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Extrusion mechanisms of intracellular Ca^{2+} in human aortic endothelial cells

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Abstract

Although participation of the plasma membrane Ca^{2+} pump in Ca^{2+} efflux has been generally accepted, the existence of Na^+-Ca^{2+} exchange in endothelial cells is still controversial. In the present experiments, the role of Na^+-Ca^{2+} exchange and Ca^{2+}-ATPase were examined in cultured human aortic endothelial cells loaded with fura-2. In Ca^{2+}-free solution, the declining phase of $[Ca^{2+}]_i$ in response to histamine was clearly slowed with low Na^+ solution or Ni^{2+}. Vanadate also slightly slowed the declining phase. The declining phase was much more clearly slowed with La^{3+}. The response to thapsigargin, a specific endoplasmic reticulum Ca^{2+} ATPase inhibitor, was much more clearly prolonged by those interventions. These results strongly imply the presence of Na^+-Ca^{2+} exchange and Ca^{2+} ATPase in the plasma membrane, and suggest that not only Ca^{2+} uptake into the internal stores but also Na^+-Ca^{2+} exchange and plasma membrane Ca^{2+} ATPase have a physiological role in reducing $[Ca^{2+}]_i$ elevated by receptor agonists and endoplasmic reticulum Ca^{2+}-ATPase inhibitors in cultured human aortic endothelial cells.

Keywords: Endothelial cell, aortic; Ca^{2+} concentration, intracellular, free; Na^+-Ca^{2+} exchange; Ca^{2+}-ATPase; Fura-2

1. Introduction

The importance of changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in mediating the release of vasodilatory prostaglandins and endothelium-derived relaxation factor was reported in vascular endothelial cells (Hallam et al., 1988; Lückhoff et al., 1988), but the processes across the plasma membrane of endothelial cells remain poorly understood. It has been reported that in Ca^{2+}-free solution, not only receptor agonists (Hosoki and Iijima, 1994; Ziegelstein et al., 1994) but also specific endoplasmic reticulum Ca^{2+} ATPase inhibitors (Hosoki and Iijima, 1995; Kwan et al., 1990) elicit a transient increase in $[Ca^{2+}]_i$. Even in the presence of the drugs, however, the level of $[Ca^{2+}]_i$ increased by the drugs is returned to the basal level within several minutes. The results indicate that Ca^{2+} can be taken up by the endoplasmic reticulum and/or extruded through the plasma membrane.

The first definitive evidence for the existence of Ca^{2+} pumps in a number of diverse excitable and non-excitable plasma membranes was provided by Dipolo (1978) and Caroni and Carafoli (1980). And participation of the plasma membrane Ca^{2+} pump in Ca^{2+} efflux has been generally accepted. In endothelial cells, it was suggested that the Na^+-Ca^{2+} exchange does not participate in Ca^{2+} efflux (Schilling et al., 1988; Cannell and Sage, 1989; Laskey et al., 1990). Recently, however, there are observations suggesting the existence of Na^+-Ca^{2+} exchange in bovine aortic (Hansen et al., 1991), bovine pulmonary (Sage et al., 1991) and rabbit cardiac valve endothelial cells (Li and Van Breemen, 1995). Therefore, the present study was carried out to investigate the physiological role of the plasma membrane Na^+-Ca^{2+} exchange and Ca^{2+}-ATPase in resting condition and in the declining phases of $[Ca^{2+}]_i$ elevated by histamine or thapsigarginin in cultured human aortic endothelial cells.

2. Materials and methods

2.1. Endothelial cell culture

Human aortic endothelial cells at passage 4 were purchased from Clonetics (San Diego, CA, USA). Cells were

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subcultured from a single primary culture in MCDB131 solution supplemented with 2% fetal bovine serum, 10 ng/ml epidermal growth factor, 10 μg/ml heparin, 50 μg/ml gentamicin and 0.25 μg/ml amphotericin B as previously described (Hosoki and Iijima, 1994). The cells were plated out in 25 cm^2 tissue culture flasks and incubated at 37°C in 5% CO_2. The cells were removed from the flasks with 0.025% trypsin-0.01% EDTA solution and seeded onto glass coverslips (9 × 25 mm) coated with fibronectin in 35 mm tissue culture dishes. All experiments were performed with human aortic endothelial cells in passages 5 or 6 and at 1–5 days post-confluency.

2.2. Fluorescence measurements of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured using the fluorescent indicator fura-2 as previously described (Hosoki and Iijima, 1994). Briefly, confluent monolayers of cells on coverslips were loaded with fura-2 by incubation in Hepes-buffered saline (HBS) containing 5 μM fura-2 acetoxymethyl ester (fura-2/AM) for 30 min at 37°C. At the end of the loading period, the coverslips were washed several times with HBS. For each experiment, a coverslip was mounted in a chamber placed on the thermostated stage on an inverted microscope (TMD-1SJ, Nikon, Tokyo, Japan) equipped with a x40 fluorite objective. The cells were superfused at 1.5 ml/min with HBS. Fluorescence measurements of $[Ca^{2+}]_i$ from single cells were performed using a spectrophotofluorimeter (CAM-230, Japan Spectroscopic, Tokyo, Japan). Excitation wavelength alternated at 400 Hz between 340 and 380 nm during recording of emission fluorescence at 500 nm. Emitted light was directed through a rectangular iris which limited the light collected to a single cell under observation. The emission intensities at 340 and 380 nm excitation (F340 and F380) and the ratio (F340/F380) were recorded on a chart recorder (WR7700, Graphtec, Tokyo, Japan). Control experiments were performed by measuring the dye loss over a 50 min period (the longest duration of a Ca^{2+} experiment) from untreated, fura-2-loaded cells. Background fluorescence from unloaded cells was subtracted from values for the fura-2-loaded cells. $[Ca^{2+}]_i$ can be calculated using the following relationship: $[Ca^{2+}]_i = K_d \times [(R - R_{min})/(R_{max} - R)] \times S_{f2}/S_{b2}$, where K_d is the dissociation constant of fura-2-Ca^{2+} complex, R is the ratio of relative fluorescence determined as described above, R_{min} and R_{max} are the ratios measured in Ca^{2+}-free solution and by the addition of 10 μM of the Ca^{2+} ionophore ionomycin to normal HBS respectively, S_{f2}/S_{b2} is the ratio of fluorescence measured at excitation wavelength 380 nm in Ca^{2+}-free and ionomycin-containing solution. We found that basal $[Ca^{2+}]_i$ of the human aortic endothelial cells in normal HBS ranged from 50 to 100 nM with the above equation. Absolute values of $[Ca^{2+}]_i$ could not be calculated because the dissociation constant of fura-2 for Ca^{2+} in the cytosol might be different from that measured in the absence of protein (Konishi et al., 1988). Therefore, in the present experiments, the ratio F340/F380 was used as a relative measurement of $[Ca^{2+}]_i$ except for the quantitative comparison of $[Ca^{2+}]_i$ at resting condition.

The results are expressed as means ± S.E.M. Student's unpaired t-test was applied; differences were considered significant if $P < 0.05$.

2.3. Solutions and drugs

Normal HBS contained (in mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.0, $MgSO_4$ 1.0, glucose 11.1, and Hepes 5.0, pH adjusted to 7.4 with NaOH. For experiments in low extracellular Na^+ (13.7 mM Na^+), the solutions contained (in mM): NaCl 13.7, *N*-methyl-D-glucamine (NMDG) 123.2, $CaCl_2$ 1.0, $MgSO_4$ 1.0, glucose 11.1, and Hepes 5.0, pH adjusted to 7.4 with HCl. Nominally Ca^{2+}-free solution was the same as normal HBS without $CaCl_2$. To eliminate the influence of the entry of Ca^{2+} from the extracellular space, experiments were carried out in Ca^{2+}-free solution apart from those examined under resting conditions.

The following drugs were used: thapsigargin, histamine dihydrochloride, lanthanum chloride heptahydrate and sodium orthovanadate (Wako Pure Chemical, Osaka, Japan), nickel chloride hexahydrate (Nacalai Tesque, Kyoto, Japan) NMDG (Sigma, St. Louis, MO, USA), fura-2/AM, Hepes and EGTA (Dojin Chemical, Kumamoto, Japan), ionomycin (Calbiochem, San Diego, CA, USA) and fibronectin (from human plasma, Koken, Tokyo, Japan).

3. Results

3.1. Effects of low Na^+, Ni^{2+}, vanadate and La^{3+} on resting $[Ca^{2+}]_i$ in single human aortic endothelial cells

To examine the role of the Na^+-Ca^{2+} exchange mechanism for maintaining the resting level of $[Ca^{2+}]_i$, endothelial cells were superfused with low Na^+ solution. Low Na^+ (13.7 mM) solution was made by replacing 90% Na^+ with equimolar *N*-methyl-D-glucamine. This may force a Na^+-Ca^{2+} exchange to stop and thereby increase $[Ca^{2+}]_i$ (Chapman, 1974; Allen et al., 1983). In Ca^{2+}-containing solution, low Na^+ solution caused an increase in $[Ca^{2+}]_i$ from a basal level of 54.5 ± 4.6 nM ($n = 5$) to a peak value of 206.3 ± 42.0 nM at 6.48 ± 1.46 min, then $[Ca^{2+}]_i$ slowly declined back to the basal level (Fig. 1A upper). In Ca^{2+}-free solution, low Na^+ solution did not cause a significant change in resting $[Ca^{2+}]_i$ for period of 30 min (Fig. 1A lower).

The role of the Na^+-Ca^{2+} exchange mechanism was also examined with 5 mM Ni^{2+}, which is reported to be an inhibitor of Na^+-Ca^{2+} exchange (Kimura et al., 1987; Kaczorowski et al., 1989). In Ca^{2+}-containing solution, Ni^{2+} caused an increase in $[Ca^{2+}]_i$ from a basal level of

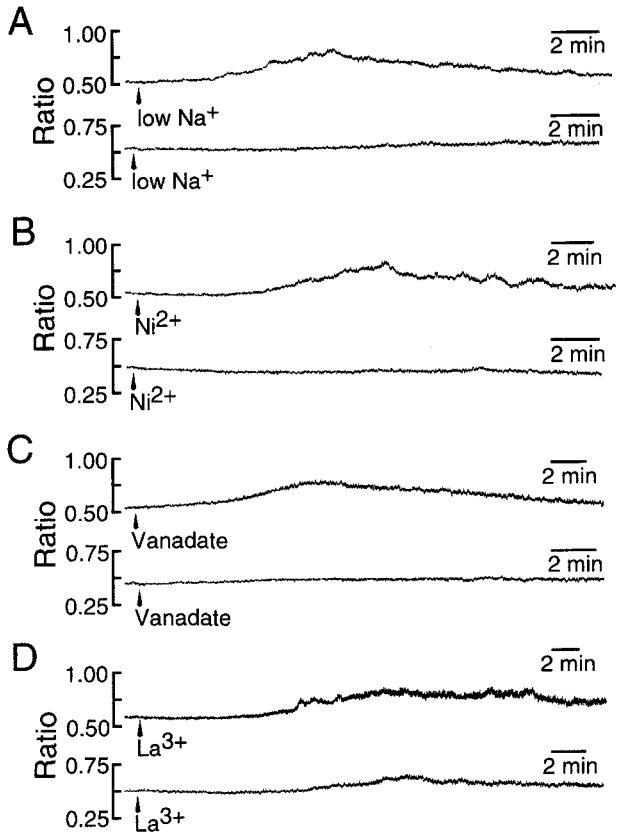

Fig. 1. Effect of low Na^+, Ni^{2+}, vanadate, and La^{3+} on resting $[Ca^{2+}]_i$ in single human aortic endothelial cells. Changes in $[Ca^{2+}]_i$ were monitored by measuring fura-2 fluorescence ratio. Each trace shows the ratio of emitted fluorescence at 340 and 380 nm excitation wavelengths. Responses of resting level $[Ca^{2+}]_i$ to low Na^+ (A) in which 90% of Na^+ were replaced with equimolar *N*-methyl-D-glucamine, 5 mM Ni^{2+} (B), 2 μM vanadate (C), and 1 mM La^{3+} (D) were obtained in the presence of Ca^{2+} (upper traces) and in nominally Ca^{2+}-free solution (lower traces).

62.6 ± 3.5 nM ($n = 5$) to a peak value of 210.6 ± 48.0 nM at 9.0 ± 1.56 min, then $[Ca^{2+}]_i$ slowly declined back to the basal level (Fig. 1B upper). In Ca^{2+}-free solution, Ni^{2+} did not cause a significant change in resting $[Ca^{2+}]_i$ for 30 min (Fig. 1B lower).

Effects of 2 μM vanadate, which is reported to be an inhibitor of the plasma membrane Ca^{2+} pump (Furukawa et al., 1988; Carafoli, 1991; Sugimura et al., 1992) on $[Ca^{2+}]_i$ were examined to elucidate the role of the plasma membrane Ca^{2+}-ATPase for maintaining $[Ca^{2+}]_i$. In Ca^{2+}-containing solution, 2 μM vanadate caused an increase in $[Ca^{2+}]_i$ from a basal level of 61.0 ± 2.5 nM ($n = 5$) to a peak value of 149.7 ± 21.2 nM at 8.70 ± 1.23 min, then $[Ca^{2+}]_i$ slowly declined back to the basal level (Fig. 1C upper). In Ca^{2+}-free solution, 2 μM vanadate did not cause a significant change in resting $[Ca^{2+}]_i$ for period of 30 min (Fig. 1C lower).

Effects of 1 mM La^{3+}, which is reported to be an inhibitor of Na^+-Ca^{2+} exchange and Ca^{2+}-ATPase (Kaczorowski et al., 1989; Kwan et al., 1990, Carafoli, 1991; Toescu and Petersen, 1994), on $[Ca^{2+}]_i$ were examined. In Ca^{2+}-containing solution, 1 mM La^{3+} caused an increase in $[Ca^{2+}]_i$ from a basal level of 62.6 ± 3.2 nM ($n = 5$) to a peak value of 218.0 ± 28.7 nM at 20.7 ± 1.31 min, and the increased level of $[Ca^{2+}]_i$ was maintained (Fig. 1D upper). In a Ca^{2+}-free solution, 1 mM La^{3+} did not cause a significant change in resting $[Ca^{2+}]_i$ up to 9.64 ± 0.48 min ($n = 5$) (Fig. 1D lower). And then $[Ca^{2+}]_i$ was slightly increased from a basal level of 57.9 ± 4.9 nM ($n = 5$) to a peak value of 137.3 ± 17.5 nM at 19.66 ± 1.76 min and the increased level of $[Ca^{2+}]_i$ was also maintained (Fig. 1D lower).

3.2. Effects of low Na^+, Ni^{2+}, vanadate and La^{3+} on histamine-induced change in $[Ca^{2+}]_i$

The roles of the plasma membrane Na^+-Ca^{2+} exchange in the declining phase of $[Ca^{2+}]_i$ elevated by histamine were examined. To eliminate the influence of the entry of Ca^{2+} from the extracellular space, experiments were carried out in Ca^{2+}-free solution. Histamine (100 μM) produced a transient increase in $[Ca^{2+}]_i$ in Ca^{2+}-free solution, and the level of $[Ca^{2+}]_i$ is returned to the basal level within a few minutes (Fig. 2A upper). In low Na^+ solution, the declining phase of $[Ca^{2+}]_i$ was slowed (Fig. 2A lower) and the time required for 50% relaxation was significantly increased by 22 s (Table 1). In the presence of 5 mM Ni^{2+}, the declining phase was also slowed (Fig. 2B lower) and the time required for 50% relaxation was significantly increased by 49 s (Table 1).

The role of the plasma membrane Ca^{2+}-ATPase in the declining phase of $[Ca^{2+}]_i$ elevated by histamine was examined with 2 μM vanadate. The declining phase of $[Ca^{2+}]_i$ elevated by histamine (100 μM) was examined in the presence of 2 μM vanadate (Fig. 3A lower). and the time required for 50% relaxation was slightly increased by 8 s (Table 1). The difference was statistically significant ($P < 0.05$).

Table 1
Effect of Ni^{2+}, low Na^+, vanadate and La^{3+} on 50% relaxation time

	50% relaxation time (s)				
	Control	Low Na^+	Ni^{2+}	Vanadate	La^{3+}
Histamine	22.9 ± 1.7	45.0 ± 5.0 [c]	71.5 ± 3.5 [c]	31.2 ± 3.1 [a]	205.2 ± 16.4 [c]
Thapsigargin	100.0 ± 5.7	145.8 ± 15.9 [b]	153.0 ± 13.5 [c]	126.7 ± 6.7 [b]	302.4 ± 23.4 [c]

Effects of 5 mM Ni^{2+}, low Na^+ solution, 2 μM vanadate and 1 mM La^{3+} on 50% relaxtion time induced by 100 μM histamine or 100 nM thapsigargin. The 50% relaxation time means the time of 50% decrease in peak fluoresence ratio induced by histamine and thapsigargin. Values are the means ± S.E.M. of 5–10 experiments. [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$.

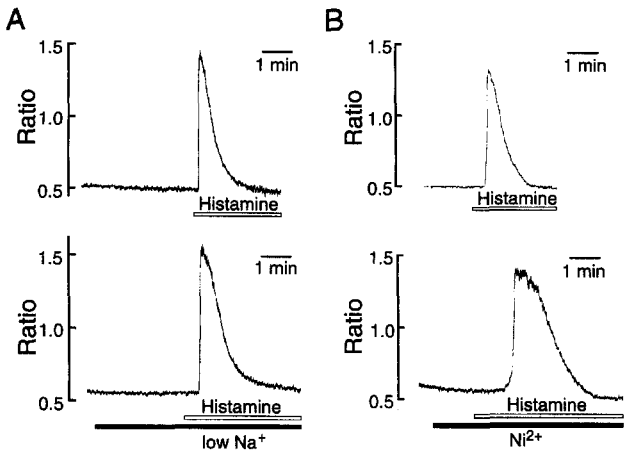

Fig. 2. Effect of low Na^+ solution and Ni^{2+} on histamine-induced change in $[Ca^{2+}]_i$ in single human aortic endothelial cells. Each trace shows the ratio of emitted fluorescence at 340 and 380 nm excitation wavelengths. Control responses (upper traces) and effects of low Na^{2+} solution (A, lower trace) and 5 mM Ni^{2+} (B, lower trace) on 100 μM histamine-induced increase in $[Ca^{2+}]_i$.

Effects of 1 mM La^{3+} on the declining phase of $[Ca^{2+}]_i$ induced by 100 μM histamine were also examined in Ca^{2+}-free solution (Fig. 3B lower). The time required for 50% relaxation was markedly increased by 182 s (Table 1).

3.3. Effects of low Na^+, and Ni^{2+}, vanadate and La^{3+} on thapsigargin-induced declining phase of $[Ca^{2+}]_i$

The physiological role of Ca^{2+} extrusion mechanisms were examined under the condition where endoplasmic

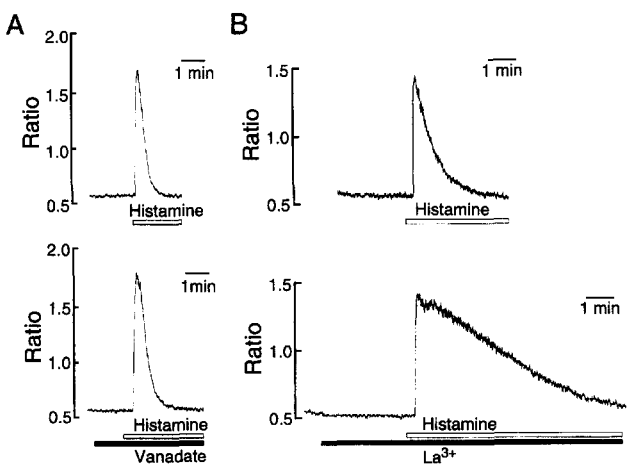

Fig. 3. Effect of 2 μM vanadate and 1 mM La^{3+} on 100 μM histamine-induced change in $[Ca^{2+}]_i$ in single human aortic endothelial cells. Each trace shows the ratio of emitted fluorescence at 340 and 380 nm excitation wavelengths. (A) Typical recording of the $[Ca^{2+}]_i$ induced by histamine in nominally Ca^{2+}-free solution in single human aortic endothelial cells (upper trace). In nominally Ca^{2+}-free solution histamine was applied in the presence of vanadate (lower trace). (B) Control response (upper trace). Histamine was applied in the presence of La^{3+} in nominally Ca^{2+}-free solution (lower trace).

Ca^{2+}-ATPase was inhibited by thapsigargin. As reported previously, thapsigargin (100 nM) elicited a transient $[Ca^{2+}]_i$ increase in Ca^{2+}-free solution (Hosoki and Iijima, 1995). Just after a peak of the thapsigargin-induced Ca^{2+} transient, the superfusion solution was changed to low Na^+ solution (Fig. 4A lower). The declining phase was clearly slowed and the time required for 50% relaxation was significantly increased by 46 s (Table 1).

Effects of 5 mM Ni^{2+} were also examined immediately after a peak of the thapsigargin-induced Ca^{2+} transient (Fig. 4B lower). The declining phase was clearly slowed and the time required for 50% relaxation was increased by 53 s (Table 1, $n = 5$).

Effects of 2 μM vanadate were examined immediately after a peak of the thapsigargin-induced Ca^{2+} transient (Fig. 5A lower). The declining phase was slightly but

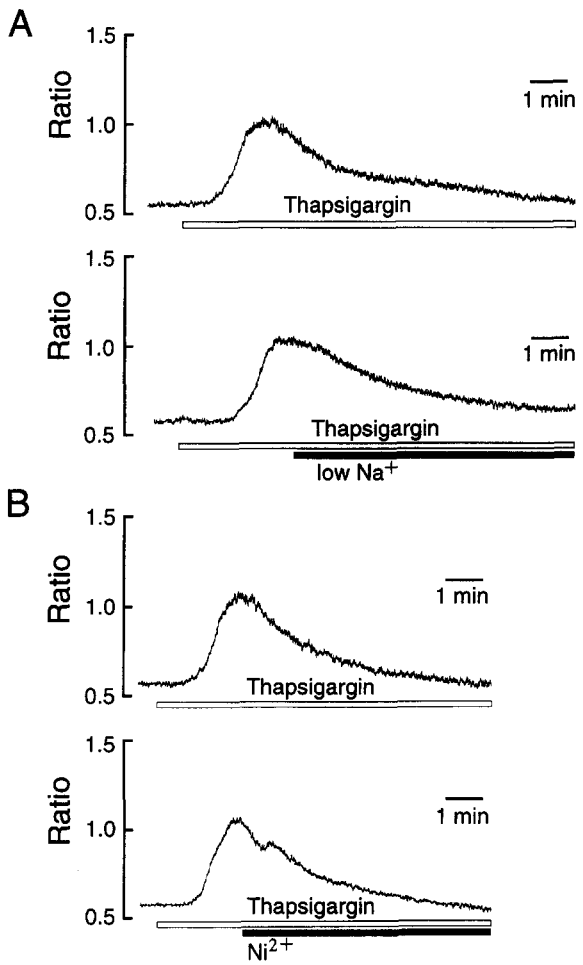

Fig. 4. Effect of low Na^{2+} solution and 5 mM Ni^{2+} on 100 nM thapsigargin-induced declining phase of $[Ca^{2+}]_i$ in single human aortic endothelial cells. Each trace shows the ratio of emitted fluorescence at 340 and 380 nm excitation wavelengths. (A) Typical recording of the $[Ca^{2+}]_i$ induced by thapsigargin in nominally Ca^{2+}-free solution in single human aortic endothelial cells (upper trace). Just after a peak of the thapsigargin-induced Ca^{2+} transient, the superfusion solution was changed to low Na^+ solution (lower trace). (B) Control response (upper trace). Ni^{2+} was added immediately after the peak of thapsigargin-induced Ca^{2+} transient (lower trace).

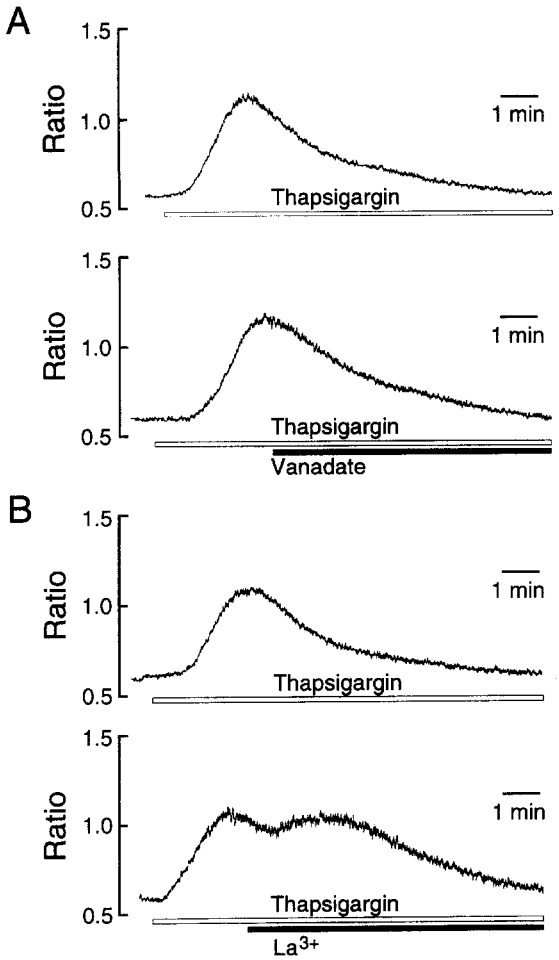

Fig. 5. Effect of 2 μM vanadate and 1 mM La^{3+} on 100 nM thapsigargin-induced declining phase of $[Ca^{2+}]_i$ in single human aortic endothelial cells. Each trace shows the ratio of emitted fluorescence at 340 and 380 nm excitation wavelengths. (A) Control response (upper trace). Vanadate was added immediately after the peak of thapsigargin-induced Ca^{2+} transient in nominally Ca^{2+}-free solution (lower trace). (B) Control response (upper trace). La^{3+} was added immediately after the peak of thapsigargin-induced Ca^{2+} transient in nominally Ca^{2+}-free solution (lower trace).

significantly slowed by 2 μM vanadate. The time required for 50% relaxation by vanadate was increased by 27 s (Table 1). The difference was statistically significant ($P < 0.01$).

Effects of 1 mM La^{3+} were tested immediately after a peak of the thapsigargin-induced Ca^{2+} transient (Fig. 5B lower). The declining phase was much more clearly slowed by La^{3+}, and the time required for 50% relaxation was prolonged by 202 s (Table 1).

4. Discussion

4.1. Regulation of $[Ca^{2+}]_i$ in endothelial cells

Participation of the plasma membrane Ca^{2+} pump in Ca^{2+} efflux has been generally accepted (Carafoli, 1991). But the existence of Na^+-Ca^{2+} exchange in endothelial cells is still controversial. The voltage dependence of resting $[Ca^{2+}]_i$, which was consistent with a simple pump-leak model, provided no evidence for the existence of Na^+-Ca^{2+} exchange in the plasma membrane of cultured bovine pulmonary artery endothelial cells (Cannell and Sage, 1989). A similar conclusion was obtained from the observation that application of the Na^+-K^+ pump inhibitor ouabain was without effect on $[Ca^{2+}]_i$ in cultured endothelial cells from the bovine pulmonary artery (Sage et al., 1991). It was reported that isotonic Li^+ substitution for Na^+ in cultured endothelial cells from bovine atria had no effect on $[Ca^{2+}]_i$ regardless of whether the cells were at rest or activated by the agonist bradykinin (Laskey et al., 1990). Similarly, bradykinin-stimulated changes in $[Ca^{2+}]_i$ in cultured bovine aortic endothelial cells were unaffected by isosmotic substitution of external Na^+ with *N*-methyl-D-glucamine (NMDG) (Schilling et al., 1988). These results suggested that the Na^+-Ca^{2+} exchange does not significantly contribute either to Ca^{2+} extrusion or entry in the cultured endothelial cells. However, it was reported that if cultured endothelial cells were first loaded with Na^+ by a Na^+ ionophore monensin and then exposed to physiological saline solution in which the external Na^+ was substituted by Li^+, a large transient increase in $[Ca^{2+}]_i$ ensued and combined pretreatment with ouabain and monensin doubled this $[Ca^{2+}]_i$ transient (Sage et al., 1991). A similar conclusion was reached from the observation that the rate of Ca^{2+} influx was increased by the application of ouabain in cultured bovine aortic endothelial cells and was increased further when the ouabain-treated endothelial cells was transferred to a solution in which external Na^+ was substituted by choline (Hansen et al., 1991). It was also reported that a selective inhibitor of Na^+-Ca^{2+} exchange, 3′,4′-dichlorobenzamil, abolished the transient $[Ca^{2+}]_i$ increase induced by Na^+ substitution, and Mg^{2+}, an inorganic inhibitor of Na^+-Ca^{2+} exchange, markedly reduced this transient $[Ca^{2+}]_i$ increase (Winquist et al., 1985; Li and Van Breemen, 1995). These results imply the presence of the Na^+-Ca^{2+} exchange mechanism for increasing $[Ca^{2+}]_i$ in intact cardiovascular endothelial cells. The physiological role of Na^+-Ca^{2+} exchange for the extrusion of Ca^{2+} is still uncertain. Moreover, the existence of Na^+-Ca^{2+} exchange in human aortic endothelial cells has not been identified yet. In the present experiments, we have examined the existence and the role of the plasma membrane Na^+-Ca^{2+} exchange and Ca^{2+}-ATPase in the regulation of $[Ca^{2+}]_i$ at resting and agonist-stimulated conditions in human aortic endothelial cells.

4.2. Regulation of $[Ca^{2+}]_i$ at resting condition in human aortic endothelial cells

Na^+-free solution should force a Na^+-Ca^{2+} exchange into the Na^+ efflux-Ca^{2+} influx mode of operation and thereby increase $[Ca^{2+}]_i$ (Chapman, 1974; Allen et al.,

1983). At resting condition, low Na^+ solution caused a significant increase in $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} (Fig. 1A). The decrease in $[Ca^{2+}]_i$ during the period of exposure to low Na^+ solution suggests that there must be a Na^+-independent Ca^{2+} extrusion mechanism, such as Ca^{2+} ATPase, in the plasma membrane of the endothelial cell. In addition, it might be likely that Na^+ leaves the cell during exposure to low Na^+ solution and this will lead to a loss of Ca^{2+} entry via the exchanger because, in the absence of extracellular Ca^{2+}, low Na^+ solution failed to modify $[Ca^{2+}]_i$. It has also been reported that Ni^{2+} (5 mM) blocks Na^+-Ca^{2+} exchange in the plasma membrane (Kimura et al., 1987; Kaczorowski et al., 1989; Levesque et al., 1994). The resting level of $[Ca^{2+}]_i$ was increased by 5 mM Ni^{2+} in the presence but not in the absence of extracellular Ca^{2+} (Fig. 1B). These results suggest the existence of the Na^+-Ca^{2+} exchange mechanism which has a physiological role in maintaining the resting level of $[Ca^{2+}]_i$, and the existence of Ca^{2+} influx from extracellular space and/or spontaneous Ca^{2+} release from intracellular store sites even in the resting condition.

Orthovanadate, which is present essentially at physiological pH as $[VO_3(OH)]^{2-}$, is a pentacoordinate stereo analogue of phosphate that is now considered as the classic inhibitor of P-type ionmotive ATPases (Carafoli, 1991). Sensitivity to vanadate is different among the various ATPases, with the inhibitor constant (K_i) values ranging from submicromolar concentrations for plasma membrane Ca^{2+} pumps of heart muscle (Caroni and Carafoli, 1981) to 1.7 μM for skeletal muscle (Michalak et al., 1984), and to 50 μM for the endoplasmic reticulum enzyme (Wang et al., 1979). In the present experiment 2 μM vanadate was used to inhibit the plasma membrane Ca^{2+} pump (Ca^{2+} ATPase) in human aortic endothelial cells because, in preliminary experiments, 10 μM vanadate caused a transient increase in $[Ca^{2+}]_i$ in Ca^{2+}-free solution. This might indicate that 10 μM vanadate also inhibit Ca^{2+} ATPase of endoplasmic reticulum. As shown in Fig. 1C, 2 μM vanadate caused an increase in $[Ca^{2+}]_i$ at the resting condition in the presence but not in the absence of extracellular Ca^{2+}. This result also suggests the existence of plasma membrane Ca^{2+} pumps in human aortic endothelial cells, and that the plasma membrane Ca^{2+} pump mechanism has a physiological role in maintaining the resting level of $[Ca^{2+}]_i$.

It has been reported that La^{3+} blocks the plasma membrane Ca^{2+} entry pathways at low (micromolar) concentrations and inhibits Ca^{2+} ATPase of the plasma membrane at millimolar concentrations in acinar cells (Wakasugi et al., 1981; Kwan et al., 1990; Toescu and Petersen, 1994). It is known that La^{3+} blocks Ca^{2+} influx by inhibiting Na^+-Ca^{2+} exchange in a number of different vesicle systems (Gill et al., 1981; Kaczorowski et al., 1984) and also blocks Ca^{2+} efflux in cultured cardiac myocytes (Barry and Smith, 1982). As shown in Fig. 1D, the increase in $[Ca^{2+}]_i$ produced by La^{3+} was slowly developing and sustained in the presence of extracellular Ca^{2+} but was transient in the absence of extracellular Ca^{2+}. La^{3+} blocks Ca^{2+} influx via Na^+-Ca^{2+} exchange much more rapidly and with high sensitivity than it does Ca^{2+} efflux (Barry and Smith, 1982).

4.3. Extrusion mechanism responsible for reducing $[Ca^{2+}]_i$ elevated by histamine or thapsigargin in human aortic endothelial cells

In Ca^{2+}-free solution, receptor agonists, such as histamine and ATP, elicit a transient increase in $[Ca^{2+}]_i$, but the increased level of $[Ca^{2+}]_i$ is returned to the basal level within several minutes (Ziche et al., 1993; Hosoki and Iijima, 1994; Ziegelstein et al., 1994). The least understood phase of the transient change in $[Ca^{2+}]_i$ is the removal of Ca^{2+} from the cytosol against its electrochemical gradient. Calcium ions can be removed from the cytosol by Ca^{2+} pump located in the plasma and the endoplasmic reticulum membrane, and by the Na^+-Ca^{2+} exchange mechanism. In the present experiment, the declining phase was significantly attenuated in low Na^+ solution and in the presence of 5 mM Ni^{2+} and 2 mM vanadate, and was more clearly slowed by 1 mM La^{3+} (Fig. 2 and Fig. 3). These results indicate that Na^+-Ca^{2+} exchange and the plasma membrane Ca^{2+} pump mechanism have a physiological role in reducing $[Ca^{2+}]_i$ elevated by histamine in human aortic endothelial cells. Prolongation of the 50% relaxation time by vanadate was statistically significant but less effective than other interventions (Table 1). This may indicate that the physiological role of the plasma membrane Ca^{2+} pump in lowering $[Ca^{2+}]_i$ increased by agonist stimulation may not be a mandatory mechanism.

Thapsigargin has been shown to inhibit the endoplasmic reticulum Ca^{2+}-ATPase without affecting the formation of inositol trisphosphate (Goeger et al., 1988; Seidler et al., 1989; Takemura et al., 1989; Thastrup et al., 1990) and to elicit a transient increase in $[Ca^{2+}]_i$ in Ca^{2+}-free solution (Ziche et al., 1993; Kwan et al., 1990; Hosoki and Iijima, 1995). It becomes possible to examine directly the role of plasma membrane Na^+-Ca^{2+} exchange and Ca^{2+}-ATPase in Ca^{2+} removal from the cytosol without the influence of the uptake into the endoplasmic reticulum. As shown in Fig. 4 and Fig. 5, even in the presence of thapsigargin, the level of $[Ca^{2+}]_i$ increased by thapsigargin was returned to the basal level within several minutes. Recovery of $[Ca^{2+}]_i$ toward the basal level could be achieved by Ca^{2+} pump and Na^+-Ca^{2+} exchange located in the plasma membrane. The declining phase was significantly slowed in low Na^+ solution and by 5 mM Ni^{2+} and 2 mM vanadate, and was more clearly prolonged by 1 mM La^{3+} (Table 1). These results much more clearly showed the physiological role of the plasma membrane Ca^{2+} pump and Na^+-Ca^{2+} exchange in reducing $[Ca^{2+}]_i$ elevated by thapsigargin in human aortic endothelial cells. It is interesting to note that,

as shown in Table 1, La^{3+}-induced prolongation of the 50% relaxation time of $[Ca^{2+}]_i$ increased by thapsigargin was more than the sum of the inhibition of Ca^{2+} pump (by vanadate) and Na^+-Ca^{2+} exchange (by low Na^+ solution or Ni^{2+}). This may indicate that La^{3+} has an additional effect on Ca^{2+} efflux pathways such as non-specific cation channels.

In conclusion, our present results showed the physiological role of plasma membrane Na^+-Ca^{2+} exchange and Ca^{2+} pump, and endoplasmic reticulum Ca^{2+} pump in extruding cytosolic Ca^{2+} at resting and agonist-stimulated conditions in cultured human aortic endothelial cells. In this study we report for the first time the physiological role of the plasma membrane Na^+-Ca^{2+} exchange and Ca^{2+}-ATPase in the extrusion of Ca^{2+} from human aortic endothelial cells. It will be crucial to characterize the Ca^{2+} transport pathways that control $[Ca^{2+}]_i$ to understand the physiology of the endothelium as well as its role in atherosclerotic and thrombotic vascular disease.

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References

Allen, D.G., D.A. Eisner, M.J. Lab and C.H. Orchard, 1983, The effects of low sodium solutions on intracellular calcium concentration and tension in ferret ventricular muscle, J. Physiol. 345, 391.

Barry, W.H. and T.W. Smith, 1982, Mechanisms of transmembrane calcium movement in cultured chick embryo ventricular cells, J. Physiol. 325, 243.

Cannell, M.B. and S.O. Sage, 1989, Bradykinin-evoked change in cytosolic calcium and membrane currents in bovine pulmonary artery endothelial cells, J. Physiol. 419, 555.

Carafoli, E., 1991, Calcium pump of the plasma membrane, Physiol. Rev. 71, 129.

Caroni, P. and E. Carafoli, 1980, An ATP-dependent Ca^{2+}-pumping system in dog heart sarcolemma, Nature 283, 765.

Caroni, P. and E. Carafoli, 1981, The Ca^{2+}-pumping ATPase of heart sarcolemma. Characterization, calmodulin dependence, and partial purification, J. Biol. Chem. 256, 3263.

Chapman R.A., 1974, A study of the contractures induced in frog atrial trabeculae by a reduction of the bathing sodium concentration, J. Physiol. 237, 295.

Dipolo, R., 1978, Ca pump driven by ATP in squid axons, Nature 274, 390.

Furukawa, K., Y. Tawada and M. Shigekawa, 1988, Regulation of the plasma membrane Ca^{2+} pump by cyclic nucleotides in cultured vascular smooth muscle cells, J. Biol. Chem. 263, 8058.

Gill, D.L., E.F. Grollman and L.D. Kohn, 1981, Calcium transport mechanisms in membrane vesicles from guinea pig brain synaptosomes, J. Biol. Chem. 256, 184.

Goeger, D.E., R.T. Riley, J.W.Dorner and R.J. Cole, 1988, Cyclopiazonic acid inhibition of the Ca^{2+}-transport ATPase in rat skeletal muscle sarcoplasmic reticulum vesicles, Biochem. Pharmacol. 37, 978.

Hallam, T.J., J.D. Pearson and L.A. Needham, 1988, Thrombin-stimulated elevation of human endothelial-cell cytoplasmic free calcium concentration causes prostacyclin production, Biochem. J. 251, 243.

Hansen, B.A., D.C. Battle and M.E. O'Donnell, 1991, Sodium-calcium exchange in bovine aortic endothelial cells, Ann. NY Acad. Sci. 639, 566.

Hosoki, E. and T. Iijima, 1994, Chloride-sensitive Ca^{2+} entry by histamine and ATP in human aortic endothelial cells, Eur. J. Pharmacol. Mol. Pharmacol. Section 266, 213.

Hosoki, E. and T. Iijima, 1995, Modulation of cytosolic Ca^{2+} concentration by thapsigargin and cyclopiazonic acid in human aortic endothelial cells, Eur. J. Pharmacol. Mol. Pharmacol. Section 288, 131.

Kaczorowski, G.J., L. Costello, J, Dethmers, M.J. Trumble and R.L. Vandlen, 1984, Mechanisms of Ca^{2+} transport in plasma membrane vesicles prepared from cultured pituitary cells, J. Biol. Chem. 259, 9395.

Kaczorowski, G.J., R.S. Slaughter, V.F. King and L. Garcia, 1989, Inhibitors of sodium-calcium exchange: identification and development of probes of transport activity, Biochim. Biophys. Acta 988, 287.

Kimura, J., S. Miyamae and A. Noma, 1987, Identification of sodium-calcium exchange current in single ventricular cells of guinea-pig, J. Physiol. 384, 199.

Konishi, M., A. Olson, S. Hollingwrth and S.M. Baylor, 1988, Myoplasmic binding of fura-2 investigated by steady-state fluorescence and absorbance measurements, Biophys. J. 54, 1089.

Kwan, C.Y., H. Takemura, J.F. Obie, O. Thastrup and J.W. Putney, JR, 1990, Effects of MeCh, thapsigargin, and La^{3+} on plasmalemmal and intracellular Ca^{2+} transport in lacrimal acinar cells, Am. J. Physiol. 258, C1006.

Laskey, R.E., D.J. Adams, A. Johns, G.M. Rubanyi and C. Van Breemen, 1990, Membrane potential and Na^+-K^+ pump activity modulate resting and bradykinin-stimulated changes in cytosolic free calcium in cultured endothelial cells from bovine atria, J. Biol. Chem. 265, 2613.

Levesque, P.C., N. Leblanc and J.R. Hume, 1994, Release of calcium from guinea pig cardiac sarcoplasmic reticulum induced by sodium-calcium exchange, Cardiovasc. Res. 28, 370.

Li, L. and C. Van Breemen, 1995, Na^+-Ca^{2+} exchange in intact endothelium of rabbit cardiac valve, Circ. Res. 76, 396.

Lückhoff, A., U. Pohl, A. Mülsch and R. Busse, 1988, Differential role of extra- and intracellular calcium in the release of EDRF and prostacyclin from cultured endothelial cells, Br. J. Pharmacol. 95, 189.

Michalak, M., K. Famulski and E. Carafori, 1984, The Ca^{2+}-pumping ATPase in skeletal muscle sarcolemma. Calmodulin dependence, regulation by cAMP-dependent phosphorylation, and purification, J. Biol. Chem. 259, 15540.

Sage, S.O., C. Van Breemen, M.B. Cannell, 1991, Sodium-calcium exchange in cultured bovine pulmonary artery endothelial cells, J. Physiol. 440, 569.

Schilling, W.P., A.K. Ritchie, L.T. Navarro and S.G. Eskin, 1988, Bradykinin-stimulated calcium influx in cultured bovine aortic endothelial cells, Am. J. Physiol. 255, H219.

Seidler, N.W., I. Jona, M. Vegh and A. Martonosi, 1989, Cyclopiazonic acid is a specific inhibitor of the Ca^{2+}-ATPase of sarcoplasmic reticulum, J. Biol. Chem. 264, 17816.

Sugimura, K., J. Abramowitz, Y. Tsukamoto and W.N. Suki, 1992, Reconstitution and partial purification of calcium transport activity from rat kidney cortex, Am. J. Physiol. 263, F192.

Takemura, H., A.R. Hughes, O. Thastrup and J.W. Putney, Jr., 1989, Activation of calcium entry by the tumor promoter thapsigargin in parotid acinar cells, J. Biol. Chem. 264, 12266.

Thastrup, O., P.J. Cullen, B.K. Drøbak, M.R. Hanley and A.P. Dawson, 1990, Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+}

stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase, Proc. Natl. Acad. Sci. USA 87, 2466.

Toescu, E.C. and O.H. Petersen, 1994, The thapsigargin-evoked increase in $[Ca^{2+}]_i$ involves an $InsP_3$-dependent Ca^{2+} release process in pancreatic acinar cells. Pflügers Arch. 427, 325.

Wakasugi, H., H. Stolze, W. Haase, and I. Sahulz, 1981, Effect of La^{3+} on secretagogue-induced Ca^{2+} fluxes in rat isolated pancreatic acinar cells. Am. J. Physiol. 240, G281.

Wang, T., L. Tsai, R.J. Solaro, A.O.G. Gende and A. Schwartz, 1979, Effects of potassium on vanadate inhibition of sarcoplasmic reticulum Ca^{2+}-ATPase from dog cardiac and rabbit skeletal muscle, Biochem. Biophys. Res. Commun. 91, 356.

Winquist, R.J., P.B. Bunting and T.L. Schofield, 1985, Blockade of endothelium-dependent relaxation by the amiloride analog dichlorobenzamil: possible role of Na^+-Ca^{2+} exchange in the release of endothelium-derived relaxant factor, J. Pharmacol. Exp. Ther. 235, 644.

Ziche, M., D. Zawieja, R.K. Hester and H. Granger, 1993, Calcium entry, mobilization, and extrusion in postcapillary venular endothelium exposed to bradykinin, Am. J. Physiol. 265, H569.

Ziegelstein, R.C., H.A. Spurgeon, R. Pili, A. Passaniti, L. Cheng, S. Corda, E.G. Lakatta and M.C. Capogrossi, 1994, A functional ryanodine-sensitive intracellular Ca^{2+} store is present in vascular endothelial cells, Circ. Res. 74, 151.