

Characterization of the Endothelin Receptor in Primary Cultures of Human Aortic Smooth Muscle Cells

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ABSTRACT—We characterized the endothelin receptor subtypes in primary cultures of human aortic smooth muscle cells (HASMCs) by binding studies. [125 I]-Endothelin (ET)-1 saturation experiments showed the existence of a homogeneous population of binding sites with the high affinity (K_D value) of 97 ± 37 pM and maximum number of binding sites (B_{max}) of 54 ± 10 fmol/mg protein. However, almost no specific [125 I]-ET-3 binding was observed. Inhibition of [125 I]-ET-1 binding in the HASMCs membrane by nonlabelled compounds showed the following order of effectiveness: ET-1=ET-2=FR139317 \gg ET-3. These results suggest that the endothelin receptor of HASMCs is of the ET_A type. We also studied the effect of ET-1 on the cytosolic [Ca^{2+}]_i in HASMCs loaded with fura-2/AM. In 1.3 mM Ca^{2+} , ET-1 produced a dose-dependent, biphasic increase in signal with a maximal effect at 10 nM. At this concentration, ET-1 produced a transient increase in [Ca^{2+}]_i that reached a peak at 1 min, which was followed by a slow but sustained increase in [Ca^{2+}]_i. This second phase was attenuated in Ca^{2+} -deficient medium. Furthermore, ET-1 increased inositol 1,4,5-triphosphate in a time- and dose-dependent manner. These results suggest that the endothelin receptors of HASMCs are of the ET_A type, which couple with Ca^{2+} channels.

Keywords: Endothelin, Receptor, Aorta (human), Smooth muscle cell

Endothelin (ET), first purified from the culture medium of porcine endothelial cells, is the most potent and longest acting constrictor of vascular and non-vascular smooth muscle cells (1). Cloning and sequencing analysis have shown that at least three genes encode the endothelin family (2). Receptors for endothelin have been studied in a variety of tissues from a number of species. At least two receptor subtypes have been identified: one has higher affinity for ET-1, ET-2-selective ET_A and non-isopeptide selective ET_B (3–5). High-affinity receptors for ET-1 have been demonstrated on cardiac membranes (6, 7) and cultured vascular smooth muscle cells (8, 9).

It is well known that activation by ET of the voltage-dependent Ca^{2+} channel plays an important role in ET-induced vasoconstriction (1, 10, 11). ET is a vasoconstrictor, exerting its effect primarily via the cytosolic free Ca^{2+} messenger system and phosphatidylinositol breakdown. Although information concerning the effect of ET on vascular smooth muscle cells has been derived primarily from cells originating from laboratory animals, several studies have used human-derived cells, including human umbilical vein smooth muscle (8, 9), human myometrial

(12) and human bronchial smooth muscle cells (13). Recently, human aortic smooth muscle cells (HASMCs) have become available for use. In the present study, we characterized the ET receptors in these cells by binding studies and measurement of intracellular Ca^{2+} content.

MATERIALS AND METHODS

Cell culture

HASMCs imported from Clonetics (San Diego, CA, USA) were purchased from Kurabo (Osaka). They were grown to confluence in monolayers on 225-cm² culture dishes in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum plus 4 μ g/ml of gentamicin under 5% CO₂–95% air atmosphere at 37°C. The medium was changed every 3 days, and the cells were subcultured every 5–7 days after trypsinization. All cells used in these experiments were under 15–25 population doubling level (PDL). HASMCs were identified by their typical "hill-and-valley" morphology and immunofluorescence using a monoclonal antibody against human α -smooth muscle actin, which is a specific marker of differentiated smooth

muscle cells.

Membrane preparation

HASMCs in 500-cm² plastic dishes were washed twice with PBS, scraped into a solution containing 250 mM sucrose, 10 mM MgCl₂ and 50 mM tris/HCl (pH 7.4), and then homogenized with a polytron at 4°C. After centrifugation at 2,000 × *g* for 10 min at 4°C, the supernatant was centrifuged at 40,000 × *g* for 20 min at 4°C and the resulting pellet was resuspended in 50 mM tris/HCl (pH 7.4), 10 mM MgCl₂ and stored at -80°C until for use.

Binding assay

Under standard conditions, incubations were carried out at 37°C for 90 min in a final volume of 0.5 ml containing 50 mM HEPES (pH=7.5), 10 mM MgCl₂, 0.1% BSA, 0.2 TIU aprotinin and 0.17 mg/ml bacitracin. The assay was started by the addition of [¹²⁵I]-ET-1 (30 pM) or [¹²⁵I]-ET-3 (30 pM), to membrane from HASMCs (50–100 µg protein). Incubation was terminated by rapid filtration through Whatman GF/C filters (Maidstone, Kent, UK) using a Brandel cell harvester (Geithersburg, MD, USA). The filter was then rinsed 3 times with 3 ml of 50 mM Tris-HCl (pH=7.4), 10 mM MgCl₂ and 0.01% BSA. Radioactivity retained on the filters was counted by a γ -counter (ARC-950; Aroka, Tokyo). Nonspecific binding was determined in the presence of 1 µM ET-1. The protein content of each membrane suspension was measured by the method of Bradford (14).

Measurements of [Ca²⁺]_i

Cells were incubated for 30 min with 5 µM fura-2/AM in HBSS containing 0.05% BSA and 10 mM glucose. They were washed twice with HBSS, and the slides were secured in a quartz cuvette in a CAF/100 fluorescence spectrometer (Japan Spectroscopic Co., Ltd., Tokyo) equipped with a thermostatically controlled cell holder. Excitation wavelengths were set at 340 and 380 nm and emission wavelength at 500 nm. Fluorescence was monitored for 1–3 min until the [Ca²⁺]_i signal stabilized, and a basal [Ca²⁺]_i measurement was obtained. Thereafter, the cells were subjected to specific agonists (e.g., ET-1) or experimental perturbations, and [Ca²⁺]_i signals were recorded for an additional 10 min. On each coverslip, Ca²⁺ calibration was performed by exposing the cells to 10 µM ionomycin, followed by 3 mM EGTA. From the ratio of fluorescence at 340 and 380 nm, the [Ca²⁺]_i was determined as described by Grynkiewicz et al. (15) using the following equation:

$$[\text{Ca}^{2+}]_i (\text{nM}) = K_d \times [(R - R_{\min}) / (R_{\max} - R)] \times \beta$$

The term β is the ratio of fluorescence of fura-2 at 380 nm in zero and saturating Ca²⁺. K_d is the dissociation constant of fura-2 for Ca²⁺, assumed to be 224 nM.

Inositol phosphate assay

HASMCs were grown to confluence on 6-well plates, and then serum-deprived for 18 hr. Inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) was quantitatively measured in ET-1-stimulated HASMCs by Ins(1,4,5)P₃ competition assay. Briefly, before stimulation with ET-1, HASMCs were preincubated with DMEM containing 10 mM LiCl for 5 min at 37°C. The reaction was terminated at designated times by adding 10% (W/V) HClO₄. The mixture was kept on ice for 20 min, and then neutralized with ice-cold 1.53 M KOH/75 mM Hepes for 20 min. The sample was centrifuged at 2,000 × *g* for 10 min to remove the KClO₄ precipitate. The supernatant samples (100 µl each) were assayed for Ins(1,4,5)P₃ with an Ins(1,4,5)P₃ assay kit (NEN-Du Pont, Boston, MA, USA). The standard curve was linear from 0.12 to 12.0 pmol of Ins(1,4,5)P₃.

Analyses of data

Results are expressed as the mean ± S.E.M. or the mean with 95% confidence limits. Analysis of binding data was performed as previously described (16). Statistical difference between the two means was determined by the non-paired Student's *t*-test. *P* values less than 0.05 are considered to be significant. The regression lines were calculated by the least squares method.

Materials

ET-1, ET-2 and ET-3 were purchased from Peptide, Inc. (Osaka). [¹²⁵I]-ET-1 (81.4 TBq/mmol), [¹²⁵I]-ET-3 (81.4 TBq/mmol) and Ins(1,4,5)P₃ assay kit were purchased from New England Nuclear (Boston, MA, USA). 1-[2-(5'-Carboxyoxazol-2'-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5-methyl phenoxy) ethane-*N, N, N', N'*-tetraacetic acid, pentaacetoxymethyl ester (Fura-2/AM), was purchased from Dojin (Kumamoto). FR139317, ((*R*)-2-[(*R*)-2-[(*S*)-2-[[1-(hexahydro-1*H*-azepinyl)]-carbonyl]amino-4-methyl-pentanoyl]amino-3-[3-(1-methyl-1*H*-indolyl)]propionyl]amino-3-(2-pyridyl)propionic acid), an endothelin ET_A-type-receptor antagonist (17) was prepared by Yamanouchi Pharmaceutical Co., Ltd. All culture reagents were from Gibco Laboratories (Paisley, Scotland), and culture flasks and plates were from Costar (Cambridge, MA, USA). All other chemicals were obtained from commercial sources and were of the highest quality available.

RESULTS

To characterize the specific binding site of ET-1 in the HASMC membrane, a radioligand binding assay was performed using [¹²⁵I]-ET-1 and [¹²⁵I]-ET-3 (Fig. 1a). Specific binding was >70% of the total binding at any concentration of [¹²⁵I]-ET-1 tested and was a saturable process.

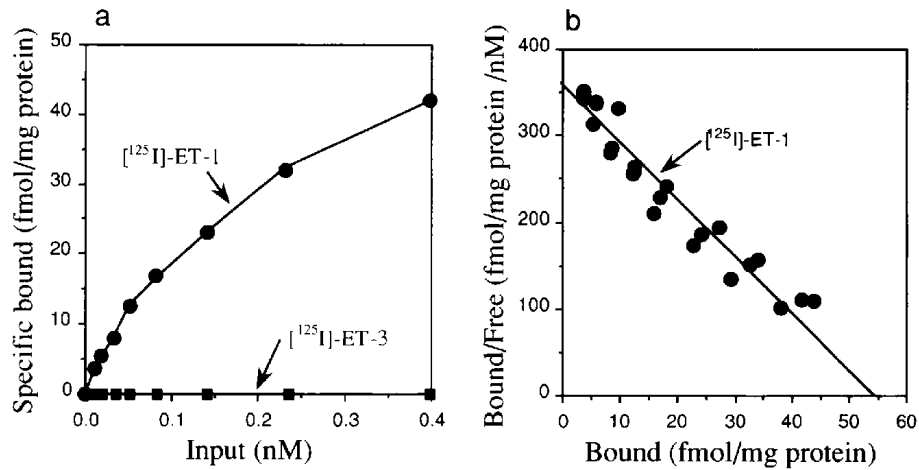


Fig. 1. Specific binding of [125 I]-ET-1 (●) and [125 I]-ET-3 (■) to HASMC membrane (a) and Scatchard plot (b). Increasing concentrations of [125 I]-ET-1 or [125 I]-ET-3 (0–0.4 nM) were added to membranes, which were then incubated for 90 min at 37°C. Specific binding is shown. Similar results were obtained from at least three different experiments.

Scatchard analysis of the data revealed a single class of high-affinity binding sites with a K_D of 97 ± 37 pM and a B_{\max} of 54 ± 10 fmol/mg protein (Fig. 1b). Specific binding of [125 I]-ET-3 was not observed in HASMC membranes. Figure 2 and Table 1 show the competitive inhibition curves and K_i values of nonlabelled ET-1, ET-2, ET-3 and the ET_A -selective antagonist, FR139317. ET-1, ET-2 and FR139317 showed similar inhibition curves and K_i values (3.8, 5.7 and 3.6 nM, respectively). ET-3, however, showed 100-fold weaker inhibition (375.3 nM). These results suggested that endothelin receptors in HASMCs are of the ET_A type.

ET-1-induced Ca^{2+} mobilization was studied by measurement of changes in $[Ca^{2+}]_i$, using fura-2 as a Ca^{2+} indi-

cator (Fig. 3). The baseline $[Ca^{2+}]_i$ was 68.6 ± 8.7 nM. The addition of 1×10^{-8} M ET-1 induced a prompt increase in $[Ca^{2+}]_i$, which peaked within 15 sec of ET-1 addition, with a peak value of 558.8 ± 31.6 nM. Figure 3 shows that this increase was dose-dependent (10^{-11} – 10^{-7} M). The response was saturated at 1×10^{-8} M ET-1, and ED_{50} was estimated to be about 3×10^{-9} M. ET-3 was a weak agonist of $[Ca^{2+}]_i$ change (Fig. 3). FR139317 abolished the ET-1-induced Ca^{2+} mobilization. The L-type Ca^{2+} channel blocker nifedipine attenuated the second plateau phase of the $[Ca^{2+}]_i$ increase at a dose of 1×10^{-4} M (Fig. 4). When extracellular Ca^{2+} was removed and supplemented by the addition of 0.1 mM EGTA, basal $[Ca^{2+}]_i$ declined to a new steady-state level (43.8 ± 1.6 nM). Under these conditions, ET-1 still induced the initial $[Ca^{2+}]_i$ increase, but not the following plateau of the response. Inhibition by caffeine and ryanodine of the ET-1-induced increase in $[Ca^{2+}]_i$ is shown in Figure 4 and Table 2. When cells were preincubated for 20 min in Ca^{2+} -free medium containing a mixture of 1×10^{-4} M EGTA and 1×10^{-2} M caffeine, there was a decline in $[Ca^{2+}]_i$ after the addition of ET-1. Similar results were obtained in Ca^{2+} -free medium containing

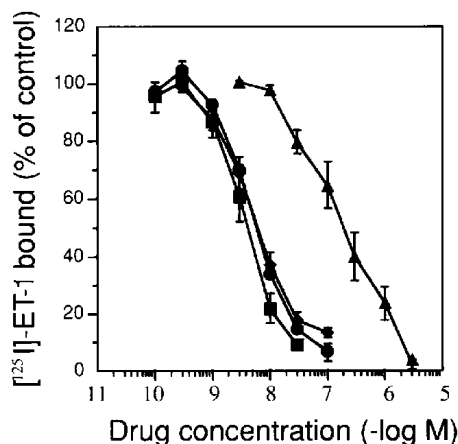


Fig. 2. Competitive inhibition curves for [125 I]-ET-1 binding to HASMC membrane. HASMC membranes were incubated with [125 I]-ET-1 in the presence of the indicated concentration of unlabeled ET-1 (■), ET-2 (●), ET-3 (▲) and FR139317 (◆). Each point represents the mean \pm S.E.M. of three to four experiments.

Table 1. Inhibition of [125 I]-ET-1 binding sites by ET-1 analogues

	K_i values (nM)	Hill slope
ET-1	3.8 (3.5– 4.2)	1.03 (0.77–1.29)
ET-2	5.7 (5.6– 5.8)	1.12 (1.03–1.21)
ET-3	375.3 (335.9–419.3)	0.82 (0.71–0.93)
FR139317	3.6 (3.6– 3.8)	1.03 (0.94–1.12)

Data are the mean and 95% CL from three separate experiments.

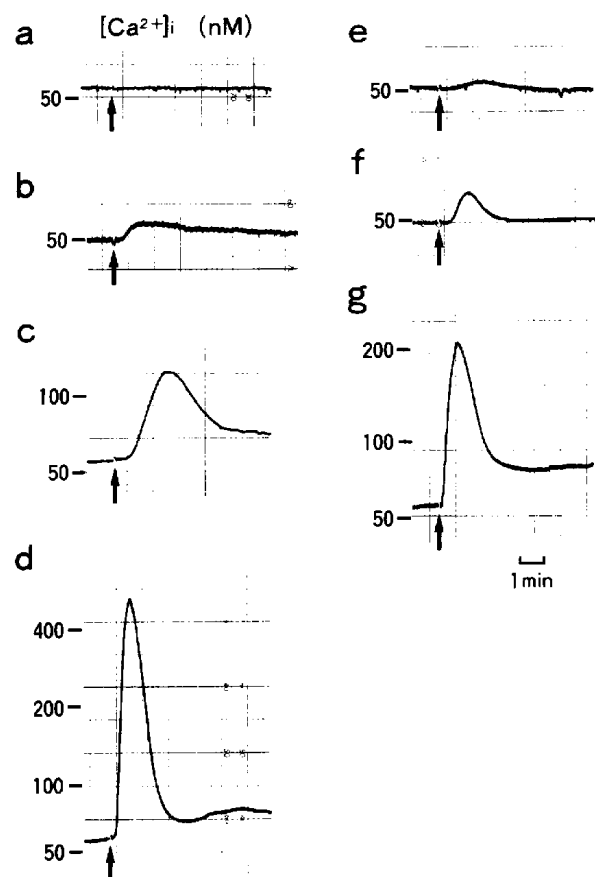


Fig. 3. The effects of ET-1 and ET-3 on intracellular free Ca^{2+} . Fura-2/AM loaded HASMCs were exposed to (a) 1×10^{-11} M, (b) 1×10^{-10} M, (c) 1×10^{-9} M and (d) 1×10^{-8} M ET-1 and (e) 1×10^{-8} M, (f) 1×10^{-7} M and (g) 1×10^{-6} M ET-3. Test substances were applied at arrows and were present throughout each recording. Representative charts are shown; similar results were obtained from at least four separate experiments.

1×10^{-4} M EGTA and 1×10^{-6} M ryanodine. These findings suggest that ET-1 induces Ca^{2+} mobilization from both caffeine-sensitive intracellular Ca^{2+} stores and extracellular pools.

As shown in Fig. 5a, the addition of 1×10^{-8} M ET-1 induced a rapid increase in $\text{Ins}(1,4,5)\text{P}_3$. Levels peaked at 15 to 30 sec, and then gradually declined over the next 90 sec. This increase in $\text{Ins}(1,4,5)\text{P}_3$ production by ET-1 was dose-dependent (Fig. 5b). The response was saturated at 1×10^{-8} M ET-1; ED_{50} value was estimated to be approximately 3×10^{-9} M. ET-1-induced $\text{Ins}(1,4,5)\text{P}_3$ production was inhibited by the addition of FR139317 (1×10^{-7} M). These results correlated well with the results of the binding study and changes in $[\text{Ca}^{2+}]_i$.

DISCUSSION

The K_D and K_i values obtained for $[^{125}\text{I}]\text{-ET-1}$ in this

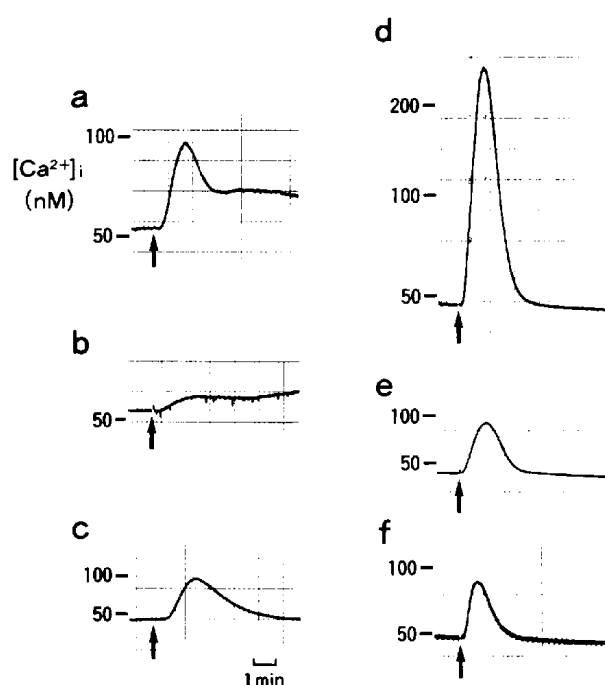


Fig. 4. Inhibition by FR139317, nicardipine, Ca^{2+} -free medium, caffeine and ryanodine of ET-1-induced increases in $[\text{Ca}^{2+}]_i$ in HASMCs. Cells were preincubated with (a) 1×10^{-8} M FR139317, (b) 1×10^{-7} M FR139317, (c) 1×10^{-6} M nicardipine, (d) Ca^{2+} -free medium supplemented 1×10^{-4} M EGTA, (e) Ca^{2+} -free medium supplemented with 1×10^{-4} M EGTA and 1×10^{-2} M caffeine and (f) Ca^{2+} -free medium supplemented with 1×10^{-4} M EGTA and 1×10^{-6} M ryanodine. ET-1 (1×10^{-8} M) was applied at arrows.

Table 2. Inhibition by caffeine and ryanodine of ET-1-induced $[\text{Ca}^{2+}]_i$ transient in HASMCs

	$[\text{Ca}^{2+}]_i$ (nM)		
	basal	1×10^{-8} M ET-1	P (vs. basal)
Control	68.6 ± 8.7	558.8 ± 31.6	<0.01
Ca^{2+} free medium	43.8 ± 1.6	249.8 ± 10.4	<0.01
+ Caffeine (1×10^{-2} M)	42.8 ± 1.9	$109.4 \pm 16.6^{**}$	NS
+ Ryanodine (1×10^{-6} M)	52.6 ± 2.2	$117.8 \pm 17.6^{**}$	NS

Values are the mean \pm S.E.M. $n=7-10$. NS=nonsignificant vs. basal. $^{**}P<0.01$ vs. the group of cells incubated with Ca^{2+} -free medium containing 1×10^{-8} M ET-1.

study agree well with those reported in the binding studies on expressed ET_A -receptors from human (18), bovine (19) and rat heart (20). Arai et al. (18) reported that the K_D value of the ET_A -type receptor was 0.18 nM in ET_A -cDNA transfected cell membrane. The results obtained in the present study (0.10 nM) matched these results well. The Scatchard plot revealed that the binding site of ET-1 to HASMCs was mono-componential, meaning that a single site exists in this cell. The binding of $[^{125}\text{I}]\text{-ET-1}$ to

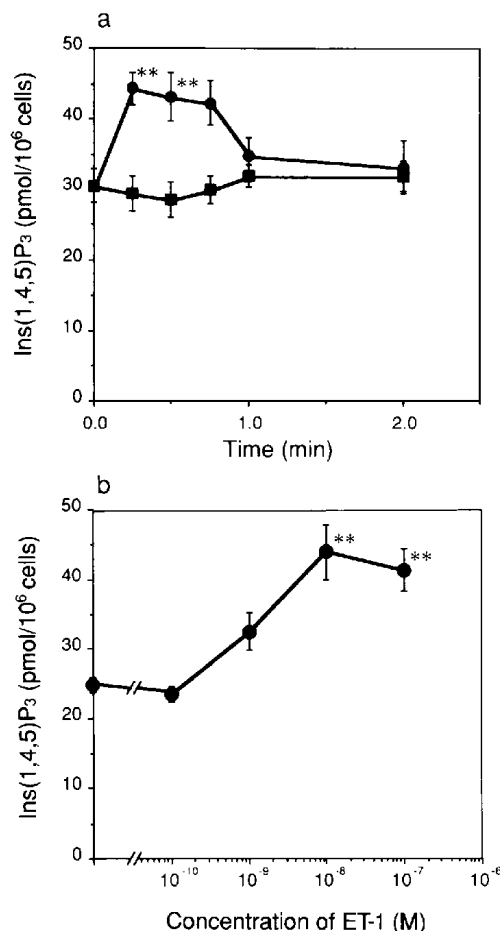


Fig. 5. Effect of ET-1 on inositol 1,4,5-triphosphate, (Ins(1,4,5)P₃) production. (a), Time course of changes in Ins(1,4,5)P₃ when HASMCs were stimulated with 1×10^{-8} M ET-1 with (■) or without 1×10^{-6} M FR139317 (●). ** $P < 0.01$ vs. the group without ET-1. (b), Dose-response curves of ET-1-induced increase in Ins(1,4,5)P₃ at 15 sec after the addition of ET-1. ** $P < 0.01$ vs. the group at time 0. The data represent the mean \pm S.E.M. of three determinations.

HASMC membrane receptors was inhibited by non-labelled ET-1 and ET-2 with K_i values of 3.8 nM and 5.7 nM, respectively. In contrast, ET-3 ($K_i = 375$ nM) showed 100-fold weaker displacement. These results suggest that the HASMC ET-receptor subtype is of the previously reported ET_A type (18, 19, 21).

Measurement of $[Ca^{2+}]_i$ with fura-2 demonstrated a biphasic pattern of ET-1 induced Ca^{2+} mobilization: an initial transient increase due to intracellular mobilization, most likely mediated by Ins(1,4,5)P₃, and a second sustained plateau phase. The second plateau phase was dependent on the presence of extracellular Ca^{2+} , and was not blocked by nicardipine (1×10^{-6} M), a dihydropyridine Ca^{2+} channel antagonist (data not shown). A high dose of nicardipine (1×10^{-4} M) attenuated the second plateau phase. This result suggests that the second

plateau phase was due mainly to Ca^{2+} influx across the plasma membrane. In rat vascular smooth muscle-derived A 10 cells, nicardipine (1×10^{-6} M) did not inhibit the plateau phase of the $[Ca^{2+}]_i$ response to ET (22). Similar results with cultured vascular smooth muscle cells were recently reported by Mitsuhashi et al. (23). Yanagisawa et al. (1) proposed that ET-1 acts primarily through dihydropyridine-sensitive Ca^{2+} channels. However, stimulation of Ca^{2+} influx insensitive to dihydropyridine Ca^{2+} channel antagonists is commonly observed with numerous Ca^{2+} -mobilizing hormones in various types of cells (24). We investigated the existence of L-type Ca^{2+} channels by the addition of KCl or Bay K 8644. These compounds have induced relatively small changes in $[Ca^{2+}]_i$ as compared with the changes they induced in other cells (data not shown). Gardner et al. (9) reported that the density or activity of voltage-sensitive Ca^{2+} channels in cultured human umbilical artery vascular smooth muscle cells is small. They suggest that the small increase in Ca^{2+} uptake induced by depolarization was taken up by the endoplasmic reticulum or recycled back to the medium. It would be interesting to know more clearly the mechanism by which ET induces activation of Ca^{2+} channels in HASMCs and other type of cells.

We demonstrated that caffeine and ryanodine abolished the ET-1-induced early mobilization of $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} . These two compounds were shown to induce the opening of Ca^{2+} channels in the endoplasmic reticulum (25, 26); in the present study, repeated exposure presumably exhausted the Ca^{2+} pool of the endoplasmic reticulum. In these conditions, ET-1-induced Ca^{2+} mobilization from intracellular Ca^{2+} stores was decreased. Several studies have demonstrated that ET-1 activates phospholipase C to produce IP₃ in rat vascular smooth muscle (27–29) and human umbilical artery (9). Our findings in the present study that ET-1 stimulates phospholipase C in cultured human aortic vascular smooth muscle cells confirm these observations.

In conclusion, the present results indicate that activation by ET-1 of the ET_A receptor is coupled to phosphoinositide hydrolysis and involves a combination of Ca^{2+} mobilization from intracellular caffeine-sensitive Ca^{2+} stores and Ca^{2+} influx from the extracellular component. These results suggest that these transmembrane signaling pathways play an important role in mechanisms of ET-1-induced vasoconstriction in human aortic vascular smooth muscle. The availability of this cell will facilitate the study of its pathology in human disease states.

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