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Comparison of the Effects of Endothelin-1, -2 and -3 (1–31) on Changes in $[Ca^{2+}]_i$ in Human Coronary Artery Smooth Muscle Cells

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ABSTRACT—We have previously found that human chymase selectively cleaves big endothelins (ETs) at the Tyr^{31}-Gly^{32} bond to produce 31-amino-acid endothelins, ETs (1–31). In the present study, we investigated the effects of ETs (1–31) on changes in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in cultured human coronary artery smooth muscle cells (HCASMCs) using confocal laser microscopy. ETs (1–31) increased $[Ca^{2+}]_i$ in a concentration-dependent manner. Phosphoramidon did not inhibit the increases in $[Ca^{2+}]_i$ caused by ETs (1–31). The $[Ca^{2+}]_i$ increases induced by ETs (1–31) were compared to those of ETs (1–21) and big ETs. ET-1 (1–21) was about 10-times more potent than big ET-1 or ET-1 (1–31), whereas big ET-2 was 10-times less potent than ET-2 (1–21) or ET-2 (1–31). ETs (1–31) may induce $[Ca^{2+}]_i$ increase through ET_A-type or ET_A-type-like receptors. The 10^{-12} M ET (1–31)-induced increases in $[Ca^{2+}]_i$ were not affected by removal of extracellular Ca^{2+}, but were inhibited by thapsigargin. These results suggested that ET-1, -2 and -3 (1–31) showed similar potencies in increasing $[Ca^{2+}]_i$ and mechanisms of ET (1–31)-induced increases in $[Ca^{2+}]_i$ may be similar among the three ETs (1–31).

Keywords: Endothelins (1–31), Endothelins (1–21), Human chymase, Confocal laser microscopy, Intracellular free Ca^{2+}

Endothelins (ET-1, -2 and -3) are 21 amino acid polypeptides that exhibit various physiological functions such as vascular contraction (1), cardiac hypertrophy (2) and mitogenesis (3). Human ETs are generated from their precursor, big endothelins (big ETs), through cleavages of the Trp^{21}-Val^{22} bond via the action of endothelin-converting enzyme (ECE). Although ECE was originally shown to be a chymotrypsin-like serine protease (1), several other enzymes have also been shown to catalyze the formation of ET-1 from big ET-1 (4). For example, rat mast cell chymase has been identified as a putative converter of big ET-1 to ET-1 (5).

Recently, we demonstrated that human mast cell chymase selectively cleaves big ETs at the Try^{31}-Gly^{32} bond to produce novel trachea-constricting 31-amino-acid length endothelins, ETs (1–31) (ET-1, -2 and -3 (1–31)), without any further degradation products (6). We have reported that ET-1 (1–31) increases intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in human coronary artery smooth muscle cells (HCASMCs) (7) and also observed that ET-1 (1–31) causes contraction of microperfused rabbit afferent and efferent arterioles (8). In the present study, we investigated the effects of ETs (1–31) on changes in $[Ca^{2+}]_i$ in HCASMCs using confocal laser microscopy and compared the potencies of three ETs (1–31). In addition, the effects of ETs (1–31) were compared to those of ETs (1–21) and big ETs. We also compared the characteristics of ETs (1–31)-induced intracellular Ca^{2+} signaling in HCASMCs.

MATERIALS AND METHODS

Cell preparation and culture

Human coronary artery smooth muscle cells at passage 4 as a commercially available product were obtained from Clonetics Corp. (San Diego, CA, USA). Cells were plated

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in 25 cm^2 tissue culture flasks at a density of 5×10^3 cells /cm^2 in MCDB131 medium supplemented with 5% heat-inactivated fetal calf serum, 0.5 ng/ml epidermal growth factor, 1 ng/ml basic fibroblast growth factor, 5 μg/ml insulin, 50 μg/ml gentamicin and 0.25 μg/ml amphotericin B. The cells were incubated at 37℃ in 5% CO_2 and the medium was replaced every other day until 60−80% confluent. Then, the cells were removed from the flasks with 0.025% trypsin plus 0.01% EDTA and washed twice with HEPES buffer solution (30 mM HEPES, 130 mM NaCl, 3.0 mM KCl, 3.0 mM Na_2SO_4, 13.3 mM NaOH, 10 mM glucose). Thereafter, the cells were seeded onto glass cover-slips attached to 35-mm tissue culture dishes coated with poly-L-lysine purchased from MatTek Corp. (Ashland, MA, USA). All experiments were performed with the cells in passages 5−15 and at 2−3 days post-confluency.

Loading of Ca^{2+} indicator fluo-3 into cells

The culture medium was removed from the dishes and replaced with modified Krebs-Henseleit bicarbonate buffer solution (K-H solution) (135 mM NaCl, 5.6 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.2 mM $CaCl_2$, and 10 mM glucose, adjusted with HCl to pH 7.40) and oxygenated with a 95% O_2 and 5% CO_2 gas mixture. Then the cells were loaded with the ester form of the dye (fluo-3/acetoxymethylester). For this purpose, the cells were incubated at 37℃ with fluo-3/AM at the final concentration of 4 μM. After a loading period of 30 min, the solution was exchanged with a dye-free K-H solution, and the cells were allowed to de-esterify the indicator for an additional 10 min. The low ester concentration was chosen to minimize problems arising from compartmentalization of the indicator (9).

Measurement of fluorescence intensity with confocal laser microscopy

The equipment for confocal fluorescence measurements has been described in detail recently (7, 10). Briefly, the confocal imaging system (RCM 8000; Nikon, Tokyo) with an Argon-ion laser was attached to an inverted microscope (Diaphot TDM 300, Nikon). Cells in the culture dish with 1 ml of K-H solution were placed on the stage of the microscope and the fluorescence in the cells excited at 488 nm by the laser. Emission at wavelengths longer than 520 nm was then detected by a photomultiplier. The system scanned full-field images at 30 frames/s, but the images were obtained by averaging eight successive frames in order to improve the signal-to-noise ratio. The objective lens used was a CF Fluor 20X/NA0.75 (Nikon). After measurement of stable baseline fluorescence intensity, 10 μl of an agent was added to the extracellular medium to yield a 1/100 concentration, and then the fluorescence intensity was recorded. After 1 min, the same cells were stimulated by 10 μM ionomicin and the relative fluorescence intensity calculated. For experiments with protease inhibitors and ET-receptor antagonists, phosphoramidon, Bowman-Birk soybean inhibitor (BBI), thiorphan, BQ123 and BQ788 were added to the incubation medium throughout dye loading and the experiments. For experiments of the inhibition of Ca^{2+} release from intracellular stores, thapsigargin, an inhibitor of sarcoplasmic reticulum Ca^{2+}-ATPase was added during dye loading and the experiments. These experiments were performed under Ca^{2+}-free conditions. Calibration of the fluo-3 fluorescence intensity, to estimate $[Ca^{2+}]_i$, was calculated from the difference between F_{max} and F_{min}. F_{min} was defined as the minimum fluorescence intensity before stimulation with each agonist. F_{max} was estimated from the maximum intensity after addition of ionomycin in each experiment. Results were expressed as percentages of the difference between F_{max} and F_{min}. For data analysis, confocal microscopic images were stored on a Panasonic magnetico-optical disk (Matsushita Electric Industrial Co., Ltd., Osaka). Sequences of digitized images were transferred to an IBM-Think Pad computer equipped with image-processing software. Data were presented as the mean±S.E.M.

Statistics

One-way ANOVA was used to determine the significance of the difference among groups, after which the modified *t*-test with the Bonferroni correction was used for comparison between individual groups. Statistic significance between two groups was examined by Student's *t*-test and Mann-Whiteney *U*-test. A value of $P<0.05$ was considered to be statistically significant.

Materials

Human ETs (1−21), big ETs and phosphoramidon were obtained from the Peptide Institute (Osaka). ETs (1−31) were synthesized by solid-phase procedures, also at the Peptide Institute. MCDB131 medium, fetal calf serum, epidermal growth factor, insulin, gentamicin, amphotericin B and trypsin were obtained from Clonetics Corp.; fluo-3/acetoxymethylester and ionomycin were purchased from Wako Pure Chemical Co. (Osaka); BQ123 and BQ788 were gifts from Banyu Pharmaceutical Co. (Tsukuba); and thapsigargin was obtained from Sigma (St. Louis, MO, USA). All other chemicals used were commercial products of reagent grade.

RESULTS

Concentration-dependent increases in $[Ca^{2+}]_i$ induced by ETs (1−31)

Figures 1A, B and C show the concentration-response curves for the increases in $[Ca^{2+}]_i$ induced by ET-1, -2 and -3 (1−31), respectively, in comparison to those of ETs (1−21) and big ETs. ETs (1−31) caused increases in $[Ca^{2+}]_i$ in a concentration-dependent manner from 10^{-14} to 10^{-10} M. The $[Ca^{2+}]_i$ increases by these ETs reached peaks within 10 s and then gradually decreased to the baseline value within 1 min. ET-1 (1−21) was about 10-times more potent than big ET-1 or ET-1 (1−31). Big ET-2 was about 10-times less potent than ET-2 (1−21) or ET-2 (1−31). The three ETs-3 increased $[Ca^{2+}]_i$ similarly.

Possibility of the conversion of ETs (1−31) to ETs (1−21) by ECE

To investigate the possibility that ETs (1−31)-induced increases in $[Ca^{2+}]_i$ are due to the degradation of ETs (1−31) to ETs (1−21) by ECE in the incubation medium or the cells, we examined the effects of ECE inhibitors on ETs (1−31)-induced increases in $[Ca^{2+}]_i$. As shown in Table 1, phosphoramidon (10^{-5} and 10^{-4} M), which is known as a potent inhibitor of ECE (11), failed to inhibit the increases in $[Ca^{2+}]_i$ caused by 10^{-12} M of ETs (1−31). BBI (10^{-5} M) and thiorphan (10^{-5} M) also did not inhibit the increases in $[Ca^{2+}]_i$ caused by 10^{-12} M of ETs (1−31) (Table 2). These results suggest that the effects of ETs (1−31) are due not to the conversion of ETs (1−31) to ETs (1−21), but to the effects of ETs (1−31) themselves.

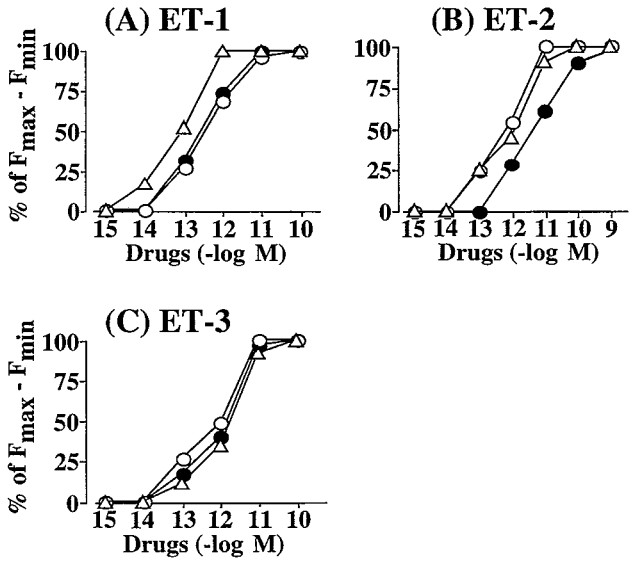

Fig. 1. Concentration-response curves for ETs (1−31)-induced increases in $[Ca^{2+}]_i$ in cultured human coronary artery smooth muscle cells (HCASMCs) in comparison with the effects of ETs (1−21) and big ETs. Values are expressed as % difference of F_{max} and F_{min} as described in the text (mean of 5 separate experiments). The maximal standard error was 6.5% of F_{max}-F_{min}. To avoid complicating the figures, error bars are omitted. ○: ETs (1−31), △: ETs (1−21), ●: big ETs.

On the other hand, big ETs-induced increases in $[Ca^{2+}]_i$ were inhibited by phosphoramidon (Table 1). From these results, it is suggested that the effects of big ETs are due in part to the conversion of big ETs to ETs (1−21).

Table 1. Effects of phosphoramidon on ETs-induced increases in $[Ca^{2+}]_i$

		ET-1	ET-2	ET-3
		Increase in $[Ca^{2+}]_i$ (% of F_{max}-F_{min})		
	Phosphoramidon (10^{-5} M)			
1−21	(−)	51.9±4.8	45.0±4.5	35.0±5.6
	(+)	50.1±5.1	43.0±4.6	35.0±6.0
big	(−)	68.5±5.6 *	61.0±5.7 *	40.0±6.9
	(+)	48.2±5.2	43.8±1.6	32.1±6.4
1−31	(−)	66.3±6.1	54.0±5.7	49.0±6.1
	(+)	62.5±6.3	52.0±5.9	47.0±5.0
	Phosphoramidon (10^{-4} M)			
big	(−)	68.8±3.8 *	65.6±9.1 *	39.7±2.0 *
	(+)	36.1±6.0	33.5±5.6	19.9±3.9
1−31	(−)	67.7±4.3	49.1±2.6	45.7±1.4
	(+)	64.8±4.3	49.1±5.5	43.2±5.0

Values are expressed as % difference of F_{max} and F_{min} as described in the text (means±S.E.M. of 5−10 separate experiments). The asterisk (*) indicates that there is a statistically significant difference between the values with or without phosphoramidon ($P<0.05$).

Table 2. Effects of protease inhibitors on ETs-induced increases in $[Ca^{2+}]_i$

	ET-1 (1−31)	ET-2 (1−31)	ET-3 (1−31)
	Increase in $[Ca^{2+}]_i$ (% of F_{max}-F_{min})		
ET (1−31) only	66.2±6.1	48.1±4.5	48.5±5.0
ET (1−31) + Thiorphan (10^{-5} M)	61.6±5.0	50.7±8.3	47.8±1.1
ET (1−31) + BBI (10^{-5} M)	62.1±5.0	53.2±6.6	45.3±2.0

Values are expressed as % difference of F_{max} and F_{min} as described in the text (means±S.E.M. of 5−9 separate experiments).

Possible involvement of ET_A- or ET_A-like receptor

To investigate whether ETs (1−31)-induced increases in $[Ca^{2+}]_i$ are a receptor-mediated phenomena, we examined the effects of known endothelin receptor antagonists on the increases in $[Ca^{2+}]_i$ evoked by ETs (1−31). Since it has been reported that there are at least two main subtypes of endothelin receptors, termed ET_A and ET_B (12), we examined the effects of a selective ET_A antagonist, BQ123 (13), and a selective ET_B antagonist, BQ788 (14), on ETs (1−31)-induced increases in $[Ca^{2+}]_i$. As shown in Fig. 2A, BQ123 at 10^{-10} M almost abolished the increase in $[Ca^{2+}]_i$ induced by 10^{-12} M ET-1 (1−31), whereas BQ788 at 10^{-10} M failed to inhibit the $[Ca^{2+}]_i$ increase. As shown in Figs. 2B and C, we also observed inhibition by BQ123, but not by BQ788, on both ET-2 and -3 (1−31)-induced increases in $[Ca^{2+}]_i$. These results suggest that the stimulatory effects of ETs (1−31) on the increase in $[Ca^{2+}]_i$ are mediated by ET_A- or ET_A-like receptors.

Ca^{2+} mobilization from intracellular stores or extracellular spaces

Figures 3A, B and C show the responses of HCASMCs to 10^{-12} M ET-1, -2 and -3 (1−31), respectively, in the presence or absence of extracellular Ca^{2+}. ETs (1−31)-induced increases in $[Ca^{2+}]_i$ were not affected by removal of Ca^{2+} from the medium. However, they were almost abolished by thapsigargin (10^{-5} M), a specific inhibitor of the sarcoplasmic reticulum Ca^{2+} pump under Ca^{2+}-free conditions. From these results, it is assumed that ETs (1−31)-induced increases in $[Ca^{2+}]_i$, at least at 10^{-12} M concentration, are attributable to the release of Ca^{2+} from intracellular stores rather than an influx from extracellular spaces. We recently reported that 10^{-12} M ET-1 (1−31) did not evoke Ca^{2+} influx into human coronary artery smooth muscle cells examined with $^{45}CaCl_2$ (15).

DISCUSSION

Human chymase is known to convert angiotensin I to angiotensin II (16). We have recently found that human mast cell chymase specifically cleaved big ETs at the Try^{31}-Gly^{32} bond and produced novel trachea-constricting 31 amino acid length peptides, ETs (1−31), which are

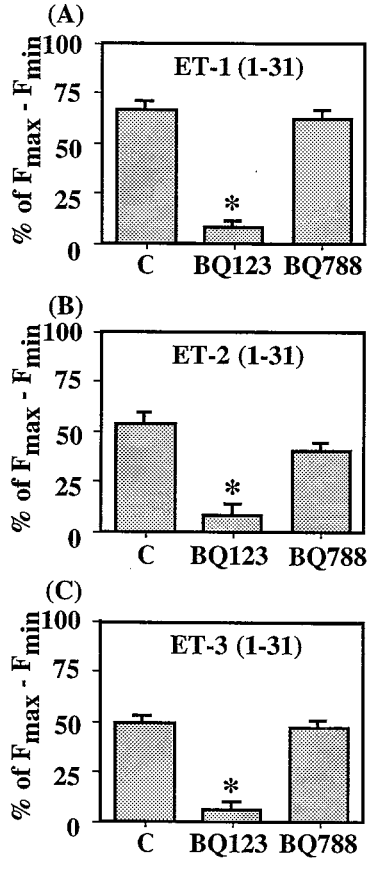

Fig. 2. Effects of BQ123 (10^{-10} M) and BQ788 (10^{-10} M) on ETs (1−31)-induced increases in $[Ca^{2+}]_i$ in HCASMCs. Values are expressed as % difference of F_{max} and F_{min} as described in the text. The ETs (1−31) concentration of 10^{-12} M was chosen as the half maximal doses for the responses of HCASMCs. Data are means± S.E.M. of 5 separate experiments. Inhibitors were added to the incubation medium throughout dye loading and experiments. C: ETs (1−31) (10^{-12} M), BQ123: ETs (1−31) (10^{-12} M) + BQ123 (10^{-10} M), BQ788: ETs (1−31) (10^{-12} M) + BQ788 (10^{-10} M). The asterisk (*) indicates that there is a statistically significant difference from the value induced by ETs (1−31) alone ($P<0.05$).

different in amino acid length from the well-known 21 amino acid ETs (1-21) (6). It has also been reported that a serine protease in the membrane fraction of human lung hydrolyses big ET-1 to ET-1 (1-31), which shows a contractile activity in the pulmonary artery (17). Recently, we found that ET-1 (1-31) causes a rise in $[Ca^{2+}]_i$ in cultured HCASMCs (7). We also observed that ET-1 (1-31) induces contraction of rabbit afferent arterioles (8). It is therefore thought that ETs (1-31) are novel and alternative vasoactive peptides produced by human chymase.

With regard to ETs (1-21), Inoue et al. (18) reported that the contractile activity of ET-1 was most potent, whereas the maximum contractile tension was greatest with ET-2 (1-21) in endothelium-denuded porcine coronary artery strips. Furthermore, they reported that ET-3 was the least potent among the three peptides. Masaki et al. (19) reported that the ranking is ET-1 = ET-2 > ET-3 for contractile activity in vessels from a variety of animal species. However, our results revealed that ET-1 (1-21) was about 10-times more potent than ET-2 (1-21) or ET-3 (1-21) in increasing $[Ca^{2+}]_i$ in HCASMCs (Fig. 1). These seemingly inconsistent resuults among studies may be attributed to the agonist concentrations used, which may vary depending on the sensitivity of the procedure. Although earlier studies used relatively high concentrations (nanomolar range) of ET-1 (1-21) (20, 21) our results revealed that ETs (1-21) increased $[Ca^{2+}]_i$ at the picomolar range, as measured by confocal laser microscopy. Our results are consistent with other observations that ET-1 (1-21) causes contraction of rat renal afferent arterioles at concentrations around 10^{-12} M (22). We also observed that ET-1 (1-21) causes a contraction of microperfused rabbit afferent arterioles at 10^{-12} M (unpublished data). One alternative explanation for ETs (1-21) potency is the difference of receptor densities among species and organs. In rat tracheal smooth muscle, we observed that the contractile activity was ET-1 (1-21) > ET-2 (1-21) = ET-3 (1-21) (6), whereas its ranking was ET-1 (1-21) > ET-2 (1-21) > ET-3 (1-21) in porcine coronary artery (23). On the other hand, ET-1, -2 and -3 (1-31) showed similar potencies in increasing $[Ca^{2+}]_i$ in HCASMCs (Fig. 1). These results are consistent with our previous finding that ET-1, -2 and -3 (1-31) caused similar constriction of porcine coronary artery (23). Although the receptors for ETs (1-31) have not been identified these results suggest the existence of specific receptor(s) for ETs (1-31) distinct from ETs (1-21). Regardless of this, it should be noted that ET-2 and -3 (1-31) were almost equipotent to ET-2 and -3 (1-21) in increasing $[Ca^{2+}]_i$ in HCASMCs. In addition, we have found that ETs (1-31) have always been detected in human hearts, in concentrations similar to those of ETs (1-21) (unpublished data), suggesting that ETs (1-31) play a role as novel vasoactive peptides.

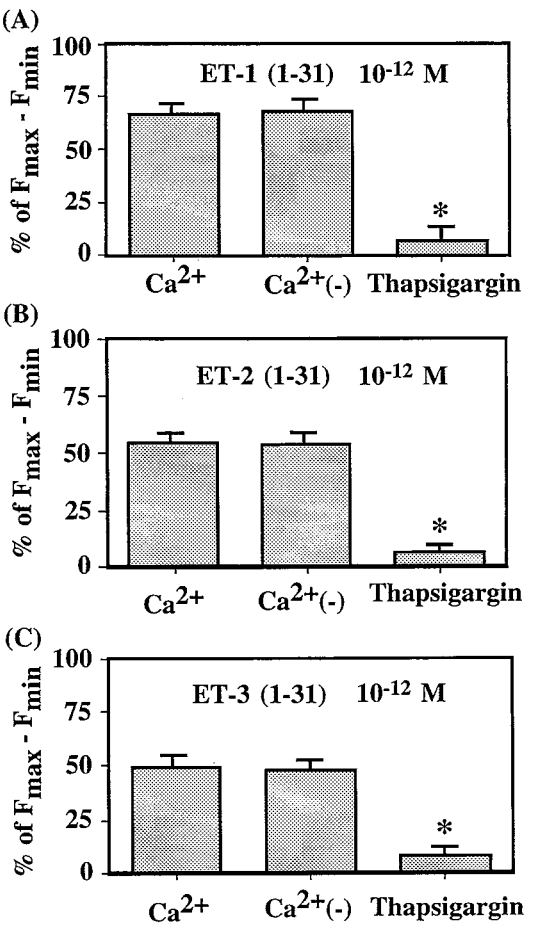

Fig. 3. Effects of extracellular Ca^{2+} removal on ETs (1-31)-induced rise in $[Ca^{2+}]_i$ in HCASMCs and their inhibition by thapsigargin, a specific inhibitor of the sarcoplasmic reticulum Ca^{2+} pump. The 10^{-12} M ETs (1-31)-induced changes in $[Ca^{2+}]_i$ were measured in the presence or absence of extracellular Ca^{2+}. Thapsigargin (10^{-5} M) was added to the incubation medium throughout dye loading and experiments, which were performed under Ca^{2+}-free conditions. Values are expressed as % difference of F_{max} and F_{min} as described in the text (mean±S.E.M. of 5 separate experiments). The values with thapsigargin in Ca^{2+}-free medium were significantly lower than the value evoked by ETs (1-31) in normal medium (*: $P<0.05$).

As shown in Table 1, the effects of ETs (1-31) are due not to the conversion of ETs (1-31) to ETs (1-21), but to the effects of ETs (1-31) themselves, because phosphoramidon, a known inhibitor of ECE, failed to inhibit ETs (1-31)-induced effects. We also confirmed that the inhibitors of other type of proteases, BBI and thiorphan, did not inhibit ETs (1-31)-induced $[Ca^{2+}]_i$ increases (Table 2). Furthermore, our results suggest that the effect of big ETs is due in part to the conversion of big ETs to ETs (1-21) (Table 1). It should be noted that ETs (1-31) themselves have biological activities in HCASMCs, regardless of whether they are degraded to ETs (1-21).

The stimulatory effects of ETs (1−31) on increases in $[Ca^{2+}]_i$ are thought to be mediated through the ET_A receptor or an ET_A-like receptor because ET_A-receptor antagonists almost completely abolished the ETs (1−31)-induced phenomena (Fig. 2). As for ET-1 (1−21), it has been reported that the ET_A receptor has a high affinity for ET-1 (1−21) and ET-2 (1−21), but not for ET-3 (1−21) in vascular smooth muscle cells (24, 25). However, as we described above, our rankings of the potencies were ET-1 (1−21) > ET-2 (1−21) = ET-3 (1−21) and ET-1 (1−31) = ET-2 (1−31) = ET-3 (1−31). These results suggest the existence of receptor(s) for ETs (1−31) as mentioned above. Although we have not determined whether or not the receptors for ETs (1−31) are identical to the ET_A receptor for ETs (1−21), preliminary experiments have indicated that ET-1 (1−31) does not compete with the binding of ^{125}I-labeled ET-1 (1−21) to ET_A and ET_B receptors expressed in CHO cells (unpublished data). Taken together, the possibility remains that ETs (1−31) exert their activities through unknown receptors, which are similar but not identical to the ET_A receptor.

As shown in Fig. 3, 10^{-12} M ETs (1−31)-induced-increases in $[Ca^{2+}]_i$ were not affected by removal of extracellular Ca^{2+}, but were inhibited by thapsigargin, a specific inhibitor of the sarcoplasmic reticulum Ca^{2+} pump. From these results, it is assumed that at picomolar concentrations, the ETs (1−31)-induced increases in $[Ca^{2+}]_i$ are attributable to the release of Ca^{2+} from intracellular stores. Considering the findings that tissue and blood contents of ET-1 (1−21) are around the picomolar range (26, 27), it is reasonable to speculate that the physiological activity of ETs (1−31) is attributable to Ca^{2+} mobilization from intracellular stores rather than influx of Ca^{2+} from the extracellular space.

In conclusion, ET-1, -2 and -3 (1−31) showed similar concentration-dependent increases in $[Ca^{2+}]_i$ in HCASMCs. The stimulatory effects of an ETs (1−31) on increases in $[Ca^{2+}]_i$ are mediated by the ET_A receptor or ET_A-like receptor, although the possibility for other receptor types cannot be excluded. At the picomolar range, ETs (1−31)-induced increases in $[Ca^{2+}]_i$ are attributable to the release of Ca^{2+} from intracellular stores. We are now investigating the tissue contents of ETs (1−31) in humans.

Acknowledgments

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