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Endothelin-1 stimulates sodium-dependent calcium efflux from bovine adrenal chromaffin cells in culture

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1 The effect of endothelin (ET)-1 on Ca^{2+} efflux from cultured bovine adrenal chromaffin cells was examined. ET-1 (10^{-7} M) significantly increased intracellular free Ca^{2+} level ($[Ca^{2+}]_i$), $^{45}Ca^{2+}$ uptake and catecholamine secretion in the cells.

2. ET-1 stimulated the efflux of $^{45}Ca^{2+}$ from the cells preloaded with $^{45}Ca^{2+}$ in a concentration-dependent manner (10^{-9}–10^{-7} M). This stimulatory effect was inhibited by ET_B receptor antagonist BQ788, but not by ET_A receptor antagonist BQ123. Selective ET_B receptor agonists Suc-[Glu^9, Ala^{11,15}]-ET-1 and sarafotoxin S6c (SRTX) also stimulated $^{45}Ca^{2+}$ efflux from the cells.

3 ET-1, Suc-[Glu^9, Ala^{11,15}]-ET-1 and SRTX increased the level of cyclic GMP in the adrenal chromaffin cells. ET-1 induced an increase in the nitric oxide (NO) level in the cells. The stimulatory effects by which ET-1 increases NO level and $^{45}Ca^{2+}$ efflux were inhibited by N^G-monomethyl-L-arginine acetate (L-NMMA), a competitive inhibitor of NO synthase.

4 The $^{45}Ca^{2+}$ efflux stimulated by ET-1 was inhibited by deprivation of extracellular Na^+, but not by deprivation of Ca^{2+}.

5 These results suggest that ET-1 stimulates an extracellular Na^+-dependent Ca^{2+} efflux through the activation of NO synthase in cultured bovine adrenal chromaffin cells.

Keywords: Endothelin; Ca^{2+} efflux; Na^+/Ca^{2+} exchange; chromaffin cell

Introduction

The adrenal medulla is one of the endocrine organs which has the vascular endothelial cells and neural crest-derived chromaffin cells. The adrenal chromaffin cells are useful for studying the mechanism of the stimulus-secretion coupling and are regarded as a model for catecholamine containing neurons. Physiological stimulations of the adrenal chromaffin cells cause an increase in the level of intracellular free Ca^{2+} ($[Ca^{2+}]_i$). The increase in $[Ca^{2+}]_i$ leads to the stimulation of catecholamine biosynthesis and secretion (Oka *et al*., 1979; Masserano *et al*., 1990). However, possible interaction between the vascular endothelial cells and the adrenal chromaffin cells is unknown.

Endothelin (ET)-1, which is a 21-residue peptide from vascular endothelial cells (Yanagisawa *et al*., 1988), is recognized as one of a family of structurally homologous peptides, which includes ET-2, ET-3 and sarafotoxins, venom from the snake *Actractaspis engadensis* (Yanagisawa & Masaki, 1989). ET-1 is well known to be a potent vasoconstrictor (Yanagisawa *et al*., 1988). In adrenal chromaffin cells, its peptide stimulates the secretion of noradrenaline and adrenaline by an increase in $[Ca^{2+}]_i$ which partially depends on activation of dihydropyridine sensitive calcium channels (Boarder & Marriott, 1991).

After stimulation of catecholamine secretion from adrenal chromaffin cells induced by various secretagogues, the increase in $[Ca^{2+}]_i$ should rapidly return to the resting level to enable response to a subsequent stimulation. In this study, to investigate the possible role of ET-1 on stimulus-secretion coupling in the adrenal chromaffin cells, we focused on the mechanism of decrease in elevated $[Ca^{2+}]_i$ and examined the effect of ET-1 on Ca^{2+} efflux from bovine adrenal chromaffin cells in culture.

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Methods

Culture of bovine adrenal chromaffin cells

Bovine adrenal chromaffin cells were dispersed enzymatically as described previously (Oka *et al*., 1979). Briefly, the medulla was sliced with a hand slicer, and the slices were digested in medium containing 0.1% collagenase, 0.01% soybean trypsin inhibitor and 0.5% bovine serum albumin in balanced salt solution [BSS: 135 mM NaCl, 5.6 mM KCl, 1.2 mM $MgSO_4$, 2.2 mM $CaCl_2$, 10 mM glucose, and 20 mM N-2-hydroxyethyl-piperazine-N′-2-ethanesulphonic acid (HEPES)/NaOH, pH 7.4]. Cells were either plated in 35 mm culture dishes at a density of 2×10^6 cells/dish for measuring $^{45}Ca^{2+}$ uptake, catecholamine secretion, $^{45}Ca^{2+}$ efflux, cyclic GMP level and NO level, or on 22×22 mm cover glasses in 35 mm culture dishes at a density of 1×10^6 cells/dish for measuring $[Ca^{2+}]_i$, and were maintained for 3 days as monolayer cultures in Eagle's basal medium supplemented with 5% heat-inactivated foetal calf serum, 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), gentamycin (40 μg/ml), fungizone (2.5 μg/ml) and 10 μM cytosine arabinoside.

Measurements of catecholamine secretion

Plated cells were washed with 1 ml of BSS and then incubated at 37°C in 1 ml of the reaction mixture. At the end of the reaction period, the medium covering the cells was saved for

catecholamine assay. The cells were then lysed by adding 1 ml of BSS and freeze-thawing, and the cell lysate was also saved for catecholamine assay. Catecholamine secretion was determined by HPLC with electrochemical detection (Yanaco model L-2000). Catecholamine secretion is expressed as a percentage of the total cellular content secreted during the incubation period.

Measurements of the $[Ca^{2+}]_i$ in single chromaffin cells

The cells cultured on cover glasses were incubated at 37°C for 30 min in 1 ml of BSS containing 4 μM fura-2 AM. Then the cells on the cover glasses were transferred to a small incubation bath (approximately 0.5 ml) on the platform of a microscope. Fluorescence was measured in single chromaffin cells on the cover glass using a fluorescence spectromicroscope (excitation, 340/380 nm, emission, 510 nm). The $[Ca^{2+}]_i$ was calculated using the equation described previously (Grynkiewicz *et al*., 1985).

Measurements of $^{45}Ca^{2+}$ uptake into cells

The cells were washed and incubated as described above in BSS containing $^{45}Ca^{2+}$ (3 μCi ml^{-1}) in the presence or absence of test agents for 10 min at 37°C. After incubation, the medium was discarded and the cells were washed five times with 1 ml of ice cold BSS. Then the intracellular $^{45}Ca^{2+}$ was extracted with 1 ml of 1% Triton X-100 and measured in a liquid scintillation counter (Houchi *et al*., 1994a).

Measurements of $^{45}Ca^{2+}$ efflux

Cells were washed and incubated as described above in BSS containing $^{45}Ca^{2+}$ (3 μCi ml^{-1}) for 1 h at 37°C. After incubation, the cells in each well were washed 16 times with 1 ml volumes of BSS for 30 s periods to remove unincorporated $^{45}Ca^{2+}$. They were next incubated 14 times with 1 ml volumes of BSS for 30 s periods to determine basal efflux levels. Then, they were incubated 15 times with 1 ml volumes of reaction mixture with or without test agents for 30 s periods to determine agonist-stimulated efflux levels. After agonist stimulation, the cells were solubilized in 1 ml of 1% Triton X-100 to determine their residual $^{45}Ca^{2+}$. Samples were counted in a liquid scintillation counter. The total radioactivity of $^{45}Ca^{2+}$ in each well was determined as the sum of the radioactivity in each fraction and the residual radioactivity, and this value was used to calculate the fractional release of Ca^{2+} in each period (Houchi *et al*., 1994b).

Measurements of cyclic GMP

3-Isobutyl-1-methylxanthine (IBMX; 5×10^{-4} M) was added to all tissue culture dishes to prevent the breakdown of cyclic GMP by phosphodiesterase. The cells were incubated for 5 min at 37°C with or without test compounds. The reactions were stopped by addition of ice-cold trichloroacetic acid solution (10%, final concentration), and then cells were scraped off the plates and centrifuged. The trichloroacetic acid was extracted from each sample with diethyl ether. The cyclic GMP level was measured by enzyme immunoassay with an Amersham cyclic GMP enzyme immunoassay system kit.

Measurements of NO

Porcine serum albumin (33 mg/ml, final concentration), $FeSO_4$ (3.3 mM), sodium *N*,*N*-diethyldithiocarbamate trihydrate (3.3 mM), the sample, and excess $Na_2S_2O_4$ (2 M) were introduced into a 4 mm diameter electron paramagnetic resonance (EPR) tube (LST-5HS, Labotec Co.), and after 5 min, the mixture was frozen at liquid nitrogen temperature (77 K). EPR spectra were measured in a JEOL FE1X spectrometer under the following conditions: microwave frequency, 9.15 GHz, microwave power, 20 mW, modulation frequency, 100 kHz, modulation amplitude, 0.63 mT, magnetic field, 320 ± 25 mT, response time, 0.1 s, sweep time 50 mT/4 min, temperature 77 K. The concentration of NO trapped was determined by comparing the intensities of the first low-field derivative EPR signal heights and their amplitudes (Tsuchiya *et al*., 1996).

Data analysis

Results are expressed as mean ± s.e.mean for all measurements. Student's *t*-test for paired data was used to test for statistical significance of differences.

Chemicals

$^{45}CaCl_2$ was obtained from Amersham Corp. (Tokyo, Japan). Endothelin-1 (human), endothelin-2 (human), endothelin-3 (human), Suc-[Glu^9, Ala^{11,15}]-endothelin-1 (8–21) and sarafotoxin S6c were obtained from the Peptide Institute (Osaka, Japan). N^G-monomethyl-L-arginine acetate and N$^\omega$-nitro-L-arginine methyl ester were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). BQ123 and BQ788 were donated by Banyu Pharmaceutical Co. (Tsukuba, Japan). Other chemicals used were commercial products of reagent grade.

Results

Effects of ET-1 on $[Ca^{2+}]_i$ levels, $^{45}Ca^{2+}$ uptake and catecholamine secretion

Table 1 shows the effects of ET-1 and acetylcholine on $[Ca^{2+}]_i$ levels, $^{45}Ca^{2+}$ uptake and catecholamine secretion in cultured bovine adrenal chromaffin cells. ET-1 (10^{-7} M) significantly increased the levels of $[Ca^{2+}]_i$ to a peak of approximately 360 nM from 100 nM, the uptake of $^{45}Ca^{2+}$ to 1.5 nmoles/dish from 0.8 nmoles/dish and the secretion of catecholamine to 3.1% from 1.8% of the total cellular content. These effects of ET-1 were weaker when compared with 10^{-4} M acetylcholine, a physiological stimulator of adrenal chromaffin cells, which increases approximately 890 nM, 2.9 nmoles/dish and 10.1% of the total cellular content, respectively. Thus, we confirmed

Table 1 Effects of ET-1 and acetylcholine on $[Ca^{2+}]_i$, $^{45}Ca^{2+}$ uptake and catecholamine secretion in cultured bovine adrenal chromaffin cells

	$[Ca^{2+}]_i$ level (nM)	*$^{45}Ca^{2+}$ uptake* (nmoles/dish)	*Catecholamine secretion* (%)
Control	98 ± 8	0.8 ± 0.1	1.8 ± 0.2
ET-1 (10^{-7} M)	360 ± 29*	1.5 ± 0.2*	3.1 ± 0.3*
Acetylcholine (10^{-4} M)	890 ± 90*	2.9 ± 0.3*	10.1 ± 1.1*

Cells were incubated for 5 min with or without ET-1 (10^{-7} M) and acetylcholine (10^{-4} M). $[Ca^{2+}]_i$ level, $^{45}Ca^{2+}$ uptake and catecholamine secretion were determined as described in methods. Data are means for three to six separate experiments. *Significantly greater than the control value ($P < 0.01$).

previous reports that ET-1 has slight effects on $[Ca^{2+}]_i$ level, $^{45}Ca^{2+}$ uptake and catecholamine secretion in adrenal chromaffin cells (Rasmussen & Printz, 1989; Boarder & Marriott, 1991).

Effects of ET-1 on $^{45}Ca^{2+}$ efflux through ET receptors

Figure 1 shows the efflux of $^{45}Ca^{2+}$ from adrenal chromaffin cells in culture induced by various concentrations of ET-1. The stimulatory effect of ET-1 on $^{45}Ca^{2+}$ efflux was dose-dependent at concentrations of 10^{-9} to 10^{-7} M. The efflux of $^{45}Ca^{2+}$ increased to a peak value within about 1 min after ET-1 addition. The peak value with 10^{-7} M ET-1 was $10.9 \pm 1.2\%$ ($n=6$) of the total $^{45}Ca^{2+}$ in the cells. After the peak, the efflux decreased rapidly over the following 5 min. To determine whether the stimulatory action of ET-1 on $^{45}Ca^{2+}$ efflux is mediated through its peptide receptor, the effects of ET receptors agonists and antagonists on $^{45}Ca^{2+}$ efflux from adrenal chromaffin cells were examined (Figure 2). The efflux of $^{45}Ca^{2+}$ from the cells was stimulated by 10^{-8} M ET-2 or 10^{-8} M ET-3 as well as 10^{-8} M ET-1. The order of $^{45}Ca^{2+}$ efflux potency of the isopeptides ET-2 or ET-3 was similar to that of ET-1. The selective ET_B receptor agonists Suc-[Glu^9, $Ala^{11,15}$]-ET-1 or sarafotoxin S6c (SRTX) also stimulated the efflux of $^{45}Ca^{2+}$ from the cells. Moreover, ET-1-induced $^{45}Ca^{2+}$ efflux was inhibited by ET_B receptor antagonist BQ788, but was not inhibited by ET_A receptor antagonist BQ123. These results suggest that ET-1-induced $^{45}Ca^{2+}$ efflux from adrenal chromaffin cells is mediated through the stimulation of ET_B receptor.

Effects of ET-1 on cyclic GMP and NO formation

Next, the effect of ET-1 on cyclic GMP formation in cultured bovine adrenal chromaffin cells were investigated. We have already reported that an increase in cyclic GMP level in adrenal chromaffin cells using dibutyryl cyclic GMP (DB-cyclic GMP) and nitroprusside, an activator of guanylate cyclase, stimulates the efflux of $^{45}Ca^{2+}$ from the cells (Houchi *et al*., 1995). As shown in Figure 3, the level of cyclic GMP in the cells was increased by ET-1, Suc-[Glu^9, $Ala^{11,15}$]-ET-1 or SRTX. ET-1-induced cyclic GMP production was reduced by N^G-monomethyl-L-arginine acetate (L-NMMA) or N^{ω}-nitro-L-arginine methyl ester (L-NAME), a competitive inhibitor of NO synthase.

Furthermore, to determine whether ET-1 produces nitric oxide (NO) in cultured bovine adrenal chromaffin cells, we tried to measure the levels of NO in the cells using electron paramagnetic resonance (EPR) spectroscopy. As shown in Figure 4a, ET-1 increased the level of NO in the adrenal chromaffin cells. NO production induced by ET-1 was inhibited by L-NMMA, a concentration-dependent manner. In addition, ET-1-induced $^{45}Ca^{2+}$ efflux from the cells was partially inhibited by L-NMMA or L-NAME (Figure 4b). Unfortunately, L-NAME had a serious interference to measure the levels of NO by EPR spectroscopy (Tsuchiya *et al*., 1996). These results indicated that the production of NO induced by ET-1 may partially regulate the efflux of $^{45}Ca^{2+}$ from adrenal chromaffin cells.

Effects of extracellular Na^+ on ET-1 induced $^{45}Ca^{2+}$ efflux

We examined the influences of extracellular Ca^{2+} and Na^+ on ET-1-induced $^{45}Ca^{2+}$ efflux from adrenal chromaffin cells (Figure 5). The stimulatory action of ET-1 on $^{45}Ca^{2+}$ efflux was not influenced by the absence of extracellular Ca^{2+}. However, ET-1-stimulated $^{45}Ca^{2+}$ efflux was progressively inhibited by reduction of the extracellular Na^+ concentration. Therefore, this ET-1-induced efflux was extracellular Na^+ dependent, leading to an acceleration of the Na^+/Ca^{2+} exchange on the cell membrane.

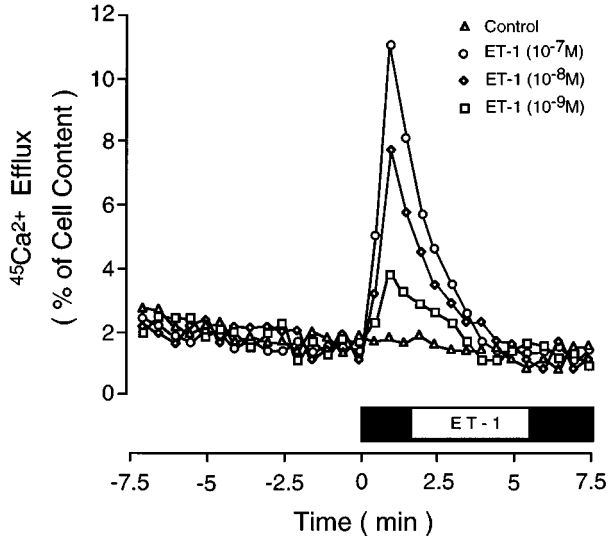

Figure 1 Effects of different concentrations of ET-1 on $^{45}Ca^{2+}$ efflux from cultured bovine adrenal chromaffin cells. Cells were preloaded with $^{45}Ca^{2+}$ as described in Methods, and then incubated with the indicated concentrations of endothelin-1 (ET-1; $10^{-9}-10^{-7}$ M). Data are shown as percentages of cell contents, and are means for four to six separate experiments. All peak levels with ET-1 were significantly greater than the control level ($P<0.01$).

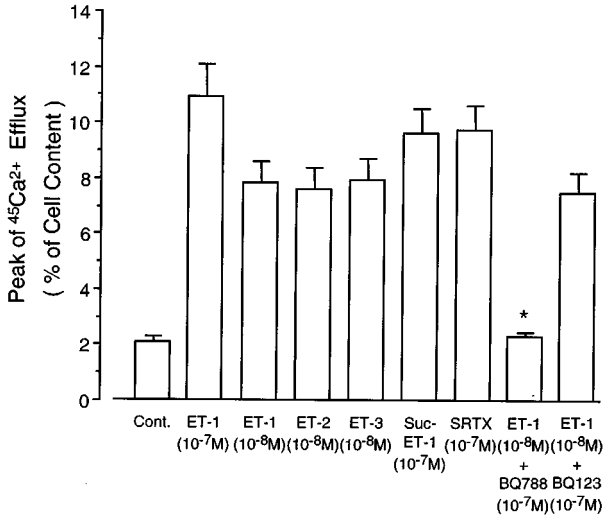

Figure 2 Effects of ET receptors agonists and antagonists on $^{45}Ca^{2+}$ efflux from cultured bovine adrenal chromaffin cells. Cells were preloaded with $^{45}Ca^{2+}$ as described in Methods and then incubated in the presence or absence of endothelin-1 (ET-1), endothelin-2 (ET-2), endothelin-3 (ET-3), Suc-[Glu^9, $Ala^{11,15}$]-endothelin-1 (Suc-ET-1) or sarafotoxin S6c (SRTX). The endothelin antagonists BQ123 (ET_A receptor antagonist; 10^{-7} M) or BQ788 (ET_B receptor antagonist; 10^{-7} M), was added 150 s before ET-1 (10^{-8} M). Data are means $\pm$ standard errors for three to four separate experiments. *Significant difference from 10^{-8} M ET-1 stimulated $^{45}Ca^{2+}$ efflux ($P<0.01$).

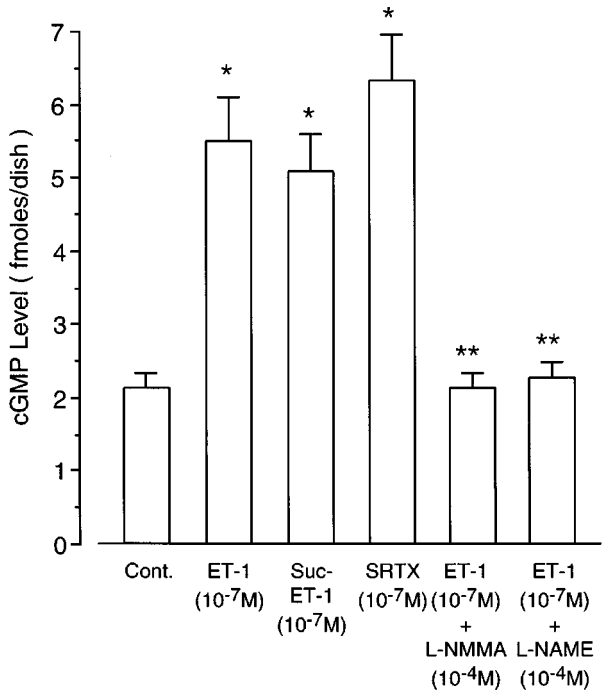

Figure 3 Effects of ET receptors antagonists on cyclic GMP formation in cultured bovine adrenal chromaffin cells. Cells were preincubated for 10 min at 37°C with 5×10^{-4} M IBMX and then incubated for 5 min with or without ET-1, Suc-[Glu^{9}, $Ala^{11,15}$]-endothelin-1 (Suc-ET-1) or sarafotoxin S6c (SRTX) with IBMX. In case of using NO synthase inhibitors, cells were preincubated for 30 min at 37°C presence of 10^{-4} M N^{G}-monomethyl-L-arginine acetate (L-NMMA) or N^{ω}-nitro-L-arginine methyl ester (L-NAME). Cyclic GMP levels in the cells were determined as described in Methods. Data are means±s.e.mean for three separate experiments. *Significantly greater than the control value ($P<0.01$). **Significantly less than the ET-1-induced cyclic GMP level ($P<0.01$).

Discussion

We examined for the first time the mechanism(s) by which ET-1 stimulates the efflux of Ca^{2+} from bovine adrenal chromaffin cells in culture. Physiological stimulation such as acetylcholine for adrenal chromaffin cells increases the level of $[Ca^{2+}]_i$ originated from intracellular pools and extracellular spaces, leading to biosynthesis and secretion of catecholamine in the cells (Oka *et al*., 1979; Masserano *et al*., 1990). The adrenal medulla is rich containing vascular endothelial cells and chromaffin cells. Therefore, ETs may have the interaction between these two cell types. The increased $[Ca^{2+}]_i$ in adrenal chromaffin cells which initiates stimulus-secretion coupling should be restored to a physiological level for response to further stimulus. Thus, ET-1 may play a role in the modulation of the intracellular signal transduction system.

A previous report indicated that ET-1 increased the secretion of noradrenaline and adrenaline from adrenal chromaffin cells (Boarder & Marriott, 1991). This peptide also significantly increased the level of $[Ca^{2+}]_i$, uptake of $^{45}Ca^{2+}$ and secretion of catecholamine (Table 1). Therefore, ET-1 may regulate stimulus-secretion coupling in adrenal chromaffin cells.

ETs are known to act on specific receptors. These receptors are classified into the two receptor subtypes, ET_A and ET_B (Arai *et al*., 1990; Sakurai *et al*., 1990; Goto *et al*., 1996). A previous report suggested that adrenal gland has these two receptor subtypes (Hagiwara *et al*., 1993). However, little is known of the special function which is coupled with ET_A or ET_B receptor on adrenal chromaffin cells. On the binding assay system for these receptors, ET_A receptor is specific for ET-1, whereas ET_B receptor binds all ETs with an equal affinity (Sakurai *et al*., 1992). As shown in Figure 2, ET-1, ET-2 or ET-3 at submaximal concentration for $^{45}Ca^{2+}$ efflux have approximately equal effects on the efflux from adrenal

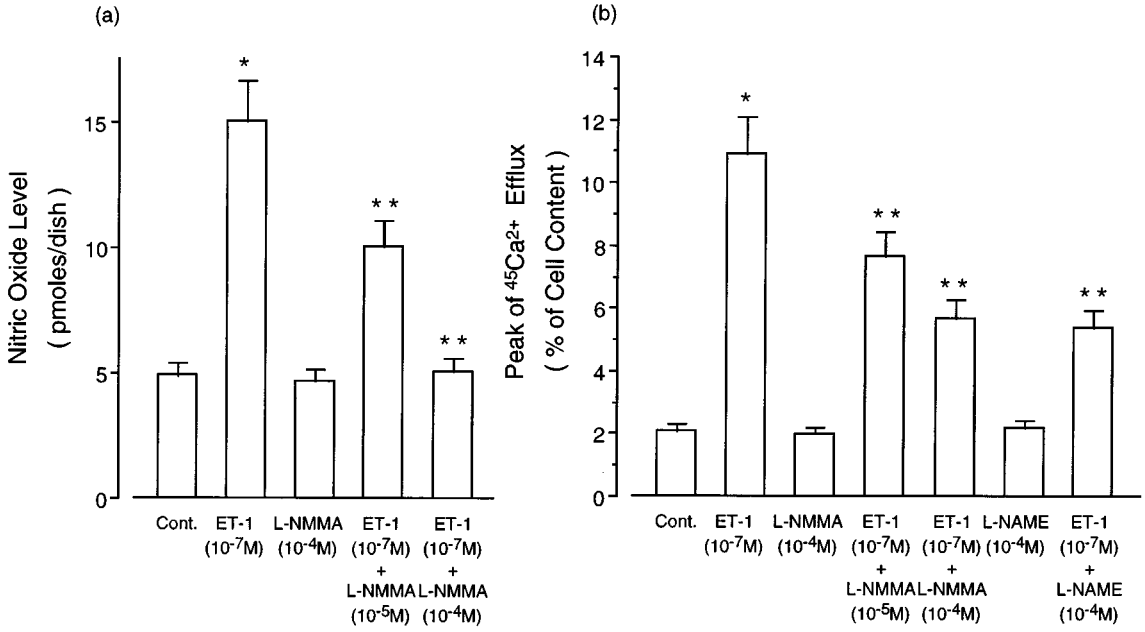

Figure 4 Effects of ET-1 on NO formation and $^{45}Ca^{2+}$ efflux in cultured bovine adrenal chromaffin cells. Cells were preincubated for 30 min at 37°C presence or absence of 10^{-5} M or 10^{-4}M N^{G}-monomethyl-L-arginine acetate (L-NMMA) or N^{ω}-nitro-L-arginine methyl ester (L-NAME) and then incubated for 5 min with or without 10^{-7} M ET-1. (a) NO levels in the cells were determined as described in Methods. Data are means±s.e.mean for three separate experiments. *Significantly greater than the control value ($P<0.01$). **Significantly less than the ET-1-induced NO level ($P<0.01$). (b) $^{45}Ca^{2+}$ efflux levels from the cells were determined as described in Methods. Data are means±s.e.mean for three separate experiments. *Significantly greater than the control value ($P<0.01$). **Significantly less than the ET-1-induced $^{45}Ca^{2+}$ efflux level ($P<0.01$).

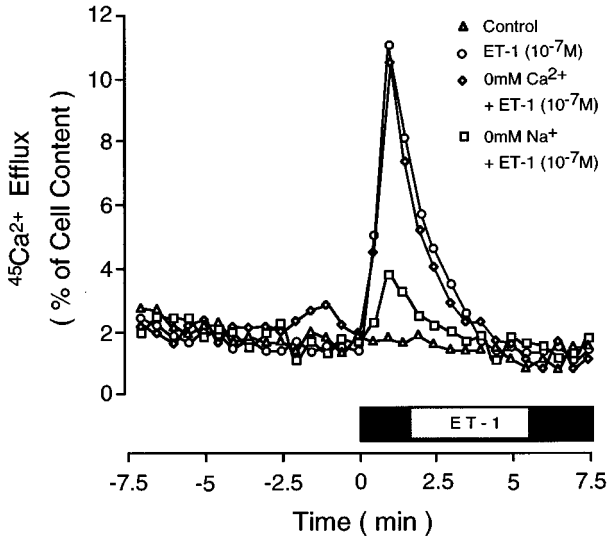

Figure 5 Effects of Ca^{2+}- and Na^{+}-deficient medium on ET-1-induced $^{45}Ca^{2+}$ efflux from cultured bovine adrenal chromaffin cells. Cells were preloaded with $^{45}Ca^{2+}$ as described in Methods. The medium was changed to Ca^{2+}- or Na^{+}-deficient medium for 2.5 min. Then 10^{-7} M ET-1 was added, and the cells were incubated for 7.5 min. Na^{+}-deficient medium was prepared with sucrose instead of Na^{+}. Data are means $\pm$ s.e.mean for three to six separate experiments. The maximal standard error was $\pm 1.5\%$. The peak level with ET-1 in Na^{+}-deficient medium was significantly less than that with ET-1 in normal medium ($P<0.01$).

chromaffin cells. Moreover, the selective ET_B receptor agonists Suc-[Glu^9, $Ala^{11,15}$]-ET-1 or SRTX (Goto *et al*., 1996) stimulated the efflux of $^{45}Ca^{2+}$ from the cells (Figure 2). ET-1-induced $^{45}Ca^{2+}$ efflux was inhibited by BQ788, a ET_B receptor antagonist, but was not inhibited by BQ123, an ET_A receptor antagonist (Goto *et al*., 1996). These results suggest that the ET-1-induced $^{45}Ca^{2+}$ efflux from adrenal chromaffin cells is mediated through the stimulation of ET_B receptor.

To investigate the intracellular mechanism(s) of ET-1-induced $^{45}Ca^{2+}$ efflux from the cells, we next observed the change in cyclic GMP level and NO level induced by ET-1. Cyclic GMP was reported as a second messenger in adrenal chromaffin cells, especially in the pathway of catecholamine biosynthesis (Tsutsui *et al*., 1994). Previously, it was indicated that an increase in the cyclic GMP level, which is caused by DB-cyclic GMP or nitroprusside (an activator of guanylate cyclase), in adrenal chromaffin cells may regulate Ca^{2+} efflux from the cells through acceleration of Na^{+}/Ca^{2+} exchange (Houchi *et al*., 1995). In this report, ET-1, Suc-[Glu^9, $Ala^{11,15}$]-ET-1 and SRTX stimulated the formation of cyclic GMP in adrenal chromaffin cells (Figure 3). ET-1-induced cyclic GMP formation was inhibited by the NO synthase inhibitor L-NMMA or L-NAME (Figure 3). NO is known to activate guanylate cyclase in adrenal glands, leading to an increase in the production of cyclic GMP (Palacios *et al*., 1989). Using our new NO assay method EPR spectroscopy, which is detectable at an order of 0.1 pmoles/ml NO (Tsuchiya *et al*., 1996), ET-1 stimulated NO production in adrenal chromaffin cells, and this stimulatory effect was almost abolished by L-NMMA, a inhibitor of NO synthase (Figure 4a). On EPR spectroscopy, another inhibitor of NO synthase L-NAME interfered in measurement of NO levels (Tsuchiya *et al*., 1996). However, L-NMMA and L-NAME influenced the efflux of $^{45}Ca^{2+}$ from the cells induced by ET-1 (Figure 4b). Therefore, the mechanism(s), in part, by which ET-1 stimulates Ca^{2+} efflux from the cells may be involved in cyclic GMP-dependent fashion.

The possibility of contamination of endothelial cells in isolation of adrenal chromaffin cells was remained. However, endothelial cells were not found in our cell culture condition by immunocytochemical techniques using DAKO EPOS anti-Von Willebrand Factor antibody (data not shown). Near recently, the presence of NO synthase has been shown in bovine adrenal chromaffin cells (Oset-Gasque *et al*., 1998). Thus, it has been shown that ET-1 stimulates NO production in adrenal chromaffin cells in culture.

Although a role of ET receptors on adrenal chromaffin cells is unknown, both ET_A and ET_B receptors are capable of coupling to GTP-binding proteins in other cell types (Goto *et al*., 1996). Diacylglycerol is produced concurrently with inositol 1,4,5-trisphosphate (IP_3) on breakdown of phosphatidyl inositol 4,5-bisphosphate (PIP_2) by phospholipase C and is thought to activate protein kinase C by increasing the affinity of the enzyme for calcium (Nishizuka, 1984). Activation of protein kinase C using phorbol ester 12-*O*-tetradecanoyl phorbol 13-acetate suggested an inhibitory action on the efflux of $^{45}Ca^{2+}$ from adrenal chromaffin cells (Houchi *et al*., 1995). However, IP_3 is an important intracellular signaling molecule that stimulates the release of Ca^{2+} from intracellular stores. It has been recognized that the activity of NO synthase is regulated by Ca^{2+}/calmodulin which is dependent on the levels of $[Ca^{2+}]_i$ (Schulz & Triggle, 1994). Therefore, an increase in $[Ca^{2+}]_i$ may act as an initiator of the formation of NO in adrenal chromaffin cells.

Recently, enhancement of NO synthase activity under shear stress via a Ca^{2+}-independent mechanism in endothelial cells was reported (Ayajiki *et al*., 1996). Moreover, this enzyme activity was regulated by the activation of one or more tyrosine kinases (Ayajiki *et al*., 1996). It was also reported that activation of tyrosine kinase is involved in the mechanism of Ca^{2+} efflux from bovine adrenal chromaffin cells (Tokumura *et al*., 1998). Thus, the possibility of Ca^{2+}-independent NO formation coupled with Ca^{2+} efflux in bovine adrenal chromaffin cells was remained.

The level of increase in $[Ca^{2+}]_i$ might influence Ca^{2+} efflux from the cells. However, for example, treatments with 10^{-7} M ET-1 and 10^{-4} M acetylcholine increased $[Ca^{2+}]_i$ in cultured bovine adrenal chromaffin cells to 360 nM and 890 nM, respectively, as measured with fura-2 (Table 1). The $[Ca^{2+}]_i$ after acetylcholine stimulation was higher than that after ET-1 stimulation, whereas the increase in $^{45}Ca^{2+}$ efflux from the cells after acetylcholine treatment was less than that after ET-1 treatment (Figure 1, Houchi *et al*., 1994b). Furthermore, ET-1-induced $^{45}Ca^{2+}$ efflux was not dependent on extracellular Ca^{2+} which is a main source of $[Ca^{2+}]_i$ through calcium channels on chromaffin cell membrane (Figure 5). Our previous reports showed no relationship between increase in $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ efflux induced by various secretagogues (Houchi *et al*., 1994b, 1995; Minakuchi *et al*., 1997). Therefore, the stimulatory effect of ET-1 on Ca^{2+} efflux cannot be simply explained as a result of increase in $[Ca^{2+}]_i$.

In this study, we observed that ET-1-induced Ca^{2+} efflux from adrenal chromaffin cells was inhibited by deprivation of extracellular Na^{+}, but was not inhibited by deprivation of extracellular Ca^{2+} (Figure 5). ET-1 may stimulate the efflux of Ca^{2+} from cultured bovine adrenal chromaffin cells through the mechanism of Na^{+}/Ca^{2+} exchange. Some other mechanism(s), such as Ca^{2+} pumps, may also be involved in ET-1-induced Ca^{2+} efflux from the cells, because the deprivation of extracellular Na^{+} treatment did not completely abolish this efflux (Figure 5) and L-NMMA inhibited NO production induced by ET-1 much more effectively than $^{45}Ca^{2+}$ efflux (Figure 4).

We conclude from this study that ET-1 increases Ca^{2+} efflux from bovine adrenal chromaffin cells in culture, and that this efflux may be explained by an extracellular Na^{+}-dependent mechanism, probably through acceleration of Na^{+}/Ca^{2+} exchange. These results suggest that ET-1 plays a role in termination of Ca^{2+}-signal transduction through the stimulation of Ca^{2+} efflux from the cells.

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