British Journal of Pharmacology (1999) 126, 1107–1114 © 1999 Stockton Press All rights reserved 0007–1188/99 $12.00
http://www.stockton-press.co.uk/bjp

Activation of three types of voltage-independent Ca^{2+} channel in A7r5 cells by endothelin-1 as revealed by a novel Ca^{2+} channel blocker LOE 908

[1]Yasushi Iwamuro, *,[2]Soichi Miwa, [2]Xiao-Feng Zhang, [1]Tetsuya Minowa, [3]Taijiro Enoki, [2]Yasuo Okamoto, [2]Hiroshi Hasegawa, [3]Hidekatsu Furutani, [2]Makoto Okazawa, [1]Masatsune Ishikawa, [1]Nobuo Hashimoto & [4]Tomoh Masaki

[1]Department of Neurosurgery, Kyoto University Faculty of Medicine, Kyoto 606-8501, Japan; [2]Department of Pharmacology, Kyoto University Faculty of Medicine, Kyoto 606-8501, Japan; [3]Department of Anesthesiology, Kyoto University Faculty of Medicine, Kyoto 606-8501, Japan; [4]National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan

1 We have shown that in addition to voltage-operated Ca^{2+} channel (VOC), endothelin-1 (ET-1) activates two types of Ca^{2+}-permeable nonselective cation channel (NSCC) in A7r5 cells: its lower concentrations ($\leqslant$1 nM; lower [ET-1]) activate only an SK&F 96365-resistant channel (NSCC-1), whereas its higher concentrations ($\geqslant$10 nM; higher [ET-1]) activate an SK&F 96365-sensitive channel (NSCC-2) as well.

2 We now characterized the effects of a blocker of Ca^{2+} entry channel LOE 908 on NSCCs and store-operated Ca^{2+} channel (SOCC) in A7r5 cells, and using two drugs, clarified the involvement of these channels in the ET-1-induced increase in the intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$). Whole-cell recordings and $[Ca^{2+}]_i$ monitoring with fluo-3 were used.

3 LOE 908 up to 10 μM had no effect on increases in $[Ca^{2+}]_i$ induced by thapsigargin or ionomycin, but SK&F 96365 abolished them.

4 In the cells clamped at -60 mV, both lower and higher [ET-1] induced inward currents with linear iv relationships and the reversal potentials of -15.0 mV. Thapsigargin induced no currents.

5 In the presence of nifedipine, lower [ET-1] induced a sustained increase in $[Ca^{2+}]_i$, whereas higher [ET-1] induced a transient peak and a sustained increase. The sustained increases by lower and higher [ET-1] were abolished by removal of extracellular Ca^{2+}, and they were suppressed by LOE 908 to 0 and 35%, respectively, with the LOE 908-resistant part being abolished by SK&F 96365.

6 These results show that LOE 908 is a blocker of NSCCs without effect on SOCC, and that the increase in $[Ca^{2+}]_i$ at lower [ET-1] results from Ca^{2+} entry through NSCC-1 in addition to VOC, whereas the increase at higher [ET-1] involves NSCC-1, NSCC-2 and SOCC in addition to VOC.

Keywords: Endothelin-1; voltage-independent calcium channel; SK&F 96365; LOE 908; A7r5 cell

Abbreviations: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulphonic acid; ET-1, endothelin-1; IP_3, D-*myo*-inositol-1,4,5-trisphosphates; NSCC, nonselective cation channel; SOCC, store-operated Ca^{2+} channel; VOC, voltage-operated Ca^{2+} channel; VSMCs, vascular smooth muscle cells

Introduction

Endothelin-1 (ET-1) is a 21-amino-acid peptide and it is one of the most potent endogenous vasoconstricting agents yet discovered (Yanagisawa *et al*., 1988). ET-1 binds to its receptors (typically ET_A receptor) on vascular smooth muscle cells (VSMCs) and subsequently raises the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), which is essential for contraction of the cells. It is generally accepted that the major part of the sustained contraction by ET-1 requires the persistent entry of extracellular Ca^{2+} (Rubanyi & Polokoff, 1994; Komuro *et al*., 1997; Zhang *et al*., 1998). The voltage-operated Ca^{2+} channel (VOC) is a well-known Ca^{2+} entry channel activated by ET-1 (Goto *et al*., 1989; Inoue *et al*., 1990), but it becomes increasingly clear that involvement of this channel in ET-1-induced vasocontractions is minimal (Huang *et al*., 1990; Simpson *et al*., 1990; Komuro *et al*., 1997; Zhang *et al*., 1998). Several researchers have shown that a Ca^{2+}-permeable nonselective cation channel (NSCC) is activated by stimulation of the native ET_A receptor in VSMCs (Van Reterghem *et al*., 1988; Chen & Wagoner, 1991; Enoki *et al*., 1995; Minowa *et al*., 1997) and of recombinant human ET_A receptors expressed in L*tk*$^-$ cells (Enoki *et al*., 1995). Recently, we have shown that ET-1 activates two types of Ca^{2+}-permeable NSCC in cultured vascular smooth muscle cells (A7r5 cells) depending on its concentrations: the channel activated by lower concentrations ($\leqslant$1 nM) of ET-1 is resistant to SK&F 96365 (designated NSCC-1), whereas the SK&F 96365-sensitive channel (designated NSCC-2) becomes activated at higher concentrations ($\geqslant$10 nM) of ET-1 in addition to NSCC-1 (Iwamuro *et al*., 1998).

The store-operated Ca^{2+} channel (SOCC) is another important class of Ca^{2+} entry channel which is activated by depletion of intracellular Ca^{2+} store (Putney, 1990; Hoth & Penner, 1992; Irvine, 1992; Fasolato *et al*., 1994; Berridge, 1995). Store depletion usually occurs following mobilization of Ca^{2+} from the store *via* increased formation of D-*myo*-inositol-1,4,5-trisphosphates (IP_3) upon stimulation of G-protein-coupled receptors (Berridge, 1993). Since the ET_A

* Author for correspondence.

receptor is a G-protein-coupled receptor, its stimulation results in increased formation of IP_3 and hence, mobilization of Ca^{2+} from the intracellular store (Rubanyi & Polokoff, 1994; Sokolovsky, 1995). Thus it is highly probable that SOCC is activated following stimulation of the ET_A receptor.

Taken together, these data indicate that stimulation of the ET_A receptor activates at least three classes of voltage-independent Ca^{2+} channel in addition to VOC: two types of Ca^{2+}-permeable NSCC, and SOCC. However, it is totally unknown whether these voltage-independent Ca^{2+} channels actually contribute to the ET-1-induced increase in $[Ca^{2+}]_i$. This seems to be mainly due to the lack of blockers specific for these Ca^{2+} entry channels (Felder *et al*., 1994; Clementi & Meldolesi, 1996).

SK&F 96365 was initially introduced as a blocker of receptor-mediated Ca^{2+} influx in a broad sense and its pharmacological actions were well-characterized (Merritt *et al*., 1990). However, it is accepted that the actions of SK&F 96365 are nonspecific: it suppresses both NSCC and SOCC (Chung *et al*., 1994; Franzius *et al*., 1994; Koch *et al*., 1994).

In the present study using $[Ca^{2+}]_i$ measurement and whole-cell recordings of patch-clamp technique, we present the data that a novel blocker of receptor-mediated Ca^{2+} influx, LOE 908, selectively blocks Ca^{2+} entry through NSCC (NSCC-1 and NSCC-2) in A7r5 cells without inhibition of Ca^{2+} entry through SOCC. Using this drug and SK&F 96365, we show that the increase in $[Ca^{2+}]_i$ in A7r5 cells induced by ET-1 involves three types of voltage independent Ca^{2+} channel in addition to VOC. Namely the increase in $[Ca^{2+}]_i$ induced by lower concentrations of ET-1 results exclusively from Ca^{2+} entry through NSCC-1 which is sensitive to LOE 908 but resistant to SK&F 96365. In contrast, the increase in $[Ca^{2+}]_i$ induced by higher concentrations of ET-1 results from Ca^{2+} entry through NSCC-2 (which is sensitive to both LOE 908 and SK&F 96365) and SOCC (which is sensitive to SK&F 96365 but resistant to LOE 908), in addition to NSCC-1.

Methods

Cell culture

A7r5 cells derived from rat aortic smooth muscle cell were cultured in monolayer in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (Hyclone), 100 u ml^{-1} penicillin G and 100 μg ml^{-1} streptomycin at 37°C in a humidified 5% CO_2/95% air atmosphere.

Measurement of $[Ca^{2+}]_i$ in A7r5 cells

$[Ca^{2+}]_i$ was measured using fluorescent probes fura-2 and fluo-3 as described (Enoki *et al*., 1995; Minowa *et al*., 1997; Iwamuro *et al*., 1998). Fluo-3 was used for experiments with LOE 908, because LOE 908 was found to disturb measurement of $[Ca^{2+}]_i$ by absorbing the light at excitation wavelengths for fura-2. For loading of the probes, the cultured A7r5 cells were incubated in Ca^{2+}-free Krebs-HEPES solution containing 5 μM fura-2/AM (acetoxymethyl ester) or fluo-3/AM for 45 min at 37°C. Normal Krebs-HEPES solution contained (mM): NaCl 140, KCl 3, $CaCl_2$ 2, $MgCl_2$ 1, glucose 11 and HEPES 10; pH 7.3, adjusted with NaOH. After centrifugation, the cells were resuspended at a density of approximately 1.25×10^6 cells ml^{-1} in Ca^{2+}-free Krebs-HEPES solution. Immediately before measurement of $[Ca^{2+}]_i$, $CaCl_2$ was added to the suspension at the final concentration of 2 mM, and 0.5 ml aliquots of the suspension were used for measurement of fluorescence by a CAF 110 spectrophotometer (JASCO, Tokyo, Japan). For measurement of $[Ca^{2+}]_i$, fura-2 was excited at two wavelengths of 340 nm and 380 nm, and emission was monitored at 510 nm. Changes in the $[Ca^{2+}]_i$ were recorded as changes in the Ca^{2+}-sensitive fluorescence ratio (Minowa *et al*., 1997; Iwamuro *et al*., 1998). Wavelengths of excitation and emission for fluo-3 were 490 nm and 540 nm, respectively, and $[Ca^{2+}]_i$ was calculated as described (Enoki *et al*., 1995).

Electrophysiology

A7r5 cells were perfused with Krebs-HEPES solution, visualized with Nomarski optics (Carl-Zeiss Axioskop) and whole-cell recordings were made with thin-wall borosilicate glass patch pipettes (resistance, 3–5 MΩ) as described previously (Iwamuro *et al*., 1998). Pipettes were filled with Cs-aspartate solution containing (in mM): Cs-aspartate 120, CsCl 20, $MgCl_2$ 2, HEPES 10, EGTA 10 (pH 7.3, adjusted with CsOH). EGTA was added to the pipette solution at a final concentration at 10 mM, a concentration having enough buffering capacity for Ca^{2+} to prevent a transient increase in $[Ca^{2+}]_i$ (Neher, 1988), and the concentration of Ca^{2+} in the solution was maintained at 100 nM by adding the amount of $CaCl_2$ as described by Van Heeswijk *et al*. (1984). Where indicated, the concentration of EGTA in the pipette solution was reduced to 0.2 mM. Tight seal whole-cell currents were recorded with an EPC 7 patch-clamp amplifier (List, Darmstadt, Germany). Perfusion rate was maintained at 2.2–2.5 ml min^{-1} and the bath volume was ~1.0 ml. All experiments were done under voltage-clamp at a holding potential of −60 mV at room temperature (22–24°C). To test the contribution of Cl^- current, the bath solution was switched from Krebs-HEPES to low Cl^- solution containing (in mM): sodium gluconate 140, KCl 3, $CaCl_2$ 2, $MgCl_2$ 1, glucose 11 and HEPES 10; pH 7.3, adjusted with NaOH. In all experiments, the bath solution was supplemented with 1 μM nifedipine to block Ca^{2+} entry through VOC. Current-voltage relationships were obtained by applying voltage steps of 100-ms duration ranging from −100 to +80 mV in 20-mV increments before and after application of drugs. The drug-induced currents at each membrane potential were determined by subtracting currents before application of the drug from currents after its application.

Drugs

LOE 908 was kindly provided by Boehringer Ingelheim K.G. (Ingelheim, Germany). Other chemicals were commercially obtained from the following sources: ET-1 from Peptide Institute (Osaka, Japan); SK&F 96365 from Biomol (Plymouth Meeting, PA, U.S.A.); fura-2/AM, fluo-3/AM, EGTA and 4,4′-diisothiocyano-2,2′-stilbenedisulphonic acid (DIDS) from Dojindo Laboratories (Kumamoto, Japan); nifedipine from Sigma (St. Louis, Missouri, U.S.A.). Nifedipine was dissolved in dimethyl sulphoxide and the final concentration of dimethyl sulphoxide was 0.1%.

Statistical analysis

Data were presented as means ± s.e.mean. The data were subjected to a two-way analysis of variance, and when a significant F value was encountered, Newman-Keuls' multiple-range test was used to test for significant differences between treatment means. A probability level of $P < 0.05$ was considered statistically significant.

Results

Effects of LOE 908 on the elevation of $[Ca^{2+}]_i$ in A7r5 cells induced by ET-1

Lower concentrations ($\leqslant$1 nM) of ET-1 in the presence of 1 μM nifedipine induced a monophasic increase in $[Ca^{2+}]_i$ in A7r5 cells monitored by a Ca^{2+} indicator fluo-3 (Figure 1a), whereas higher concentrations ($\geqslant$10 nM) of ET-1 induced a biphasic increase in $[Ca^{2+}]_i$ consisting of an initial transient peak and the subsequent sustained phase (Figure 1b). The sustained increase induced by either concentration of ET-1 was abolished by removal of extracellular Ca^{2+} (Figures 1c and d).

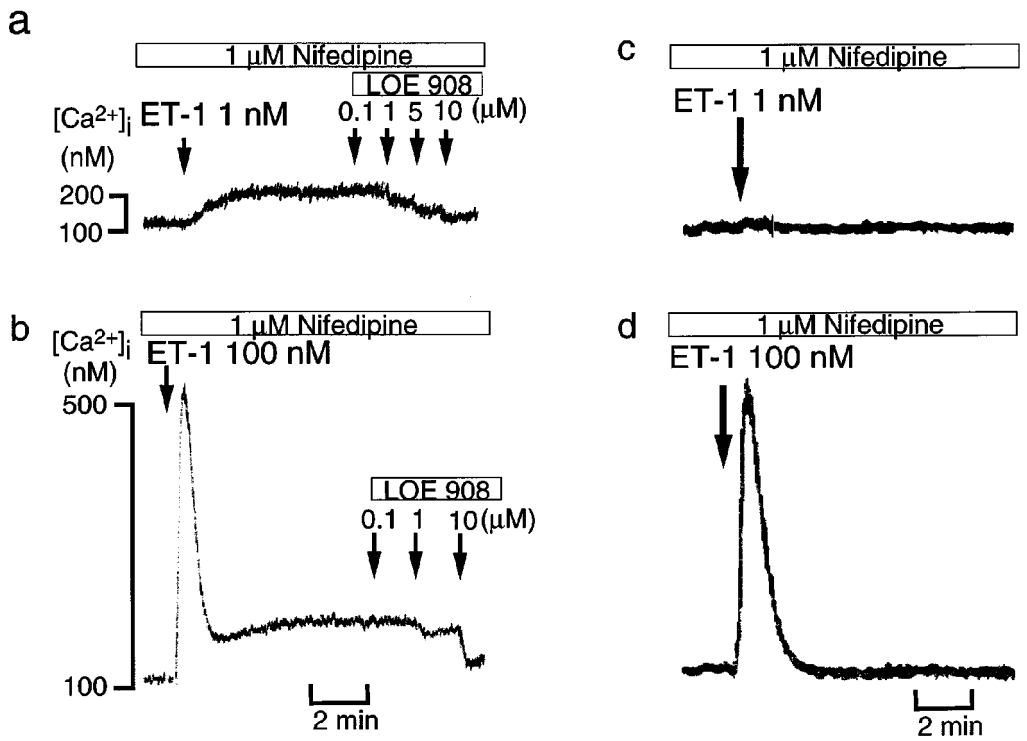

Figure 1 Original tracings illustrating the effects of varying concentrations of LOE 908 and removal of extracellular Ca^{2+} on the increase in the intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in A7r5 cells induced by either a lower (1 nM) or higher (100 nM) concentration of endothelin-1 (ET-1). Fluo-3-loaded A7r5 cells were stimulated by either 1 nM (a and c) or 100 nM ET-1 (b and d) in the presence (a and b) or absence (c and d) of extracellular Ca^{2+}. After the response to ET-1 reached a steady-state, varying concentrations of LOE 908 were added to the medium. In all experiments, nifedipine was added to the medium at the final concentration of 1 μM.

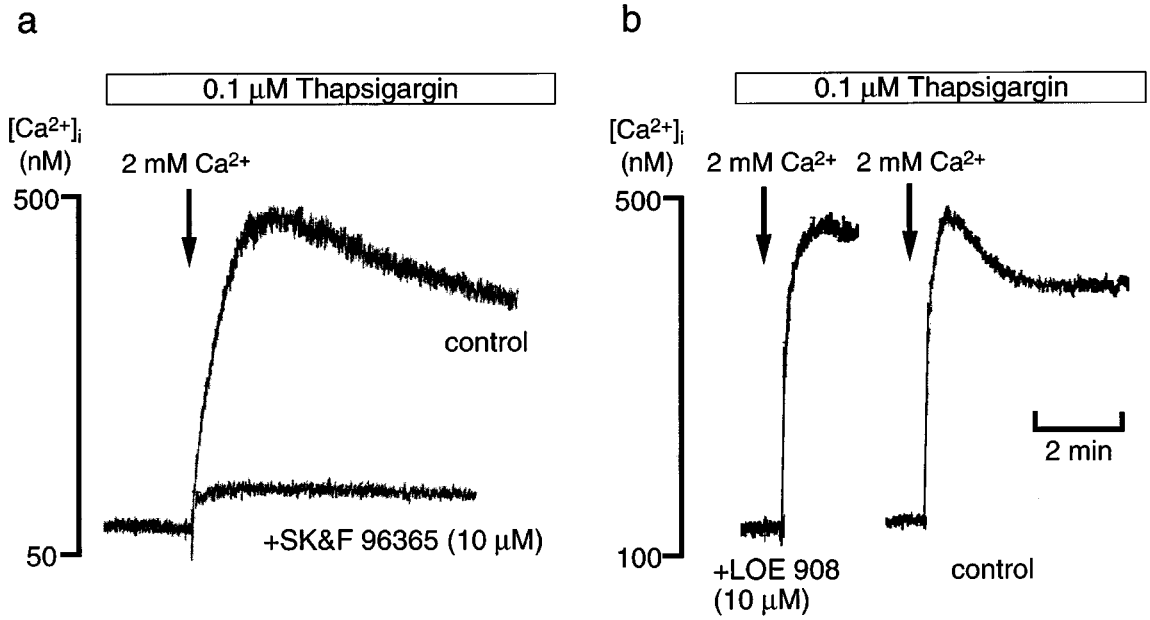

Figure 2 Original tracings illustrating the effects of SK&F 96365 and LOE 908 on the increase in $[Ca^{2+}]_i$ following addition of Ca^{2+} in A7r5 cells pretreated with thapsigarigin. A7r5 cells were loaded with fura-2 for SK&F 96365 (a) and with fluo-3 for LOE 908 (b). The A7r5 cells were preincubated in Ca^{2+}-free Krebs-HEPES solution containing 0.1 μM thapsigargin for 10 min and subsequently for further 5 min in the same solution with or without either SK&F 96365 or LOE 908. The activities of Ca^{2+} influx were studied by addition of 2 mM Ca^{2+} to the bath solution.

The increase induced by 1 nM ET-1 was suppressed by LOE 908 in a concentration-dependent manner and the maximal inhibition of about 100% was observed at concentrations higher than 10 μM (Figures 1a and 3). The sustained increase induced by 100 nM ET-1 was also suppressed by LOE 908 concentration-dependently but the extent of inhibition was smaller than that for 1 nM ET-1: about 35% of the increase remained uninhibited after administration of 10 μM LOE 908 (Figure 1b and 3).

These results indicate that the response of the ET-1-induced increase in $[Ca^{2+}]_i$ to LOE 908 is different from that to SK&F 96365. That is, the increase induced by lower concentrations of ET-1 was totally sensitive to LOE 908, although it was resistant to SK&F 96365 as previously indicated (Iwamuro *et al*., 1998). Therefore, we began to characterize the action of LOE 908.

Effects of LOE 908 on the increase in $[Ca^{2+}]_i$ induced by thapsigargin or ionomycin as an index of Ca^{2+} entry through SOCC

To assess the effects of LOE 908 on Ca^{2+} entry through SOCC, we attempted to obtain elevations of $[Ca^{2+}]_i$ resulting from selective activation of SOCC. For this purpose, A7r5 cells were treated with an inhibitor of sarcoplasmic reticulum Ca^{2+}-ATPase, thapsigargin, or with a Ca^{2+} ionophore ionomycin.

Addition of 2 mM Ca^{2+} to A7r5 cells pretreated with 0.1 μM thapsigargin markedly increased $[Ca^{2+}]_i$ in the presence of 1 μM nifedipine (Figure 2). After addition of a calcium chelator EGTA at 5 mM to medium during the sustained elevation, the increase was abolished (data not shown). SK&F 96365 at 10 μM almost abolished the increase in $[Ca^{2+}]_i$ induced by addition of Ca^{2+} in the thapsigargin-treated cells (Figure 2a).

In contrast with SK&F 96365, LOE 908 up to the concentration of 10 μM had no effect on the increase in $[Ca^{2+}]_i$ as an index of Ca^{2+} entry through SOCC (Figures 2b and 3). However, when the concentration of LOE 908 was elevated even higher, a slight but significant inhibition became apparent: the inhibition was about 15% at 20 μM (Figure 3). Essentially similar results were obtained when 0.1 μM ionomycin was used instead of thapsigargin (Figure 3).

Effects of LOE 908 on cation currents induced by ET-1

Stimulation with lower (1 nM) or higher (10 nM, 100 nM) concentrations of ET-1 induced slow inward currents in A7r5 cells held at -60 mV with whole-cell configuration of patch-clamp (Figure 4). The currents induced by 1 nM, 10 nM or 100 nM ET-1 showed linear current-voltage relationships with the reversal potentials of -17.6 ± 9.6 mV ($n=30$) (Figure 4e), -14.7 ± 3.7 mV ($n=18$) (Figure 4f) and -16.3 ± 5.8 mV

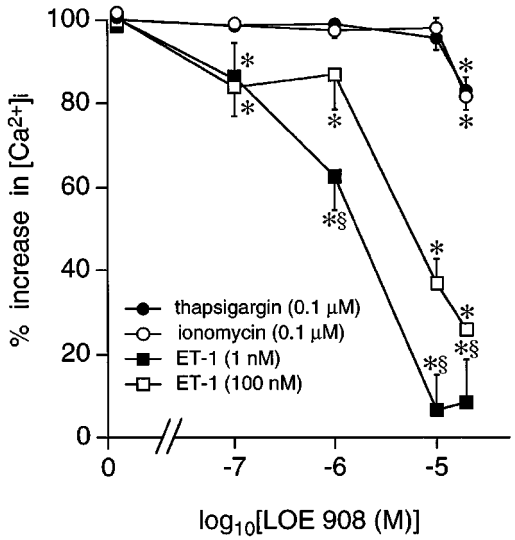

Figure 3 Concentration-response curves for inhibitory effects of LOE 908 on Ca^{2+} influx through store-operated Ca^{2+} channel (SOCC) activated by either thapsigargin or ionomycin and through Ca^{2+}-permeable nonselective cation channel (NSCC) activated by ET-1. For measurement of Ca^{2+} influx through SOCC, A7r5 cells loaded with fluo-3 were preincubated in Ca^{2+}-free Krebs-HEPES solution containing 0.1 μM thapsigargin or 0.1 μM ionomycin for 10 min and subsequently for further 5 min in the same solution with or without varying concentrations of LOE 908. The activities of Ca^{2+} influx were studied by addition of 2 mM Ca^{2+} to the solution. For measurement of Ca^{2+} influx through NSCC, fluo-3-loaded A7r5 cells were preincubated in normal Krebs-HEPES with or without varying concentrations of LOE 908 for 5 min, and stimulated with 1 nM or 100 nM ET-1. The increases in $[Ca^{2+}]_i$ in the presence of LOE 908 were represented as percentages of those in its absence. Each point represents mean value$\pm$s.e.mean of six experiments. *$P<0.01$; significantly different from control values without pre-treatment by LOE 908. §$P<0.01$; significantly different from the value with 100 nM ET-1.

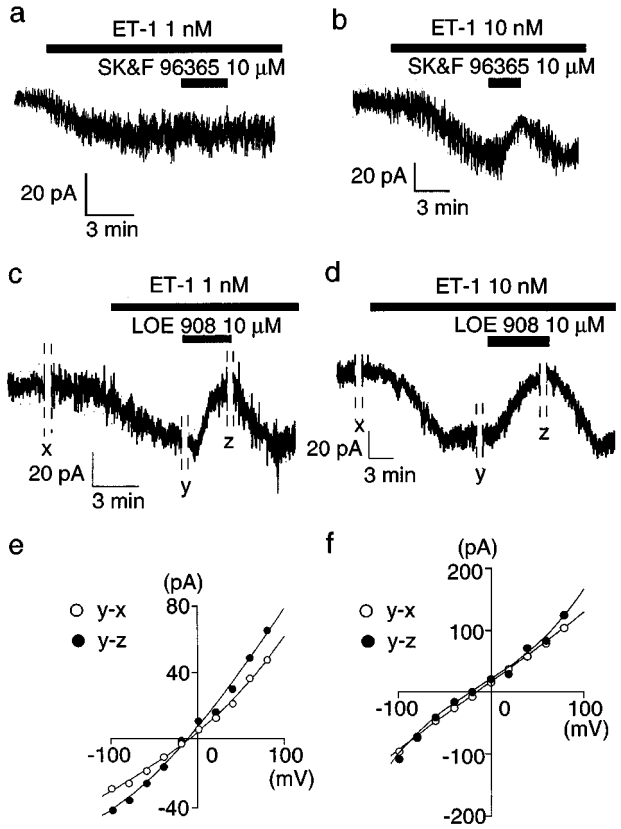

Figure 4 Effects of LOE 908 or SK&F 96365 on whole-cell currents induced by a lower (1 nM) or higher (10 nM) concentration of ET-1 in A7r5 cells in the presence of 1 μM nifedipine. The cells were clamped at a holding potential of -60 mV with the whole-cell configuration, and ET-1 was added to the bath solution at the final concentration of 1 nM (a, c and e) or 10 nM (b, d and f). After the ET-1-induced currents reached a steady-state, SK&F 96365 (a and b) or LOE 908 (c and d) was added at 10 μM during the time intervals indicated by horizontal bars. At the time indicated by x, y and z in (c and d), voltage steps ranging from -100 to $+80$ mV in 20 mV increments were applied. (e and f) represent the current-voltage relationships obtained from the data in (c and d), respectively. The currents induced by ET-1 were obtained by subtracting currents at x from those at y, and the currents inhibited by LOE 908 were obtained by subtracting currents at z from those at y.

($n=14$), respectively. The current-voltage relationships of the currents induced by 1 nM and 10 nM ET-1 were unaffected by reducing the concentration of Cl^- in the bath solution to 9 mM: the reversal potentials were -13.3 ± 9.6 mV ($n=4$) and -11.3 ± 4.4 mV ($n=4$), respectively.

The current induced by 1 nM ET-1 was resistant to SK&F 96365 (Figures 4a and 5). On the other hand, the major part of the currents induced by 10 nM or 100 nM ET-1 was suppressed by 10 μM SK&F 96365 (Figures 4b and 5): the inhibition amounted to $54.8\pm6.4\%$ ($n=6$) and $52.4\pm9.5\%$ ($n=6$), respectively. The characteristics of the currents inhibited by SK&F 96365 were essentially similar to the ET-1-induced currents, in terms of the linear current-voltage relationships and the reversal potentials (-12.3 ± 7.6 mV for 10 nM ET-1, $n=6$; -16.1 ± 6.4 mV for 100 nM ET-1, $n=6$).

The currents induced by 1 nM, 10 nM or 100 nM ET-1 were completely suppressed by 10 μM LOE 908: the inhibition amounted to $103.6\pm3.9\%$ ($n=5$), $96.5\pm4.9\%$ ($n=4$), and $99.8\pm4.9\%$ ($n=4$), respectively (Figures 4c,d and 5). The currents inhibited by LOE 908 showed linear current-voltage relationships and the reversal potentials of -16.8 ± 2.1 mV ($n=5$), -11.3 ± 4.9 mV ($n=4$) and -14.9 ± 4.8 mV ($n=4$), respectively (Figures 4e and f).

Monitoring of currents through SOCC by whole-cell recordings of patch-clamp technique

To assess whether Ca^{2+} currents through SOCC actually contribute to the whole-cell currents induced by ET-1, we attempted to monitor currents induced by thapsigargin. At a holding potential of -60 mV, addition of thapsigargin at 0.1 μM produced no significant currents (Figure 6a). However, when the concentration of EGTA in the pipette solution was decreased from 10 mM to 0.2 mM, a transient inward current became apparent following treatment with thapsigargin (Figure 6b). The currents were abolished after treatment with a blocker of Cl^--channel DIDS at 1 mM (Figure 6c), showing that the currents induced by thapsigargin are conducted through Ca^{2+}-activated Cl^--channel.

Finally we examined the effects of LOE 908 on the increase in $[Ca^{2+}]_i$ induced by a lower (1 nM) or higher (100 nM) concentration of ET-1 (Figures 7 and 8). The increase in $[Ca^{2+}]_i$ induced by 1 nM ET-1 was unaffected by pretreatment with 10 μM SK&F 96365 (Figures 7a and 8a) but it was abolished by pretreatment with 10 μM LOE 908 alone or in combination with SK&F 96365 (Figures 7b and 8a). The major part of the increase in $[Ca^{2+}]_i$ induced by 100 nM ET-1 was suppressed by 10 μM LOE 908, but about 35% ($34.2\pm4.6\%$, $n=5$) was left unsuppressed (Figures 7c and 8b). SK&F 96365 suppressed the increase to the same extent as LOE 908 ($36.7\pm4.7\%$, $n=5$) (Figure 8b). The increase induced by 100 nM ET-1 was abolished ($4.4\pm5.4\%$, $n=5$), when the cells were pretreated with SK&F 96365 together with LOE 908 (Figures 7c and 8b).

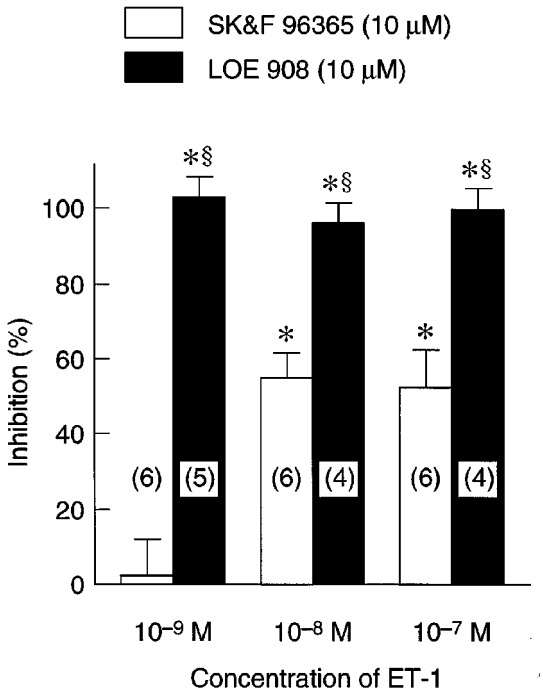

Figure 5 Summary of the effects of LOE 908 or SK&F 96365 on whole-cell currents induced by lower (1 nM) or higher (10 nM and 100 nM) concentrations of ET-1 in A7r5 cells in the presence of 1 μM nifedipine. Experimental protocols were the same as those in Figure 4, except that stimulation with 100 nM ET-1 was also performed. Each bar represents mean value$\pm$s.e.mean of the number of experiments shown in parentheses. *$P<0.01$, significantly different from control values without blockers. §$P<0.01$; significantly different from the value after pretreatment with SK&F 96365 alone.

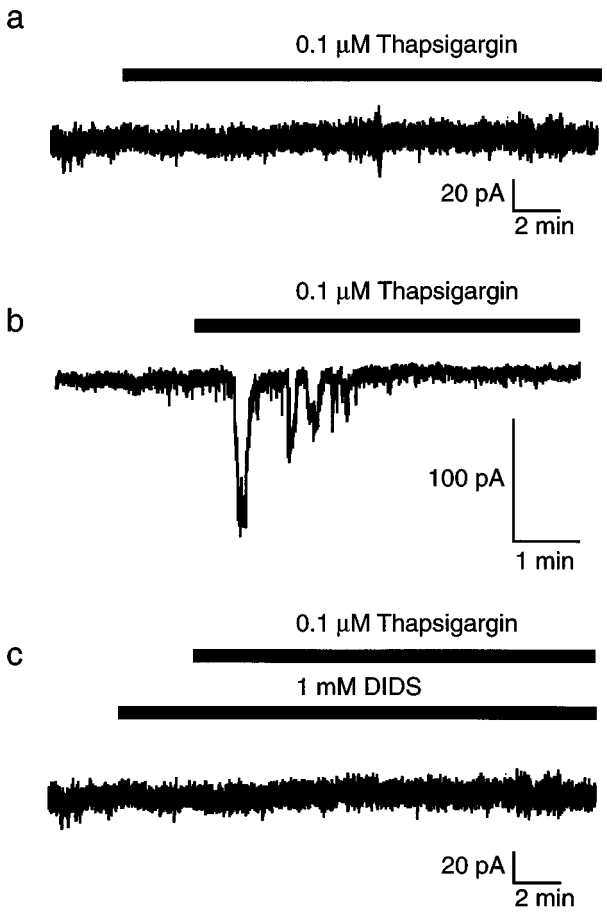

Figure 6 Original tracings illustrating activation of whole-cell currents in A7r5 cells by thapsigargin. A7r5 cells were clamped at a holding potential of -60 mV with the whole-cell configuration: the pipette solution contained 10 mM (a) or 0.2 mM EGTA (b and c). Thapsigargin was added to the bath solution at the final concentration of 0.1 μM during the time indicated by horizontal bars. In the experiment (c) a blocker of the Cl^{-1} channel, DIDS, was added to the bath solution at 1 mM.

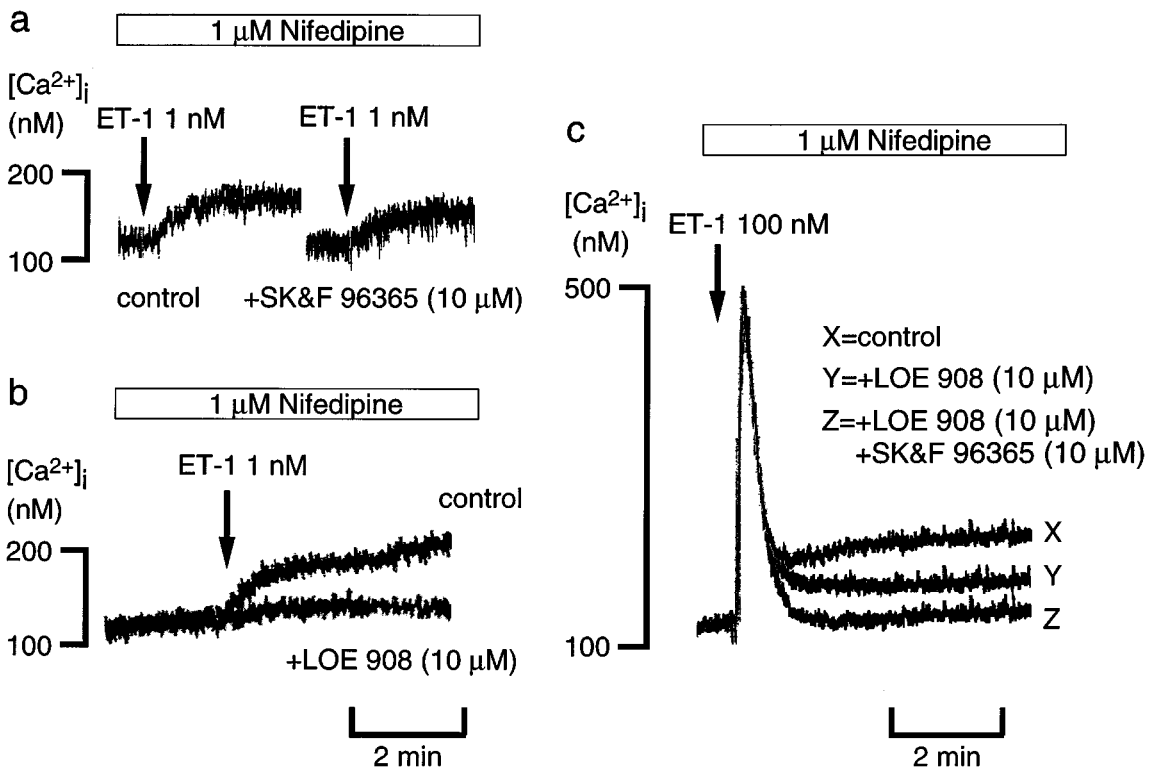

Figure 7 Original tracings illustrating the effects of LOE 908, SK&F 96365 and their combination on the increase in $[Ca^{2+}]_i$ in A7r5 cells induced by either a lower (1 nM) or higher (100 nM) concentration of ET-1. A7r5 cells loaded with fura-2 (a) or fluo 3 (b and c) were stimulated by either 1 nM (a and b) or 100 nM ET-1 (c) in the presence or absence of 10 μM SK&F 96365, 10 μM LOE 908 or their combination. In all experiments, nifedipine was added to the medium at the final concentration of 1 μM.

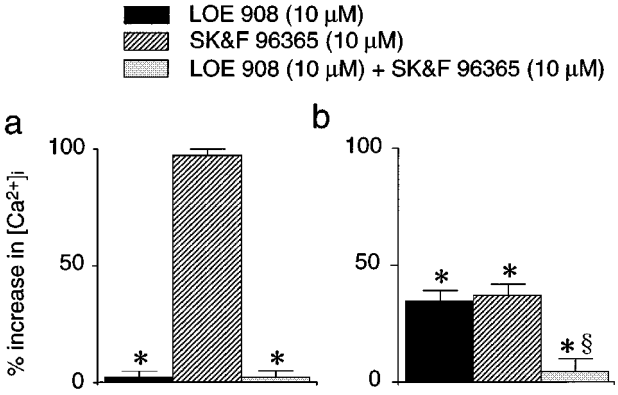

Figure 8 Effects of LOE 908, SK&F 96365 and their combination on the increase in $[Ca^{2+}]_i$ in A7r5 cells induced by either a lower (1 nM) (a) or higher (100 nM) concentration of ET-1 (b). Experiments were performed as described in Figure 7. The ET-1-induced increases in $[Ca^{2+}]_i$ in the presence of channel blockers were represented as percentages of those in their absence. Each bar represents mean value $\pm$ s.e.mean of five experiments. $^*P<0.01$, significantly different from control values without blockers. §$P<0.01$; significantly different from the value after pretreatment with either LOE 908 or SK&F 96365 alone.

Discussion

LOE 908, which had been developed as an inhibitor of the so-called receptor-activated Ca^{2+} influx (Krautwurst *et al*., 1993; 1994; Encabo *et al*., 1996), had little effect on the increase in $[Ca^{2+}]_i$ induced by treatment with thapsigargin or ionomycin up to the concentration of 10 μM (Figures 2 and 3). Since thapsigargin and ionomycin selectively deplete Ca^{2+} of the intracellular store to activate SOCC (Takemura *et al*., 1989; Thastrup *et al*., 1990; Morgan & Jacob, 1994), the present findings show that LOE 908 possesses no inhibitory effect on Ca^{2+} entry through SOCC. This result is discrepant with a previous report which indicates that the increase in $[Ca^{2+}]_i$ in endothelial cells induced by treatment with thapsigargin is blocked by LOE 908 (Encabo *et al*., 1996). The precise reason for this discrepancy is unknown, but it may be due to their use of different cells (i.e., endothelial cells), which might express SOCC with different sensitivities to the drug.

In contrast, the present study shows that SK&F 96365 is a blocker of SOCC, judging from abolition of the thapsigargin-induced increase in $[Ca^{2+}]_i$ (Figure 2). This result is consistent with the previous report by Mason *et al*. (1993).

As we have shown (Iwamuro *et al*., 1998), ET-1 can activate two types of Ca^{2+}-permeable NSCC in A7r5 cells: NSCC-1 and NSCC-2 characterized by insensitivity and sensitivity to SK&F 96365, respectively (Figures 4a,b and 5; see also 'Introduction'). In the present study, LOE 908 was found to be a blocker of both types of NSCC activated by ET-1 (Figures 4c,d and 5). This property of LOE 908 contrasts well with SK&F 96365 which is a blocker of NSCC-2 (Figures 4a,b and 5) and SOCC (Figure 2). Taken together with the recent data (Iwamuro *et al*., 1998), we can now pharmacologically discriminate among three voltage-independent Ca^{2+} channels (NSCC-1, NSCC-2 and SOCC) using LOE 908 and SK&F 96365 (Table 1). NSCC-1 (activated by both lower and higher concentrations of ET-1) is sensitive to LOE 908 but resistant to SK&F 96365; NSCC-2 (activated by higher concentrations of ET-1) is sensitive to both LOE 908 and SK&F 96365; SOCC (activated by higher concentrations of ET-1) is resistant to LOE 908 but sensitive to SK&F 96365.

Table 1 Three types of voltage-independent Ca^{2+} channel activated by lower ($\leqslant$1 nM) or higher ($\geqslant$10 nM) concentrations of ET-1

	Concentration of ET-1	*Sensitivity to*	
		SK&F 96365	*LOE 908*
NSCC-1	low & high	no	yes
NSCC-2	high	yes	yes
SOCC	high	yes	no

From the data in Figure 1 using EGTA, it is apparent that the sustained increase in $[Ca^{2+}]_i$ induced by lower or higher concentrations of ET-1 is due to entry of extracellular Ca^{2+}. The increase in $[Ca^{2+}]_i$ which is induced by lower concentrations of ET-1 in the presence of nifedipine seems to be the result of Ca^{2+}entry through NSCC-1, judging from its sensitivity to LOE 908 and insensitivity to SK&F 96365 (Figures 7 and 8).

On the other hand, the increase in $[Ca^{2+}]_i$ induced by higher concentrations of ET-1 in the presence of nifedipine seems to be the result of Ca^{2+} entry through NSCC-1, NSCC-2 and SOCC. That is, the LOE 908-sensitive part (amounting to about 65%) of the increase (Figure 8) is apparently due to Ca^{2+} entry through NSCC-1 and NSCC-2, since higher concentrations of ET-1 activate both NSCC-1 and NSCC-2 (Figures 4 and 5). The LOE 908-insensitive part and SK&F 96365-insensitive part (each part amounting to about 35% of the increase) are considered to be due to Ca^{2+} entry through SOCC and NSCC-1, based on their further sensitivity to SK&F 96365 and LOE 908, respectively (Figures 7 and 8). Therefore, taking the quantitative data into consideration, Ca^{2+} entry through NSCC-1, NSCC-2 and SOCC contributes to 35, 30 and 35%, respectively, of the increase in $[Ca^{2+}]_i$ induced by higher concentrations of ET-1.

The elevation of $[Ca^{2+}]_i$ following treatment with thapsigargin (Figure 2) is far larger than that following treatment with 100 nM ET-1 (Figure 7) which activates both NSCCs and SOCC. This is probably due to inhibition of Ca^{2+} uptake into the sarcoplasmic reticulum by thapsigargin. Thus, the contribution of Ca^{2+} entry through SOCC to the ET-1-induced elevation of $[Ca^{2+}]_i$ is smaller than expected from the elevation of $[Ca^{2+}]_i$ following treatment with thapsigargin.

The sensitivity to LOE 908 of the whole-cell currents induced by higher concentrations of ET-1 (Figures 4 and 5) is apparently higher than that of the increase in $[Ca^{2+}]_i$ (Figures 7 and 8). This phenomenon results from the failure of detection of the LOE 908-resistant current through SOCC with the present whole-cell recordings (Figure 6). Indeed, the current through SOCC is too small and it can be indirectly monitored as Ca^{2+}-activated Cl^- currents, only when the Ca^{2+} buffering capacity (i.e. the concentration of EGTA) in the patch pipette is lowered (Figure 6). This result is consistent with the notion that SOCC is highly selective for Ca^{2+} (Hoth & Penner, 1992; Gericke *et al*., 1994).

In summary, the present study clearly showed that LOE 908 is a blocker of both types of NSCC (NSCC-1 and 2) without inhibitory effect on SOCC. These properties of LOE 908 were clearly different from those of SK&F 96365, which is a blocker of NSCC-2 and SOCC. Combining LOE 908 and SK&F 96365, it was shown that the nifedipine-resistant increase in $[Ca^{2+}]_i$ induced by lower concentrations of ET-1 results exclusively from Ca^{2+} entry through NSCC-1, whereas that induced by higher concentrations of ET-1 results from Ca^{2+} entry through NSCC-1, NSCC-2 and SOCC. Now that three types of voltage-independent Ca^{2+} channel can be pharmacologically discriminated using SK&F 96365 and LOE 908, it has become possible to determine voltage-independent Ca^{2+} channels involved in the elevation of $[Ca^{2+}]_i$ induced by other agonists.

We thank Boehringer Ingelheim K.G. (Ingelheim, Germany) for a kind donation of LOE 908. This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan, by a grant from the Smoking Research Foundation, Japan and by the Uehara Memorial Foundation Fellowship, Tokyo, Japan.

References

BERRIDGE, M.J. (1993). Inositol triphosphate and calcium signalling. *Nature,* **361,** 315–325.

BERRIDGE, M.J. (1995). Capacitative calcium entry. *Biochem. J.,* **312,** 1–11.

CHEN, C. & WAGONER, P.K. (1991). Endothelin induces a nonselective cation current in vascular smooth muscle cells. *Circ. Res.,* **69,** 447–454.

CHUNG, S.C., MCDONALD, T.V. & GARDNER, P. (1994). Inhibition by SK&F 96365 of Ca^{2+} current, IL-2 production and activation in T lymphocytes. *Br. J. Pharmacol.,* **113,** 861–868.

CLEMENTI, M. & MELDOLESI, J. (1996). Pharmacological and functional properties of voltage-independent Ca^{2+} channels. *Cell Calcium,* **19,** 269–279.

ENCABO, A., ROMANIN, C., BIRKE, F.W., KUKOVETZ, W.R. & GROSCHNER, K. (1996). Inhibition of a store-operated Ca^{2+} entry pathway in human endothelial cells by the isoquinoline derivative LOE 908. *Br. J. Pharmacol.,* **119,** 702–706.

ENOKI, T., MIWA, S., SAKAMOTO, A., MINOWA, T., KOMURO, T., KOBAYASHI, S., NINOMIYA, H. & MASAKI, T. (1995). Long-lasting activation of cation current by low concentration of endothelin-1 in mouse fibroblasts and smooth muscle cells of rabbit aorta. *Br. J. Pharmacol.,* **115,** 479–485.

FASOLATO, C., INNOCENTI, B. & POZZAN, T. (1994). Receptor-activated Ca^{2+} influx: how many mechanisms for how many channels? *Trends Pharmacol. Sci.,* **15,** 77–83.

FELDER, C.C., SINGER-LAHAT, D. & MATHES, C. (1994). Voltage-independent calcium channels: Regulation by receptors and intracellular calcium stores. *Biochem. Pharmacol.,* **48,** 1997–2004.

FRANZIUS, D., HOTH, M. & PENNER, R. (1994). Non-specific effects of calcium entry antagonists in mast cells. *Pflügers Arch.,* **428,** 433–438.

GERICKE, M., OIKE, M., DROOGMANS, G. & NILIUS, B. (1994). Inhibition of capacitative Ca^{2+} entry by a Cl^- channel blocker in human endothelial cells. *Eur. J. Pharmacol.,* **269,** 381–384.

GOTO, K., KASUYA, Y., MATSUKI, N., TAKUWA, Y., KURIHARA, H., ISHIKAWA, T., KIMURA, S., YANAGISAWA, M. & MASAKI, T. (1989). Endothelin activates the dihydropyridine-sensitive, voltage-dependent Ca^{2+} channel in vascular smooth muscle. *Proc. Natl. Acad. Sci. U.S.A.,* **86,** 3915–3918.

HOTH, M. & PENNER, R. (1992). Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature,* **355,** 353–358.

HUANG, X.N., HISAYAMA, T. & TAKAYANAGI, I. (1990). Endothelin-1 induced contraction of rat aorta: contributions made by Ca^{2+} influx and activation of contractile apparatus associated with no change in cytoplasmic Ca^{2+} level. *Naunyn Schmiedebergs Arch. Pharmacol.,* **341,** 80–87.

INOUE, Y., OIKE, M., NAKAO, K., KITAMURA, K. & KURIYAMA, H. (1990). Endothelin augments unitary calcium channel currents on the smooth muscle cell membrane of guinea-pig portal vein. *J. Physiol.,* **423,** 171–191.

IRVINE, R.F. (1992). Inositol phosphates and Ca^{2+} entry: toward a proliferation or a simplification? *FASEB J.,* **6,** 3085–3091.

IWAMURO, Y., MIWA, S., MINOWA, T., ENOKI, T., ZHANG, X.-F., ISHIKAWA, M., HASHIMOTO, N. & MASAKI, T. (1998). Activation of two types of Ca^{2+}-permeable nonselective cation channel by endothelin-1 in A7r5 cells. *Br. J. Pharmacol.,* **124,** 1541–1549.

KOCH, B.D., FAUROT, G.F., KOPANITSA, M.V. & SWINNEY, D.C. (1994). Pharmacology of a Ca^{2+}-influx pathway activated by emptying the intracellular Ca^{2+} stores in HL-60 cells: evidence that a cytochrome P-450 is not involved. *Biochem. J.,* **302,** 187–190.

KOMURO, T., MIWA, S., ZHANG, X.-F., MINOWA, T., ENOKI, T., KOBAYASHI, S., OKAMOTO, Y., NINOMIYA, H., SAWAMURA, T., KIKUTA, K., IWAMURO, Y., FURUTANI, H., HASEGAWA, H., UEMURA, Y., KIKUCHI, H. & MASAKI, T. (1997). Physiological role of Ca^{2+}-permeable nonselective cation channel in endothelin-1-induced contraction of rabbit aorta. *J. Cardiovasc. Pharmacol.,* **30,** 504–509.

KRAUTWURST, D., DEGTIAR, V.E., SCHULTZ, G. & HESCHELER, J. (1994). The isoquinoline derivative LOE 908 selectively blocks vasopressin-activated nonselective cation currents in A7r5 aortic smooth muscle cells. *Naun-Schmiedeberg's Arch. Pharmacol.,* **349,** 301–307.

KRAUTWURST, D., HESCHELER, J., ARNDTS, D., LÖSEL, W., HAMMER, R. & SCHULTZ, G. (1993). A novel potent inhibitor of receptor-activated nonselective cation currents in HL-60 cells. *Mol. Pharmacol.,* **43,** 655–659.

MASON, M.J., MAYER, B. & HYMEL, L. (1993). Inhibition of Ca^{2+} transport pathways in thymic lymphocytes by econazole, miconazole, and SK&F 96365. *Am. J. Physiol.,* **264,** C654–C662.

MERRITT, J.E., ARMSTRONG, W.P., BENHAM, C.D., HALLAM, T.J., JACOB, R., JAXA-CHAMIEC, A., LEIGH, B.K., MCCARTHY, S.A., MOORES, K.E. & RINK, T.J. (1990). SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. *Biochem. J.,* **271,** 515–522.

MINOWA, T., MIWA, S., KOBAYASHI, S., ENOKI, T., ZHANG, X-F., KOMURO, T., IWAMURO, Y. & MASAKI, T. (1997). Inhibitory effect of nitrovasodilators and cyclic GMP on ET-1-activated Ca^{2+}-permeable nonselective cation channel in rat aortic smooth muscle cells. *Br. J. Pharmacol.,* **120,** 1536–1544.

MORGAN, A.J. & JACOB, R. (1994). Ionomycin enhances Ca^{2+} influx by stimulating store-regulated cation entry and not by a direct action at the plasma membrane. *Biochem. J.,* **300,** 665–672.

PUTNEY JR. J.W. (1990). Capacitative calcium entry revisited. *Cell Calcium,* **11,** 611–624.

RUBANYI, G.M. & POLOKOFF, M.A. (1994). Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *Pharmacol. Rev.,* **46,** 325–415.

SIMPSON, A.W., STAMPFL, A. & ASHLEY, C.C. (1990). Evidence for receptor-mediated bivalent-cation entry in A10 vascular smooth-muscle cells. *Biochem. J.,* **267,** 277–280.

SOKOLOVSKY, M. (1995). Endothelin receptor subtypes and their role in transmembrane signaling mechanisms. *Pharmac. Ther.,* **68,** 435–471.

TAKEMURA, H., HUGHES, A.R., THASTRUP, O. & PUTNEY JR. J.W. (1989). Activation of calcium entry by the tumor promoter thapsigargin in parotid acinar cells. *J. Biol. Chem.,* **264,** 12266–12271.

THASTRUP, O., CULLEN, P.J., DROBAK, B.K., HANLEY, M.R. & DAWSON, A.P. (1990). Thapsigargin, a tumour promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Pro. Natl. Acad. Sci. U.S.A.,* **87,** 2466–2470.

VAN HEESWIJK, M.P.E., GEERTSEN, J.A.M. & VAN OS, C.H. (1984). Kinetic properties of the ATP-dependent pump and the exchange system in basolateral membranes from rat kidney cortex. *J. Memb. Biol.,* **79,** 19–31.

VAN RENTERGHEM, C., VIGNE, P., BARHANIN, J., SCHMID ALLIANA, A., FRELIN, C. & LAZDUNSKI, M. (1988). Molecular mechanism of action of the vasoconstrictor peptide endothelin. *Biochem. Biophys. Res. Commun.,* **157,** 977–985.

YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature,* **332,** 411–415.

ZHANG, X.-F., KOMURO, T., MIWA, S., MINOWA, T., IWAMURO, Y., OKAMOTO, Y., NINOMIYA, H., SAWAMURA, T. & MASAKI, T. (1998). Role of nonselective cation channels as Ca^{2+} entry pathway in endothelin-1-induced contraction and their suppression by nitric oxide. *Eur. J. Pharmacol.,* **352,** 237–245.

(Received August 5, 1998
Revised December 7, 1998
Accepted December 10, 1998)