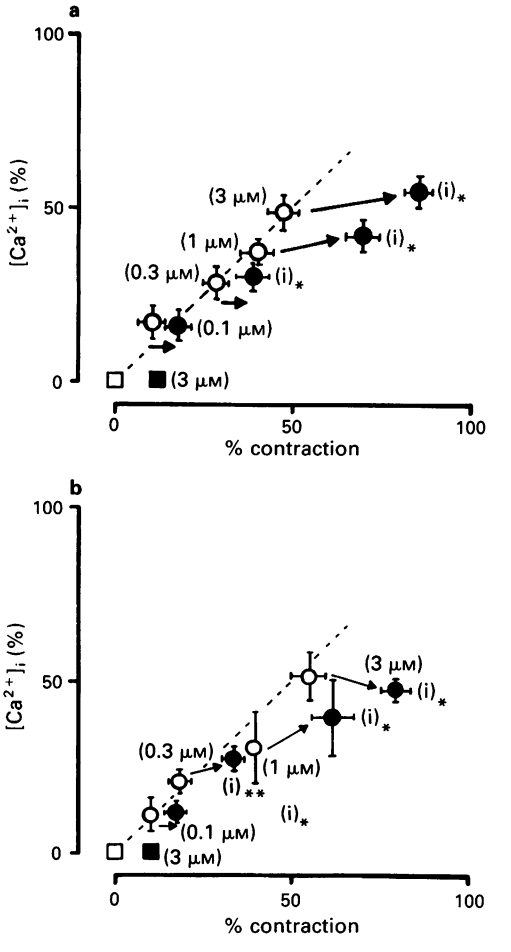

Figure 7 Changes in tension-$[Ca^{2+}]_i$ in 5-hydroxytryptamine (5-HT)-induced contractions before (○) and after (●) treatment with either endothelin-1 (ET-1, 30 pM) (a) or 12-deoxyphorbol 13-isobutylate (DPB) (3 nM) (b). Arrows show the direction of shift. Effects of diltiazem (10 μM) on the response to 5-HT alone (□), and that to 5-HT plus ET-1 or DPB (■). (i) indicates contraction. * $P < 0.05$, ** $P < 0.01$ vs. corresponding control values of arteries. Points and bars, indicate mean ± s.e. of 4–6 preparations.

(10 nM) significantly augmented not only the 5-HT-induced increase in $[Ca^{2+}]_i$ but also tension development (closed circles). Thus, the tension-$[Ca^{2+}]_i$ relationship shifted to the upper right (Figure 8). The 5-HT (3 μM)-induced tension and

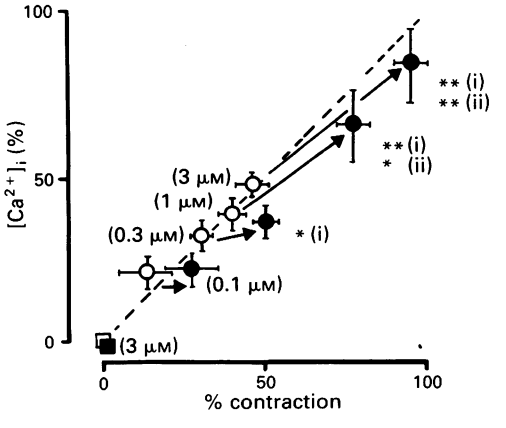

Figure 8 Changes in tension-$[Ca^{2+}]_i$ in 5-hydroxytryptamine (5-HT)-induced contractions before (○) and after (●) treatment with Bay K 8644 (10 nM). Effect of diltiazem (10 μM) on the response to 5-HT alone (□), and that to 5-HT plus Bay K 8644 (■). Arrows show the direction of shift. (i) and (ii) indicate contraction and $[Ca^{2+}]_i$, respectively. Further explanations are the same as those for Figure 7.

$[Ca^{2+}]_i$ potentiated by Bay K 8644 (10 nM) were abolished when diltiazem (10 μM) was applied (closed square in Figure 8).

Discussion

The present study showed that ET-1 *per se* (30–100 pM) produced a slight increase in basal tension, less than 10% of the 40 mM K^+-induced contraction, and strongly potentiated the 5-HT-induced contraction of the coronary artery without a significant increase in $[Ca^{2+}]_i$. The pharmacological properties of ET-1 seem to be qualitatively similar to those of a phorbol ester such as DPB. In contrast, the potentiation of the contractions produced by Bay K 8644 was considered to be attributable to Ca^{2+} agonistic augmentation of transmembrane Ca^{2+} influx (Schramm *et al.*, 1983). This is supported by the finding that the present study showed that Bay K 8644 potentiated the contraction with a concomitant increase in $[Ca^{2+}]_i$ in the coronary artery.

The present study confirmed previous reports by others (De Nucci *et al.*, 1988; Sakata *et al.*, 1989) that removal of endothelium sensitizes arterial smooth muscle and augments the contractile responses to ET-1. Although ET-1 has been reported to produce endothelium-dependent relaxation in the rat (De Nucci *et al.*, 1988), the porcine coronary artery with intact endothelium, which was preconstricted with U46619, a thromboxane A_2 analogue, did not relax in response to ET-1, while substance P relaxed the artery (our unpublished observations). Furthermore, the freshly isolated and dispersed endothelial cells of porcine coronary artery preloaded with fura-2 did not show any increase in $[Ca^{2+}]_i$ in response to 0.1 μM ET-1 ($[Ca^{2+}]_i$ at rest, 106 $\pm$ 8 nM, $n = 5$, and after 0.1 μM ET-1, 105 $\pm$ 15 nM, $n = 5$), while substance P (10 nM) increased the $[Ca^{2+}]_i$ (unpublished observations). The $[Ca^{2+}]_i$ before and at the peak after treatment with substance P was 105 $\pm$ 4 nM and 1,008 $\pm$ 8 nM ($n = 23$), respectively. The relaxation of the coronary artery produced by substance P was not inhibited by indomethacin but attenuated by methylene blue, indicating the release of EDRF concomitant with the increase in $[Ca^{2+}]_i$ (Uchida *et al.*, 1990). It has been reported that the release of vasorelaxant substances such as EDRF and PGI_2 inevitably produce a concomitant increase in $[Ca^{2+}]_i$ (Luckhoff *et al.*, 1988). Therefore, as for porcine coronary artery, it is unlikely that ET-1 can produce relaxation by stimulation of EDRF release. The removal of endothelium seems to inhibit the basal release of a relaxing substance such as EDRF; thus, this removal enhances the actions of practically all vasoconstrictor agonists used in the present study.

Earlier studies on ET-1-induced contraction of the coronary artery have suggested that nicardipine, a Ca^{2+} antagonist, shifted the concentration-response curves for ET-1 to the right in a parallel manner, indicating a competitive antagonism between ET-1 and the Ca^{2+} antagonist at the same binding site on the Ca^{2+} channel (Yanagisawa *et al.*, 1988). However, the results of radio-ligand binding assays of ET-1 binding to plasma membranes of the rat (Gu *et al.*, 1989) and chicken heart (Watanabe *et al.*, 1989) and of porcine coronary artery (Kasuya *et al.*, 1989b) have clearly shown that ET-1 does not bind to the Ca^{2+} channel receptor sensitive to Ca^{2+} antagonists of the 1,4-dihydropyridine type but to ET-1 receptor(s). Our radio-receptor binding assay on the Ca^{2+} antagonist receptor in the porcine coronary artery also showed a clear separation of the binding site between ET-1 and (+)-[^{3}H]-PN200-110. In accordance with the results of the radio-receptor binding assay, the present functional study showed that nisoldipine antagonized the ET-1-induced contraction in a non-competitive manner. Our previous radio-receptor binding study (Yamada *et al.*, 1990) and the present functional study indicate that Bay K 8644 and a Ca^{2+} antagonist of the 1,4-dihydropyridine type such as (+)-[^{3}H]-PN200-110 share the same binding site at the voltage-dependent Ca^{2+} channel in the porcine coronary artery. The functional and binding studies are thus well correlated, and both studies can aid in the elucidation of the pharmacological characteristics of ET-1 and Bay K 8644.

We can only speculate on the mechanism of the potentiation of the 5-HT-induced contraction produced by ET-1. It has been reported that in the rat aorta, 5-HT activates phospholipase C and thus promotes PI hydrolysis which leads to production of inositol 1,4,5-trisphosphate ($InsP_3$) and DAG (Nakaki *et al.*, 1985). Since $InsP_3$ releases Ca^{2+} from intracellular Ca^{2+} stores (Berridge, 1984), $InsP_3$-induced contractions are rather resistant to Ca^{2+} antagonists and withdrawal of extracellular Ca^{2+}. However, our present study showed that 5-HT- and high-K^+-induced contraction and the increase in $[Ca^{2+}]_i$ in particular at the tonic phase of contraction correlated well with contraction amplitude. The 5-HT-induced contraction was not affected by prazosin, phentolamine or chlorpheniramine, but competitively antagonized by ketanserin, indicating a pivotal role of 5-HT_2 receptors. Moreover, of various agonists including 5-HT, histamine, and ACh, the 5-HT-induced contraction of porcine coronary artery was the most susceptible to a Ca^{2+} antagonist such as nifedipine (Nakayama *et al.*, 1989) and to withdrawal of Ca^{2+}. Therefore, like high-K^+-induced contraction, the contraction produced by 5-HT in porcine coronary artery seems to be primarily dependent on the transmembrane supply of Ca^{2+}.

As to the mechanism of ET-1-induced contraction, the following two ideas have been proposed: (1) augmentation of Ca^{2+} influx (Yanagisawa *et al.*, 1989); (2) augmentation of turnover of PI and increase in intracellular messengers such as $InsP_3$ and DAG. These ideas seem to be qualitatively common to those for the mechanism of action of various agonistic stimuli including 5-HT, and tumour promoting phorbols on various biological systems such as vascular smooth muscles. In the present study, we observed that ET-1 and DPB at a low concentration (less than 1–3 nM) increased the tension and the $[Ca^{2+}]_i$ almost in a parallel manner, indicating that the first mechanism may be dominant, while the second mechanism of promotion of PI hydrolysis seems to be dominant when a large concentration of ET-1 or DPB over 10–100 nM is applied. Of the various biological roles of PKC promoted by DAG, the phosphorylation of various proteins (Nishizuka, 1984) is the first step, and as a consequence, the Ca^{2+} sensitivity of molecules such as the contractile proteins in vascular smooth muscles is increased. The PKC has also been known to promote Ca^{2+} current through the channels sensitive to Ca^{2+} antagonists of the 1,4-dihydropyridine type (Vivaudou *et al.*, 1988). In contrast, PKC is also involved in a negative feedback mechanism; i.e., it decreases receptor-coupled responses (Nishizuka, 1984). The high concentrations of ET-1 or tumour promoting phorbol esters such as DPB and 12-O-tetradecanoylphorbol-13-acetate (TPA) inhibited the contractile responses of the pig coronary artery to various agonistic stimuli, such as 5-HT in the present study, and ACh (Itoh *et al.*, 1988) or mechanical stretch (our unpublished observations).

The combined effects of ET-1 and other agonists on the $[Ca^{2+}]_i$-tension relation were complex. In addition to the direct vasoconstriction effect on vascular smooth muscle cells, 5-HT enhances the contractile effects of a number of agonists (e.g. noradrenaline) (de la Lande *et al.*, 1966) and sympathetic stimulation *in vivo* and *in vitro* (Van Nueten *et al.*, 1981; Pott *et al.*, 1986). 5-HT also accelerates platelet aggregation when combined with low, subthreshold concentrations of other agonists (e.g. ADP, collagen, adrenaline, and thromboxane A_2 or its mimic U46619; De Clerck & De Courcelles, 1989). These amplifications occur biochemically at the post receptor stage of the signal transduction system: the turnover of PI and the rise in $[Ca^{2+}]_i$ are amplified (De Clerk & De Courcelles, 1989). Nishizuka (1984) and his colleagues (Kaibuchi *et al.*, 1983) have proposed an interesting idea concerning the role of PKC in the cellular signal transduction: they suggested synergistic interaction between the PKC and Ca^{2+}-calmodulin

pathways in a variety of cellular responses to external stimuli. The interaction between PKC-mediated phosphorylation and increased Ca^{2+}-calmodulin leads to full activation of a physiological cellular response such as release of 5-HT from platelets. Zawalich *et al.* (1983) also reported that two Ca^{2+} signalling systems, i.e., transmembrane Ca^{2+} mobilization and activation of PKC, are responsible for receptor-mediated release of insulin and aldosterone. On the other hand, Naka *et al.* (1983) showed that the Ca^{2+}-calmodulin system directly activates myosin light chain kinase in a $[Ca^{2+}]_i$-dependent manner, while the PKC system, which does not require an increase in $[Ca^{2+}]_i$, rather depresses the activation of actomyosin in platelets. Thus, the two pathways act antagonistically in the regulation of platelet function.

Our present study suggests that the simultaneous stimulation of two different receptors augments contraction in the porcine coronary arterial smooth muscle. It seems possible that the combined stimulation of 5-HT- and ET-1-receptors may amplify the intracellular signalling systems more effectively, and produce a full activation of contraction without a significant increase in $[Ca^{2+}]_i$. The activation of PKC may play an important role in this regard. In contrast, the present study showed that Bay K 8644 acted as a simple promoter of transmembrane influx of Ca^{2+}, for it potentiated the contractile response, along with an increase in $[Ca^{2+}]_i$, to all the pharmacological agents tested, as is also seen with other pharmacological, electrical, and mechanical stimuli (Nakayama *et al.*, 1983; Nakayama, 1986; Nakayama & Tanaka, 1989). It has been reported that Bay K 8644 potentiates 5-HT effects at the 5-HT_2 receptor in rabbit aorta in a manner consistent with a simple increase in efficacy (Barrett *et al.*, 1986). The present results with Bay K 8644 alone, Bay K 8644 plus 5-HT, or Ca^{2+} antagonists such as nisoldipine and diltiazem are not entirely consistent with such a second messenger system, but are similar to those reported by Barrett *et al.* (1986). Since ET-1 potentiated the 5-HT-induced contraction without increasing the $[Ca^{2+}]_i$ in the present study, it is unlikely that ET-1, even at a low concentration, acts solely as a promoter of Ca^{2+} influx in the coronary artery. We are now conducting experiments in order to obtain more direct evidence of combined effects of ET-1 and other agonists on PKC activation and contractile function.

Finally, the physiological and pathological significance of the present study should be mentioned: 5-HT released from platelets has been known as a strong coronary vasoconstrictor and platelet aggregator which may be a factor in coronary vasospasm (Ashton *et al.*, 1986). The plasma ET-1 level of normotensive and spontaneously hypertensive rats (Suzuki *et al.*, 1990) and of man (Ando *et al.*, 1989) is reportedly 1–2 pg ml^{-1} as measured by use of an enzymatic immunoassay, a value corresponding to about 0.5 pM, when the plasma volume is arbitrarily taken as 1/13 of body weight (about 60 kg). Thus, a small amount of ET-1 at the picomolar level released locally *in situ* may not only regulate physiologically vascular tone, but also promote contraction of vascular smooth muscles in the coronary as well as peripheral vessels. The recent study (Matsumoto *et al.*, 1990) reporting that ET-1 in combination with 5-HT activates platelets and accelerates aggregation and the results from the present study suggest the importance of the synergistic effects of ET-1 and other endogenous spasmogens, in particular 5-HT, on the coronary circulation.

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