



Endothelin generating pathway through endothelin_{1–31} in human cultured bronchial smooth muscle cells

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1 The effects of endothelin (ET)-1_{1–31} and ET-2_{1–31}, human chymase products of the corresponding big ETs, on the intracellular free Ca²⁺ concentration ([Ca²⁺]_i) and [¹²⁵I]-ET-1 binding were investigated using human cultured bronchial smooth muscle cells (BSMC).

2 ET-1_{1–31} (10^{–8} M – 3 × 10^{–7} M) and ET-2_{1–31} (3 × 10^{–8} M – 3 × 10^{–6} M) caused an increase in [Ca²⁺]_i in a concentration-dependent manner. Big ET-1 (3 × 10^{–8} M – 10^{–6} M) also caused this increase, but not big ET-2 at concentrations up to 10^{–6} M. The [Ca²⁺]_i increase induced by ET-1 was inhibited by both BQ123, an ET_A-receptor antagonist, and BQ788, an ET_B-receptor antagonist, whereas that induced by ET-3 was inhibited by BQ788 but not by BQ123.

3 Increases in [Ca²⁺]_i caused by ET-1_{1–31}, big ET-1 and ET-2_{1–31} were completely inhibited by 10^{–4} M phosphoramidon, a dual neutral endopeptidase (NEP)/endothelin-converting enzyme (ECE) inhibitor, and 10^{–5} M thiorphan, a NEP inhibitor.

4 Scatchard plot analyses of the saturation curves of [¹²⁵I]-ET-1 and [¹²⁵I]-ET-3 showed that both ET_A- and ET_B-receptors at the ratio of 4:1 were expressed on BSMC. ET-1_{1–31}, big ET-1 and ET-2_{1–31} inhibited [¹²⁵I]-ET-1 binding in a concentration-dependent manner, and these effects were attenuated by treatment with thiorphan. On the other hand, big ET-2 slightly inhibited the binding at a high concentration and this was not affected by thiorphan.

5 These results suggest that ET-1_{1–31}, big ET-1 and ET-2_{1–31} cause an increase in [Ca²⁺]_i by being converted into the corresponding ET-1 and ET-2 by NEP, but this did not occur with big ET-2 in human BSMC. ET-2_{1–31} produced by human chymase from big ET-2 might be important for the generation of ET-2 in human bronchial tissue.

Keywords: Endothelin; big endothelin; endothelin converting enzyme; neutral endopeptidase; chymase; bronchial smooth muscle cells

Abbreviations: BSMC, bronchial smooth muscle cells; [Ca²⁺]_i, intracellular free Ca²⁺ concentration; ECE, endothelin-converting enzyme; ET, endothelin; NEP, neutral endopeptidase

Introduction

Endothelin (ET), a highly potent vasoactive peptide containing 21 amino acid residues, exists as three different isoforms as ET-1, ET-2, and ET-3 (Inoue *et al.*, 1989). All three isoforms are generated from the corresponding big forms through a specific cleavage between Trp²¹ and Val²² (ET-1, ET-2) or Trp²¹ and Ile²² (ET-3). ET converting enzyme (ECE) is a membrane-bound neutral metalloprotease. Two distinct ECEs, termed ECE-1 and ECE-2, have so far been identified (Schmidt *et al.*, 1994; Xu *et al.*, 1994; Emoto & Yanagisawa, 1995). Enzyme activities of ECE-1 and ECE-2 are inhibited by phosphoramidon, a dual neutral endopeptidase (NEP)/ECE inhibitor, but not thiorphan, a NEP inhibitor (Turner & Murphy, 1996). Both ECEs are structurally related to NEP-24.11, and enzyme activity of NEP-24.11 is inhibited by phosphoramidon and thiorphan. NEP-24.11 is a plasma membrane-bound zinc metalloprotease that was initially isolated from renal epithelial brush border cells and cleaves peptide substances at the amino side of hydrophobic amino acids (Erdös & Skidgel, 1989). It is well known that NEP-24.11 efficiently cleaves potent vasoactive peptides such as ETs, substance P, bradykinin, arterial natriuretic peptide, C-type natriuretic peptide and angiotensins (Roques *et al.*, 1993).

Recently, Hanson *et al.* (1997) reported that a serine protease in human lungs hydrolyzes big ET-1 to ET-1_{1–31}, which has a vasocontractile activity. Furthermore, it has been reported that human mast cell chymase specifically converts big ETs to ETs_{1–31} which have contractile activity in the trachea, without any further degradation products (Nakano *et al.*, 1997). In the present study, we examined the effects of synthetic ET-1_{1–31} and ET-2_{1–31} on the intracellular free calcium concentration ([Ca²⁺]_i) and [¹²⁵I]-ET-1 binding using human cultured bronchial smooth muscle cells (BSMC). In addition, we analysed the mechanisms of the response to ET-1_{1–31} and ET-2_{1–31} using various protease inhibitors.

Methods

Cell culture

Human bronchial smooth muscle cells isolated from the bronchus of a 21-year-old male donor by the explant method were purchased from Clonetics (San Diego, U.S.A.). The identity of human bronchial smooth muscle cells was verified by immunohistochemistry for α -smooth muscle actin. Greater than 95% of cells were α -actin smooth muscle immunopositive. The cells were cultured in MCDB131 medium supplemented with 5% foetal bovine serum, 0.5 ng ml^{–1} epidermal growth factor, 50 μ g ml^{–1} insulin, 2 ng ml^{–1} fibroblast growth factor,

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50 $\mu\text{g ml}^{-1}$ gentamicin and 50 ng ml^{-1} amphotericin B. All experiments were performed with the cells in passages 6–7.

Measurement of $[\text{Ca}^{2+}]_i$

Cells were grown in 75-cm² tissue culture flasks and removed from the flasks with 0.025% trypsin plus 0.01% EDTA. Cell suspensions were washed once with the culture medium. Cells were counted and resuspended in the culture medium to a final concentration of 10^6 cells ml^{-1} . The cell suspensions were incubated with 1 μM fura-2/acetoxymethyl ester (fura-2/AM) at 37°C for 10 min. The loaded cells were resuspended in 0.5 ml of Tris-HEPES buffer solution (in mM: Tris 10, HEPES 10, KCl 5, MgCl_2 1.2, CaCl_2 1, NaCl 140, glucose 15, adjusted with HCl to pH 7.4) in a cuvette (50 \times 7 mm diameter) and stirred continuously. Various concentrations of ET derivatives in 5 μl of 0.1% bovine serum albumin were added. ET receptor antagonist or protease inhibitor in 5 μl of 10% DMSO was added at 5 min before addition of ET derivatives. Fluorescence measurements were done with a spectrofluorometer (CAF-110, Japan Spectroscopy, Inc., Tokyo, Japan). $[\text{Ca}^{2+}]_i$ was calculated according to the formula described by Grynkiewicz *et al.* (1985): $[\text{Ca}^{2+}]_i = [(R - R_{\min}) / (R_{\max} - R)] \cdot (S_f / S_b) \cdot K_D$, where R is the ratio of the 340/380 nm of the fluorescence signal, R_{\min} is the 340/380 ratio in calcium-free media with 10 mM EGTA added, R_{\max} is the 340/380 ratio in the presence of saturating calcium, and S_f / S_b is the ratio of the 380-nm fluorescence measured in a calcium-free solution to that measured in a calcium-replete solution. The K_D value for fura-2/AM is 224 nM (Grynkiewicz *et al.*, 1985).

Binding studies

^{125}I -ET-1 and ^{125}I -ET-3 bindings to the cells were done as described previously (Mihara *et al.*, 1994). The cells were cultured in 24-well culture plates. After 4–6 days, the culture medium was aspirated, and the cells were washed twice with ice-cold HEPES (20 mM)-buffered Hank's solution (pH 7.4). In the saturation experiments, the cells were incubated with increasing concentrations of ^{125}I -ET-1 and ^{125}I -ET-3 (10–700 pM) in 0.3 ml of HEPES-buffered Hank's solution containing 0.1 mM PMSF, 10 $\mu\text{g ml}^{-1}$ aprotinin, 10 $\mu\text{g ml}^{-1}$ leupeptin, 10 $\mu\text{g ml}^{-1}$ pepstatin A, 250 $\mu\text{g ml}^{-1}$ bacitracin and 10 $\mu\text{g ml}^{-1}$ soybean trypsin inhibitor. Nonspecific bindings were determined with the same concentrations of iodinated ETs in the presence of 10^{-7} M unlabelled ETs. In the competition experiments, each well was incubated with 12.5 pM ^{125}I -ET-1 in the absence and presence of various concentrations of unlabelled ET-1₁₋₃₁, big ET-1, ET-2₁₋₃₁ or big ET-2. Nonspecific binding was defined in the presence of 10^{-7} M ET-1 and was about 5% of the total binding. Protease inhibitor was added at 20 min before addition of unlabelled ET derivatives. Equilibrium binding studies were performed at 37°C for 60 min. After rapid removal of the incubation medium, 0.25 ml of ice-cold HEPES-buffered Hank's solution was added. Free ligand was removed by washing the intact attached cells twice with ice-cold HEPES-buffered Hank's solution. Cells were dissolved in 0.1 N NaOH and transferred to a test tube, then the radioactivity was counted.

Drugs

Human ET-1, ET-1₁₋₃₁, big ET-1, ET-2, ET-2₁₋₃₁, big ET-2, ET-3 and phosphoramidon (*N*-(α -rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan) were obtained from Peptide Institute (Osaka, Japan). Fura-2/acetoxymethylester

was purchased from Dojindo (Kumamoto, Japan). BQ123 and BQ788 were purchased from Neosystem Laboratoire (Strasbourg, France). ^{125}I -ET-1 and ^{125}I -ET-3 (81.4 Tbecq mmol^{-1}) were obtained from New England Nuclear (Boston, MA, U.S.A.). Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Statistics

The results shown in the text, table and figures are expressed as mean values \pm s.e.mean. The potency of the agonists was assessed as the negative logarithm of the concentration required to cause 50% of the maximum response (pD_2) using the logistic equation described by DeLean *et al.* (1978). Statistical analyses were made using Student's *t*-test and Tukey's method after one-way analysis of variance.

Results

Effect of ET derivatives on $[\text{Ca}^{2+}]_i$ in human BSMC

ET-1 (3×10^{-10} M– 10^{-7} M), ET-1₁₋₃₁ (10^{-8} – 3×10^{-7} M) and big ET-1 (3×10^{-8} – 10^{-6} M) induced a concentration-depen-

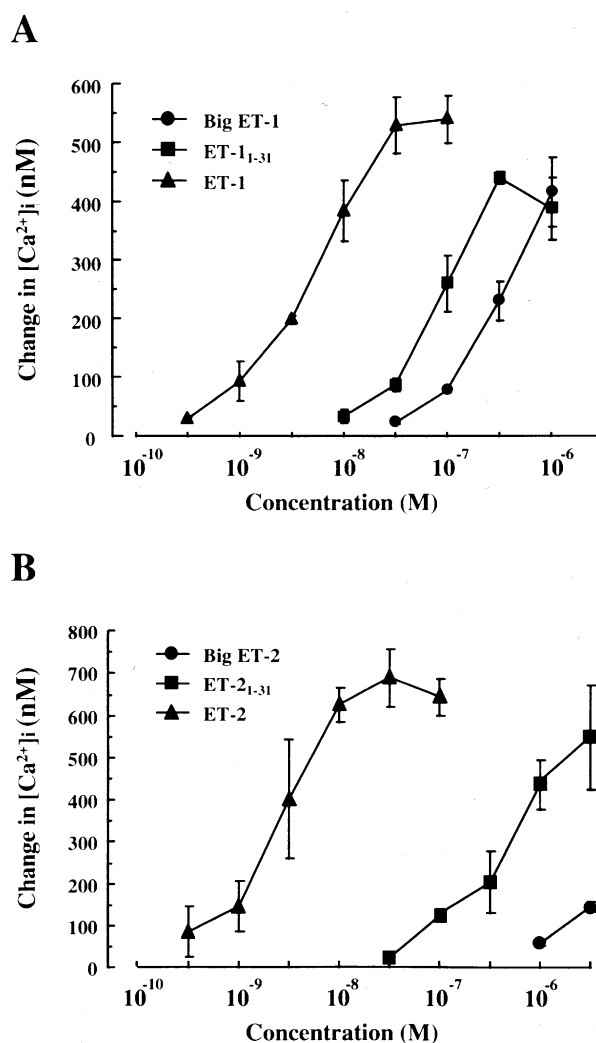


Figure 1 Concentration-response curves for increases in $[\text{Ca}^{2+}]_i$ induced by ET-1, ET-1₁₋₃₁ and big ET-1 (A) and ET-2, ET-2₁₋₃₁ and big ET-2 (B) in human cultured BSMC. Changes in $[\text{Ca}^{2+}]_i$ from basal level before addition of each stimulus were determined by fura-2/AM. Values are the mean \pm s.e.mean for three determinations.

dent increase in $[Ca^{2+}]_i$ with pD_2 values of 8.46 ± 0.08 ($n=3$), 7.23 ± 0.09 ($n=3$) and 6.76 ± 0.02 ($n=3$), respectively (Figure 1A). Thus, the effect of ET-1₁₋₃₁ was about three times more potent than big ET-1, although it was about 17 times less potent than ET-1. As shown in Figure 1B, ET-2 (3×10^{-10} – 3×10^{-8} M) and ET-2₁₋₃₁ (3×10^{-8} M– 3×10^{-6} M) also induced a concentration-dependent increase in $[Ca^{2+}]_i$, whereas big ET-2 (up to 10^{-6} M) did not show any increase in $[Ca^{2+}]_i$.

ET receptor subtypes expressed on cultured human BSMC

To analyse ET receptor subtypes expressed on BSMC, we examined the effects of ET_A- and ET_B-receptor antagonists on the increase in $[Ca^{2+}]_i$ induced by ET-1 or ET-3. As shown in Figure 2A, treatment with BQ123 (10^{-7} and 10^{-6} M) significantly inhibited the 10^{-7} M ET-1-induced increase in $[Ca^{2+}]_i$. Combined treatment with BQ123 (10^{-6} M) and BQ788 (10^{-6} M) suppressed the increase in $[Ca^{2+}]_i$ induced by ET-1 more efficiently than treatment with BQ123 alone. On the other hand, 10^{-7} M ET-3 slightly induced a $[Ca^{2+}]_i$ increase with about six times less potency than that of 10^{-7} M ET-1 (Figure 2B). BQ123 did not inhibit the increase in $[Ca^{2+}]_i$ induced by ET-3, whereas BQ788 strongly suppressed that induced by ET-3.

Effects of various protease inhibitors on $[Ca^{2+}]_i$ increases induced by ET₁₋₃₁ and big ETs

As illustrated in Figure 3A, the increases in $[Ca^{2+}]_i$ caused by 10^{-6} M ET-1₁₋₃₁ and 10^{-6} M big ET-1 were completely abolished by treatment with 10^{-4} M phosphoramidon. Similar

inhibition by phosphoramidon was observed in the $[Ca^{2+}]_i$ increase induced by the same concentration of ET-2₁₋₃₁ (Figure 3B, left). Although the effect of 10^{-6} M big ET-2 on $[Ca^{2+}]_i$ was extremely weak, it was not affected by phosphoramidon (Figure 3B, right). These results are summarized in Figure 4. Increases in $[Ca^{2+}]_i$ induced by ET-1 and ET-2 were not affected by 10^{-4} M phosphoramidon (data not shown).

To further characterize the phosphoramidon-sensitive converting enzyme that cleaves ET₁₋₃₁ and big ET-1 to corresponding ETs, the effects of various protease inhibitors on $[Ca^{2+}]_i$ increases induced by big ET-1 and ET-2₁₋₃₁ were examined. As shown in Table 1, both increases were significantly inhibited by thiorphan (an inhibitor of NEP) but not BBI (a nonpermeable inhibitor of trypsin- and chymotrypsin-type proteases) or chymostatin (a permeable inhibitor of chymotrypsin-type proteases).

$[^{125}I]$ -ET-1 and $[^{125}I]$ -ET-3 binding

As shown in Figure 5, the binding of $[^{125}I]$ -ET-1 and $[^{125}I]$ -ET-3 to cultured BSMC was saturable. Scatchard plot analyses of these saturation curves showed that the apparent dissociation constant (K_D) and maximal binding (B_{max}) values were 63.8 ± 6.9 pM and 29.6 ± 0.7 fmol per 10^5 cells for $[^{125}I]$ -ET-1 binding, and 50.9 ± 3.3 pM and 5.8 ± 0.4 fmol per 10^5 cells for $[^{125}I]$ -ET-3 binding, respectively (Figure 5, inset). These data indicated that 80% of ET receptors were ET_A-receptor subtype and 20% of those were ET_B-receptor subtype in BSMC.

In order to investigate the effect of thiorphan on the competition curves of ET-1₁₋₃₁, big ET-1, ET-2₁₋₃₁ and big ET-2, we examined the inhibition of the specific $[^{125}I]$ -ET-1 binding by various concentrations of these ET derivatives in

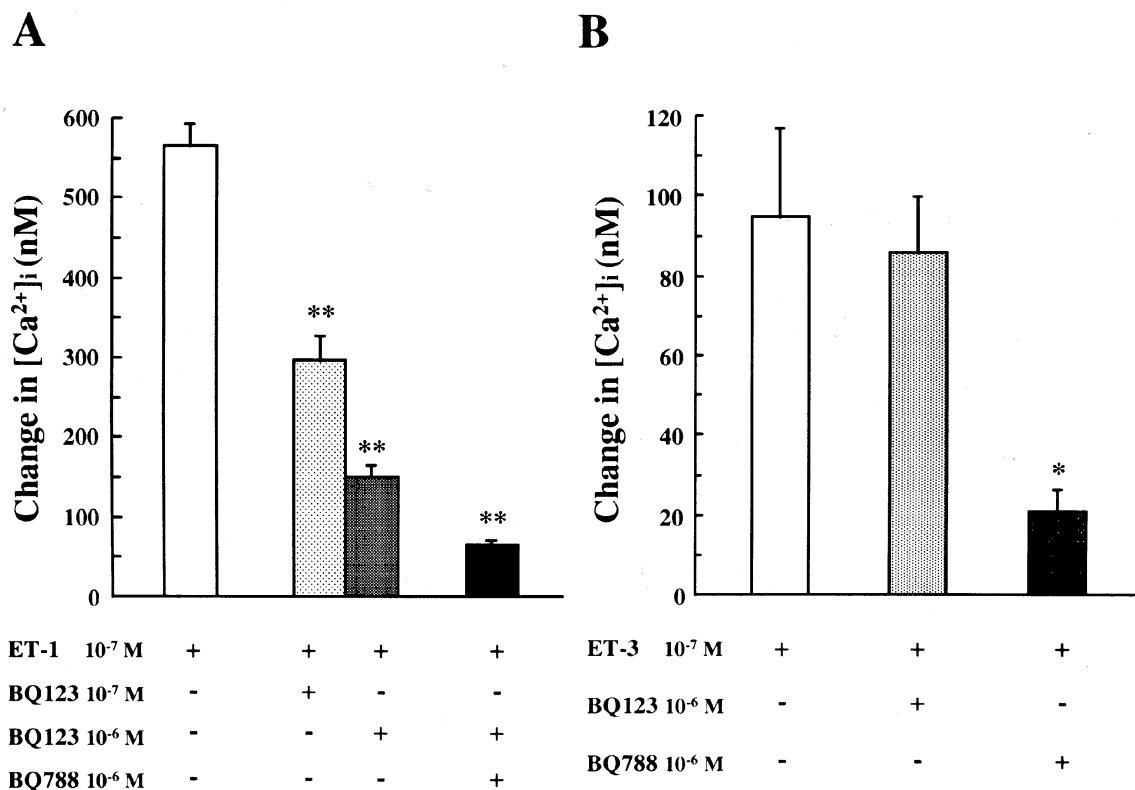


Figure 2 Effects of ET_A- and ET_B-receptor antagonists on $[Ca^{2+}]_i$ increases induced by ET-1 (A) and ET-3 (B) in human cultured BSMC. Values are expressed as the change in $[Ca^{2+}]_i$ from basal level before addition of each stimulus. The cell suspensions were treated for 5 min with BQ123 and BQ788 before addition of ET-1 (10^{-7} M) or ET-3 (10^{-7} M). Data are mean \pm s.e. mean ($n=4$). * $P<0.05$, ** $P<0.01$ vs ET-1 or ET-3 by Tukey's method.

the absence and presence of thiorphan (10^{-5} M). As shown in Figure 6A, the [¹²⁵I]-ET-1 binding was concentration-dependently inhibited by ET-1₁₋₃₁ (3×10^{-9} – 10^{-7} M) or big ET-1 (10^{-8} – 10^{-6} M) in the absence of thiorphan. In the

presence of thiorphan (10^{-5} M), these competition curves were shifted to the right. ET-2₁₋₃₁ (10^{-8} – 10^{-6} M) also inhibited the specific [¹²⁵I]-ET-1 binding concentration-dependently in the absence of thiorphan, whereas the inhibitory effect of big ET-2

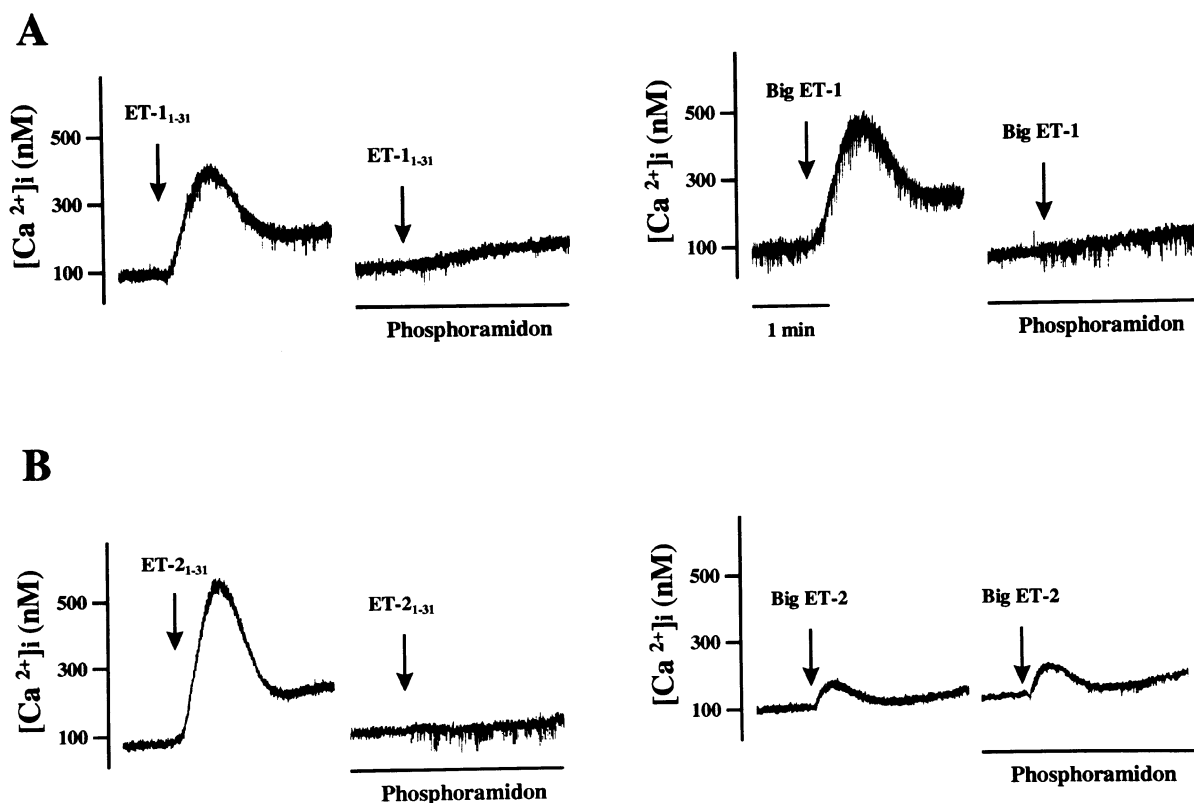


Figure 3 Representative charts for the effect of phosphoramidon on $[Ca^{2+}]_i$ responses induced by ET-1₁₋₃₁, big ET-1 (A), ET-2₁₋₃₁ or big ET-2 (B) in human cultured BSMC. The cell suspensions were treated for 5 min with phosphoramidon (10^{-4} M) before addition of each stimulus. ET derivatives were added at 10^{-6} M.

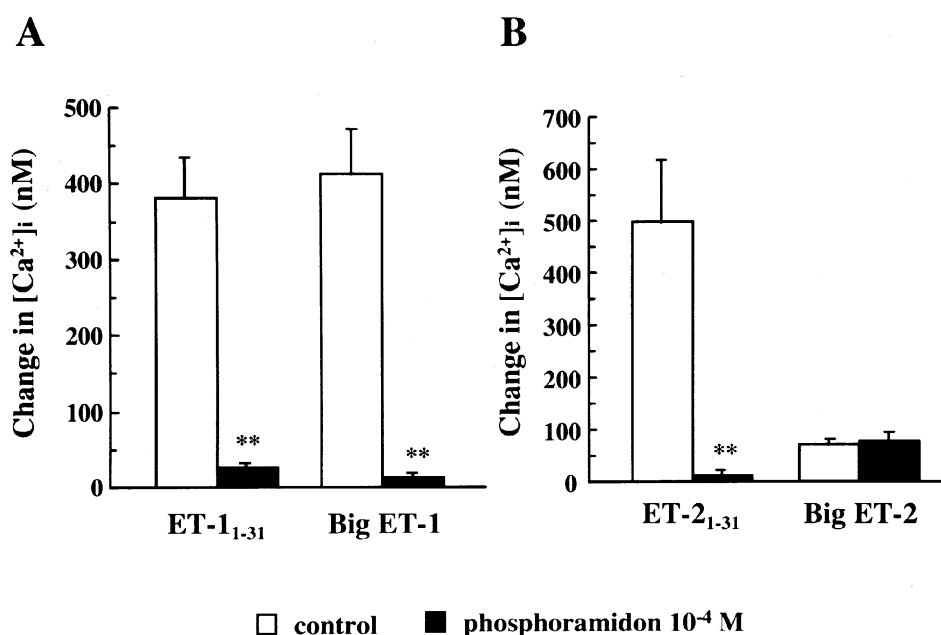


Figure 4 Effect of phosphoramidon on $[Ca^{2+}]_i$ increases induced by ET-1₁₋₃₁, big ET-1 (A), ET-2₁₋₃₁ and big ET-2 (B) in human cultured BSMC. Values are expressed as the change in $[Ca^{2+}]_i$ from the basal level before addition of each stimulus. Data are mean \pm s.e. mean ($n=4$). ** $P < 0.01$ vs control by Student's t -test.

on the binding was extremely weak compared to that of ET-2₁₋₃₁. Thiorphan (10⁻⁵ M) strongly suppressed the effect of ET-2₁₋₃₁, but did not that of big ET-2 (Figure 6B). Thiorphan (10⁻⁵ M) did not affect the competition curves of ET-1 and ET-2 (data not shown). Phosphoramidon (10⁻⁴ M) also inhibited the effects of ET-1₁₋₃₁, big ET-1₁₋₃₁ and ET-2₁₋₃₁, but not of big ET-2 (data not shown).

Discussion

The physiological actions of ETs are mediated by at least two distinct receptor subtypes, ET_A- and ET_B-receptors. ET_A-receptor has a higher affinity for ET-1 and ET-2 than for ET-3, whereas ET_B-receptor has an equal affinity for all isopeptides (Arai *et al.*, 1990; Sakurai *et al.*, 1990). In human cultured BSMC, an increase in [Ca²⁺]_i caused by a high concentration of ET-1 was inhibited by treatment with BQ123, an ET_A-receptor antagonist, in a concentration-dependent manner. Combined treatment with BQ123 and BQ788, an ET_B-receptor antagonist, suppressed the increase in [Ca²⁺]_i caused by ET-1 more efficiently than treatment with BQ123 alone. Although a high concentration of ET-3 also increased [Ca²⁺]_i, the effect was about six times less

than that of the same concentration of ET-1. The increase in [Ca²⁺]_i caused by ET-3 was not affected by treatment with BQ123 but strongly suppressed by BQ788. Furthermore, Scatchard analyses of the saturation curves of [¹²⁵I]-ET-1 and [¹²⁵I]-ET-3 bindings to the cells showed that BSMC expressed both ET_A- and ET_B-receptors, and the calculated ratio of the ET_A- to ET_B-receptor number was about 4:1. These results suggest that the increases in [Ca²⁺]_i induced by ET-1 and ET-2 are mainly mediated by ET_A-receptor on human BSMC.

ET-1₁₋₃₁ and big ET-1 caused an increase in [Ca²⁺]_i in a concentration-dependent manner, and the effect of ET-1₁₋₃₁ was about three times more potent than big ET-1 in human BSMC. On the other hand, ET-2₁₋₃₁ also caused an increase in [Ca²⁺]_i, but big ET-2 at concentrations up to 10⁻⁶ M did not. Lebel *et al.* (1996) have reported that the contractions of strips in guinea-pig lung parenchyma elicited by big ET-1 and big ET-2 result from their conversion to ET-1 and ET-2 by NEP. Laporte & Sirois (1997) have also reported that the generation of ET-1 and ET-2 from exogenous big ET-1 and big ET-2 are suppressed by phosphoramidon, a dual NEP/ECE inhibitor, and thiorphan, a NEP inhibitor, in cultured guinea-pig Clara cells. Thus, we examined the effects of these protease inhibitors on the increases in [Ca²⁺]_i caused by ET derivatives. Treatment with phosphoramidon almost completely inhibited the increases in [Ca²⁺]_i caused by ET-1₁₋₃₁, big ET-1 and ET-2₁₋₃₁. Furthermore, the increases in [Ca²⁺]_i by big ET-1 and ET-2₁₋₃₁ were strongly suppressed by thiorphan, but not by BBI or chymostatin. In addition to these functional studies, radioligand binding studies showed that the inhibitory effects of ET-1₁₋₃₁, big ET-1 and ET-2₁₋₃₁ on the [¹²⁵I]-ET-1 binding were strongly suppressed by treatment with thiorphan and phosphoramidon. Taken together with previous reports, our results strongly suggest that the increases in [Ca²⁺]_i caused by ET-1₁₋₃₁, big ET-1 and ET-2₁₋₃₁ are mediated through cleavage to these corresponding ETs by NEP in human BSMC. Our results are also supported by the reports that ECE requires the carboxyl-terminal sequence from His²⁷ to Gly³⁴

Table 1 Effects of various protease inhibitors on [Ca²⁺]_i increases induced by big ET-1 and ET-2₁₋₃₁ in human cultured BSMC

Stimulus	Inhibitor	Inhibition (%)
Big ET-1 10 ⁻⁶ M	Thiorphan 10 ⁻⁵ M	82.9 ± 2.5
	BBI 10 ⁻⁵ M	3.7 ± 5.5
	Chymostatin 5 × 10 ⁻⁵ M	22.2 ± 17.8
ET-2 ₁₋₃₁ 10 ⁻⁶ M	Thiorphan 10 ⁻⁵ M	90.5 ± 1.5
	BBI 10 ⁻⁵ M	10.1 ± 9.1
	Chymostatin 5 × 10 ⁻⁵ M	1.9 ± 1.1

Values are mean ± s.e.mean (n = 3).

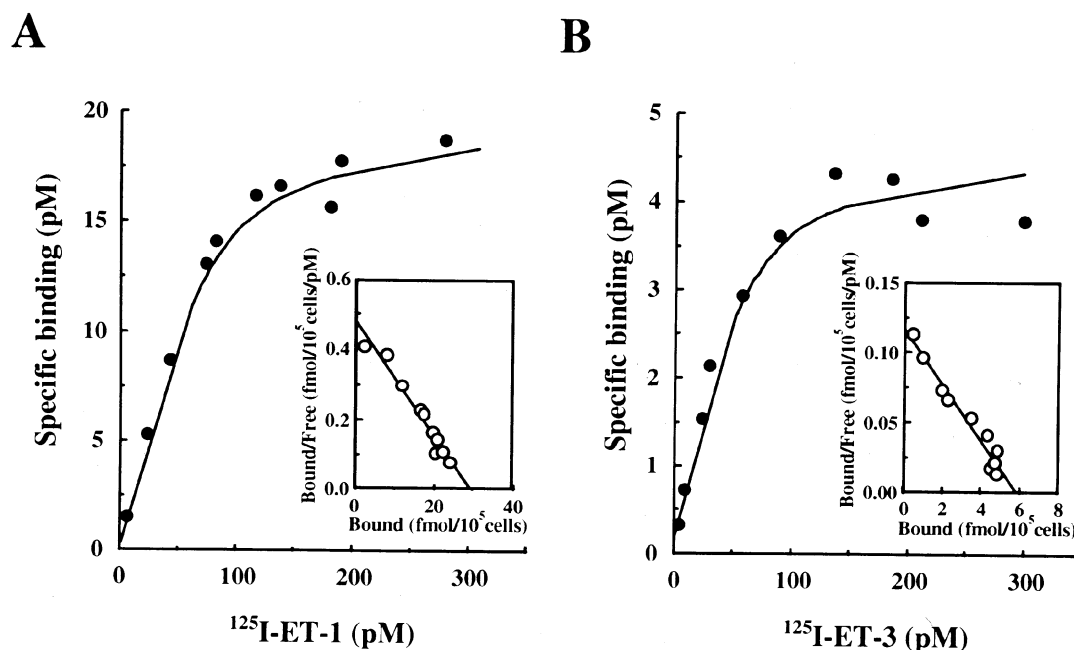


Figure 5 Saturation binding curves of [¹²⁵I]-ET-1 (A) and [¹²⁵I]-ET-3 (B) in human cultured BSMC. Scatchard plots for the specific bindings of [¹²⁵I]-ET-1 and [¹²⁵I]-ET-3 are shown in inset A and B, respectively. Binding analyses were performed on 10⁵ cells. Each point represents the mean values of triplicate measurements.

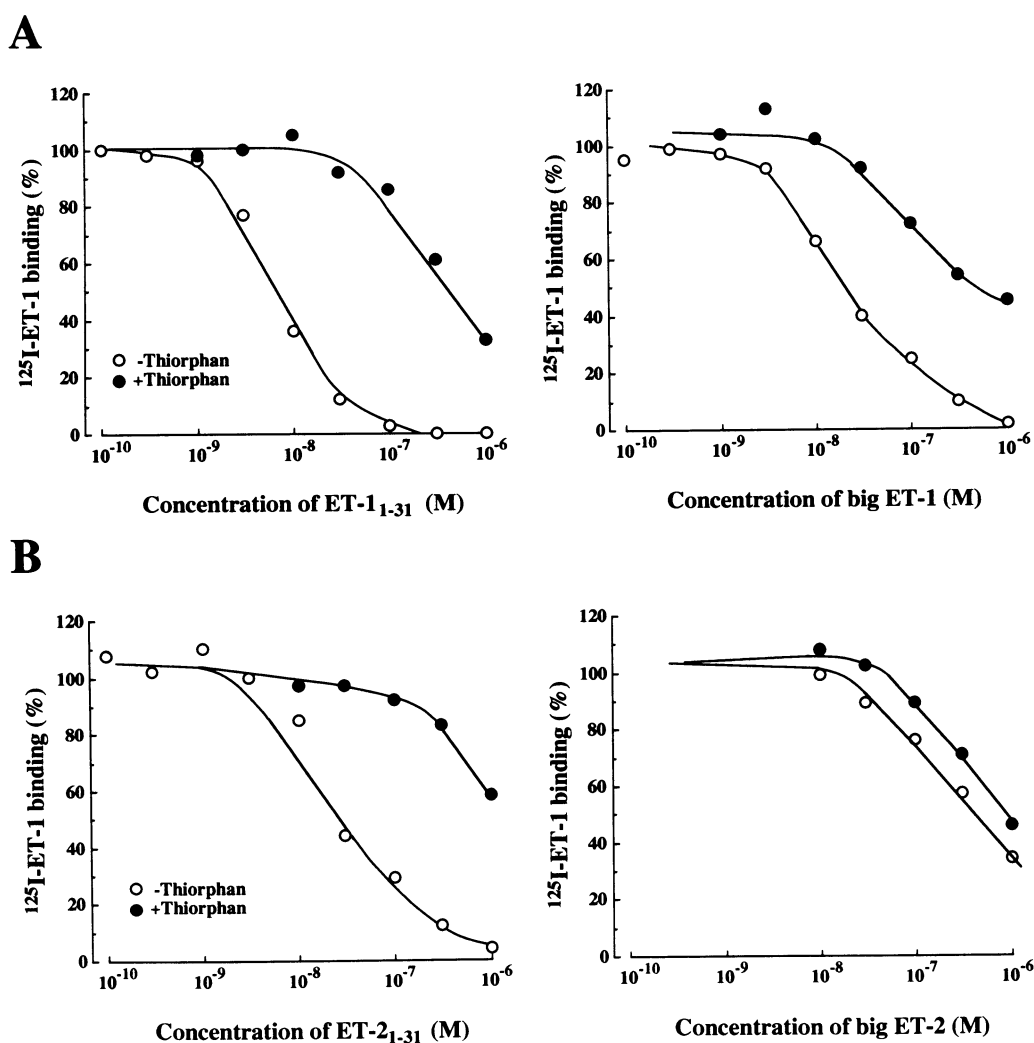


Figure 6 Effects of the absence or presence of thiophan (10^{-5} M) on the inhibition of [125 I]-ET-1 binding by ET-1₁₋₃₁, big ET-1 (A), ET-2₁₋₃₁, and big ET-2 (B) in human cultured BSMC. Each point represents the mean values of triplicate determinations and are representative of those from three separate experiments.

and Trp²¹ of big ET-1 for enzyme recognition and is not able to cleave ET-1₁₋₃₁ (Ohnaka *et al.*, 1993; Xu *et al.*, 1994).

In the present study, big ET-2 of up to 10^{-6} M did not cause any increase in $[Ca^{2+}]_i$, but ET-2₁₋₃₁ did in BSMC. Furthermore, the inhibitory effect of big ET-2 to [125 I]-ET-1 binding was not affected by treatment with thiophan. Thus, it appears that ET-2₁₋₃₁ is efficiently converted to ET-2 by NEP, but big ET-2 is not. Erdös & Skidgel (1989) reported that NEP cleaves peptide substances at the amino side of hydrophobic amino acids. Further studies are needed to clarify the substrate specificity for NEP.

In human BSMC, ET-1 and ET-2 in concentrations ranging from 3×10^{-10} – 10^{-7} M caused an increase in $[Ca^{2+}]_i$ in a concentration-dependent manner. These concentrations, needed to induce functional action in this study, agreed well with those of a previous report that ET-1 induces the elevation of $[Ca^{2+}]_i$ and causes an increase in the *c-fos* and *c-myc* mRNA levels in rat vascular smooth muscle cells (Komuro *et al.*, 1988). In human coronary artery smooth muscle cells, however, Yoshizumi *et al.* (1998) reported that ET-1 (10^{-14} – 10^{-10} M) causes an increase in $[Ca^{2+}]_i$ with a concentration-dependent manner. They showed that ET-1₁₋₃₁ (10^{-13} – 10^{-10} M) by itself causes an increase in $[Ca^{2+}]_i$ mediated through ET_A-receptor or an ET_A-like receptor since the effect

of ET-1₁₋₃₁ is not affected by phosphoramidon. The discrepancy in the sensitivity of ET-1 between their report and ours may arise from the differences in the $[Ca^{2+}]_i$ measuring method or among smooth muscle cell types. However, at least we could not observe any direct effect of ET₁₋₃₁ in human BSMC.

Human chymase has been reported to be highly efficient for converting angiotensin I to angiotensin II (Urata *et al.*, 1990b), and the chymase-dependent angiotensin II-forming pathway is recognized as a major pathway for angiotensin II formation in cardiovascular tissues (Urata *et al.*, 1990a). Recently, Nakano *et al.* (1997) reported that human mast cell chymase also specifically converts big ETs to ET₁₋₃₁, without any further degradation products. In asthmatic patients with bronchial hyper-responsiveness, ET-like immunoreactivity strikingly increases in the airway epithelium and bronchoalveolar lavage fluid, and activated mast cells also increase in number in the bronchial tissue and bronchial lavage fluid (Nomura *et al.*, 1989; Mattoli *et al.*, 1991; Springall *et al.*, 1991). Chymase-like immunoreactivity and enzymatic activity have been demonstrated in not only cardiovascular tissues but also human lung tissue (Urata *et al.*, 1993). These reports lead us to the hypothesis that human chymase released from mast cells by various stimuli converts big ETs to ET₁₋₃₁. Recently, Takai *et*

al. (1998) reported that the contractile response of big ET-2 is predominantly dependent on the conversion of big ET-2 to ET-2₁₋₃₁ by chymase in isolated monkey trachea. Human chymase seems to play an important role in the ET (especially ET-2) generating pathway in inflammatory tissues. In human BSMC, we showed that the increases in $[Ca^{2+}]_i$ caused by ET-1₁₋₃₁, big ET-1 and ET-2₁₋₃₁ are mediated through corresponding ETs cleaved by NEP. NEP has been found in various types of cells, such as human airway smooth muscle and rat

aortic fibroblasts and smooth muscle cells (Hamad *et al.*, 1997; González *et al.*, 1998). Therefore, it is likely that local production of ETs through ETs₁₋₃₁ is regulated by the NEP cleavage pathway.

In this report, we presented the first evidence that ET-1₁₋₃₁ and ET-2₁₋₃₁ cause an increase in $[Ca^{2+}]_i$ by being converted into the corresponding ET-1 and ET-2 by NEP in human BSMC. Human chymase may play an important role in the ET generating pathway in bronchial tissue.

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(Received February 1, 1999

Revised March 13, 1999

Accepted April 20, 1999)