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Pharmacological characterization of Ca^{2+} entry channels in endothelin-1-induced contraction of rat aorta using LOE 908 and SK&F 96365

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1 We have recently shown that endothelin-1 (ET-1) activates two types of Ca^{2+}-permeable nonselective cation channels (designated NSCC-1 and NSCC-2) and store-operated Ca^{2+} channel (SOCC). These channels can be pharmacologically discriminated using Ca^{2+} channel blockers such as SK&F 96365 and LOE 908. Here we characterized Ca^{2+} entry channels involved in ET-1-induced contractions of rat thoracic aortic rings and increases in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) of single smooth muscle cells using these blockers.

2 LOE 908 or a blocker of voltage-operated Ca^{2+} channel nifedipine had no effect on the contractions and increases in $[Ca^{2+}]_i$ induced by thapsigargin or ionomycin, whereas SK&F 96365 abolished them.

3 The contractions and increases in $[Ca^{2+}]_i$ induced by ET-1 depended on extracellular Ca^{2+} but were resistant to nifedipine. The responses to lower concentrations ($\leqslant$0.1 nM) of ET-1 were abolished by either SK&F 96365 or LOE 908. The responses to higher concentrations ($\geqslant$1 nM) were abolished by SK&F 96365, but were partially resistant to LOE 908.

4 SK&F 96365 inhibited the LOE 908-resistant contractions induced by higher concentrations of ET-1 with IC_{50} values similar to those for contractions induced by thapsigargin or ionomycin.

5 These results show that the contractions and increases in $[Ca^{2+}]_i$ of rat aortic smooth muscles at lower concentrations of ET-1 involve only one Ca^{2+} entry channel which is sensitive to SK&F 96365 and LOE 908 (NSCC-2), whereas those at higher concentrations of ET-1 involve another Ca^{2+} entry channel which is sensitive to SK&F 96365 but resistant to LOE 908 (SOCC) in addition to the former channel.

Keywords: Endothelin-1; vasocontraction; intracellular free Ca^{2+} concentration; voltage-independent calcium channel; SK&F 96365; LOE 908; vascular smooth muscle cells; rat thoracic aorta

Abbreviations: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; ET-1, endothelin-1; IP_3, D-*myo*-inositol-1,4,5-trisphosphates; NA, noradrenaline; NSCC, nonselective cation channel; SOCC, store-operated Ca^{2+} channel; SR, sarcoplasmic reticulum; VOCC, voltage-operated Ca^{2+} channel; VSMC, vascular smooth muscle cell

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). It is generally accepted that the major part of the sustained contraction by ET-1 requires the persistent entry of extracellular Ca^{2+} and the resulting increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Rubanyi & Polokoff, 1994; Komuro *et al*., 1997b; Zhang *et al*., 1998). One of the Ca^{2+} entry channels activated by ET-1 (Goto *et al*., 1989; Inoue *et al*., 1990) is voltage-operated Ca^{2+} channel (VOCC), but it becomes increasingly clear that involvement of this channel in ET-1-induced contractions and increases in $[Ca^{2+}]_i$ is minimal (Huang *et al*., 1990; Simpson *et al*., 1990; Komuro *et al*., 1997b; Zhang *et al*., 1998). Several researchers have shown that Ca^{2+}-permeable nonselective cation channels (NSCC) as a Ca^{2+} entry pathway is activated by ET-1 in vascular smooth muscle cells (VSMCs) (Van Renterghem *et al*., 1988; Chen & Wagoner, 1991; Enoki *et al*., 1995; Minowa *et al*., 1997) and in L*tk*$^-$ cells expressing recombinant human ET_A receptors (Enoki *et al*., 1995).

Store-operated Ca^{2+} channel (SOCC) activated by depletion of intracellular Ca^{2+} store (Putney, 1990; Hoth & Penner, 1992; Irvine, 1992; Fasolato *et al*., 1994; Berridge, 1995) is also one of the candidates for Ca^{2+} entry channels activated by ET-1, because stimulation of ET receptors induces increased formation of D-*myo*-inositol-1,4,5-trisphosphates (IP_3) and subsequent depletion of the Ca^{2+} store (Rubanyi & Polokoff, 1994).

Indeed, we have recently shown that in A7r5 cells derived from rat thoracic aortic smooth muscle cells, ET-1 can activate three types of voltage-independent Ca^{2+} entry channel, which are pharmacologically discriminated using Ca^{2+} channel blockers such as SK&F 96365 and LOE 908: two types of

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Ca^{2+}-permeable NSCC (designated NSCC-1 and NSCC-2) and SOCC (Iwamuro *et al*., 1998; 1999). NSCC-1 is resistant to SK&F 96365 but sensitive to LOE 908, whereas NSCC-2 is sensitive to both SK&F 96365 and LOE 908. SOCC is sensitive to SK&F 96365 but resistant to LOE 908. Furthermore, activation of these channels is dependent on the concentrations of ET-1: lower concentrations of ET-1 activate only NSCC-1, whereas its higher concentrations activate all of the three channels (Iwamuro *et al*., 1998; 1999).

In the present study, we attempted to clarify Ca^{2+} entry channels involved in the contractions of rat thoracic aorta and increases in $[Ca^{2+}]_i$ of enzymatically dispersed VSMCs induced by lower or higher concentrations of ET-1 using SK&F 96365 and LOE 908.

Methods

Preparation of tissues for measurement of tension

Preparation of rat thoracic aortic rings and measurement of isometric tension were performed as described recently (Komuro *et al*., 1997a,b; Zhang *et al*., 1998). Briefly, male Wistar rats (150–200 g) were anaesthetized with diethylether and exsanguinated. The thoracic aortae were removed quickly, and placed in Krebs solution which contained (in mM) : NaCl 120, KCl 5.4, $CaCl_2$ 2.2, $MgCl_2$ 1.0, $NaHCO_3$ 25 and glucose 5.6. Blood was rinsed from the lumen, adherent connective tissue was removed carefully and rings, approximately 1–2 mm in width, were cut from each aorta. Endothelial cells were removed from ring specimens by gently rubbing the intimal surface with a cotton bud moistened with Krebs solution. Successful removal of endothelial cells was confirmed by the inability of acetylcholine (1 μM) to induce relaxation. The aortic rings were mounted using a pair of stainless steel hooks under a resting tension of 1 g in organ baths containing 5 ml of Krebs solution which was maintained at 37°C and bubbled with a 95% O_2 and 5% CO_2 mixture: one of the hooks was connected to a force transducer (Orientec, Tokyo, Japan) and the developed tension was displayed on a Nihon Kohden (Tokyo, Japan) RJG4128 polygraph.

A change of the bath fluid and readjustment of resting force were done every 20 min until a stable baseline was attained (usually after about 60 min). After readjustment, the preparations were challenged at hourly intervals with 0.3 μM noradrenaline (NA). When two NA contractions gave reproducible results, the actual experiment was started. At the beginning of each experiment, each preparation was challenged with 1 μM NA as a measure of contractile force of each preparation, and the contractile responses to stimulations such as ET-1, high K^+ or thapsigargin/Ca^{2+} were represented as percentages of the tension induced by 1 μM NA. In some experiments, the aortic rings were stimulated by high K^+ solution in which the concentration of KCl in Krebs solution was increased to 45 mM with the equimolar concentration of NaCl being reduced to maintain isotonicity. Unless specified otherwise, Ca^{2+} channel blockers were added 60 min before stimulation.

For stimulation by ET-1 in the absence of extracellular Ca^{2+}, Ca^{2+}-free Krebs solution which contained 2 mM EGTA instead of Ca^{2+} was used: a switch from normal Krebs solution to Ca^{2+}-free solution was done 60 min before stimulation by ET-1. To deplete the intracellular Ca^{2+} store, the aortic rings were preincubated for 90 min in Ca^{2+}-free Krebs solution containing 1 μM thapsigargin or 1 μM ionomycin. In this case, the concentration of EGTA was 0.2 mM instead of 2 mM.

Preparation and primary culture of VSMCs for measurement of $[Ca^{2+}]_i$

Isolated VSMCs were prepared from rat thoracic aortae as described previously (Inoue & Kuriyama 1993; Enoki *et al*., 1995). The rat thoracic aortae were placed in Ca^{2+}-free Krebs-HEPES solution which contained (in mM): NaCl 140, KCl 3, $MgCl_2$ 1, glucose 11 and HEPES 10 (pH 7.4, adjusted with NaOH). After thoracic aortae were cut longitudinally, endothelial cells were removed. The thoracic aortae were cut into rectangular strips of 3×4 mm and incubated for 6 h at 4°C in Ca^{2+}-free Krebs-HEPES solution containing papain (2.84 units ml^{-1}) and 0.5 mM dithiothreitol (DTT). Thereafter, the strips were resuspended and incubated in Ca^{2+}-free Krebs-HEPES solution containing collagenase (250 units ml^{-1}) at 37°C for 7 min. The digested strips were cut into pieces with fine scissors and triturated with a blunt-tipped pipette until a sufficient number of single cells were released. The dispersed VSMCs were seeded on 35-mm glass-bottomed plastic dishes (MatTek Corporation, Ashland, MA, U.S.A.), cultured in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum, 100 u ml^{-1} penicillin G and 100 mg ml^{-1} streptomycin at 37°C in a humidified 5% CO_2/95% air atmosphere, and 4–5 days later, the cells were used for $[Ca^{2+}]_i$ measurement.

Microfluorimetry of fluo-3

For loading of fluo-3, the cultured VSMCs were incubated in Ca^{2+}-free Krebs-HEPES solution containing 10 μM fluo-3/AM (acetoxymethyl esters) for 30 min at 37°C (Minta *et al*., 1989; Enoki *et al*., 1995; Iwamuro *et al*., 1999). After washing with normal Krebs-HEPES solution (2.2 mM $CaCl_2$ was added to Ca^{2+}-free Krebs-HEPES solution), they were kept in fresh Krebs-HEPES solution at 37°C for at least 30 min. In experiments using the cultured VSMCs, Ca^{2+}-free Krebs-HEPES solution contained 0.1 mM EGTA instead of 2 mM EGTA. Fluo-3 microfluorimetry was done at 25°C by an Attofluor Ratio- Vision real-time digital fluorescence analyzer (Atto Instruments, Potomac, MD, U.S.A.) equipped with a Carl-Zeiss Axiovert-100 inverted epifluorescent microscope. A 100-W mercury burner served as the source of excitation. In measurements of $[Ca^{2+}]_i$, fluo-3 was excited at 450–490 nm and fluorescence was detected at 515–565 nm. At the end of the experiment, ionomycin and subsequently EGTA were added at final concentrations of 10 μM and 10 mM, respectively, to obtain the fluorescence intensity maximum (F_{max}) and the fluorescence intensity minimum (F_{min}). $[Ca^{2+}]_i$ was determined from the equilibrium equation, $[Ca^{2+}]_i = K_D (F-F_{min})/(F_{max}-F)$, where F was the experimental value of fluorescence and K_D was defined as 0.40 μM (Minta *et al*., 1989)

Statistical analysis

Data were presented as means ± s.e.mean. The EC_{50} or IC_{50} values were estimated for individual concentration-response curves by use of nonlinear least-squares regression analysis. A pair of means were compared with Student's *t*-test. Groups of data were subjected to a two-way analysis of variance (ANOVA), 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.

Drugs

LOE 908 was kindly provided by Boehringer-Ingelheim (Ingelheim, Germany). Other chemicals were commercially obtained from the following sources: ET-1 from Peptide Institute (Osaka, Japan); SK&F 96365 from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.); fluo-3/AM, HEPES and EGTA from Dojindo Laboratories (Kumamoto, Japan); NA bitartrate and collagenase (177 units per mg protein) from Wako Pure Chemical Industries Ltd. (Osaka, Japan); dithiothreitol (DTT) and ionomycin from Nacalai Tesque Inc. (Kyoto, Japan); thapsigargin from Research Biochemicals International (Natick, MA, U.S.A.); nifedipine and papain (32 units per mg protein) from Sigma (St. Louis, MO, U.S.A.). Nifedipine, thapsigargin and LOE 908 were dissolved in dimethyl sulphoxide, and SK&F 96365 was dissolved in water.

Results

Contractions resulting from Ca^{2+} entry through SOCC

To confirm that LOE 908 has no inhibitory effect on SOCC in isolated rat aorta as in A7r5 cells (Iwamuro *et al.*, 1999), we examined the effects of this drug on contractions of rat thoracic aortic rings without endothelium which result exclusively from selective activation of SOCC. For this purpose, the aortic rings were pretreated for 90 min with 1 μM thapsigargin (an inhibitor of sarcoplasmic reticulum Ca^{2+}-ATPase) or 1 μM ionomycin (a Ca^{2+} ionophore) in the absence of extracellular Ca^{2+} to deplete the intracellular Ca^{2+} store. Subsequent addition of Ca^{2+} to the bath solution at 2.2 mM induced contractions comparable to those by 1 μM NA (Figure 1b). Without pretreatment by thapsigargin, addition of Ca^{2+} produced no contractions (Figure 1a). When extracellular Ca^{2+} was removed by adding a Ca^{2+} chelator EGTA during the plateau phase of the contractions, the developed tension returned to the resting level (3.6 ± 0.4% of Ca^{2+}-induced contractions, $n=8$; see also Figure 1c).

To determine the degree of depletion of the intracellular Ca^{2+} store, we examined the changes in the amplitude of the contractions induced by addition of 2.2 mM Ca^{2+} or 1 μM NA following pretreatment with 1 μM thapsigargin (Figure 2). The contractions induced by addition of Ca^{2+} progressively increased after pretreatment with thapsigargin, and reached a plateau level at around 90 min (Figure 2a). In contrast, the NA-induced contractions as an index of Ca^{2+} mobilization from the store decreased with time and reached a steady-state at around 90 min (Figure 2b). A lower (0.1 μM) or higher (5 μM) concentration of thapsigargin gave similar results (data not shown). Essentially similar results were obtained when 1 μM ionomycin was used instead of thapsigargin (data not shown). In the following experiments, Ca^{2+} was added 90 min after treatment with 1 μM thapsigargin or 1 μM ionomycin.

As shown in Figures 1 and 3, the Ca^{2+}-induced contractions of the aortic rings pretreated with 1 μM thapsigargin or 1 μM ionomycin were unaffected by a specific blocker of L-type VOCC nifedipine up to 10 μM or LOE 908 up to 100 μM. In contrast, SK&F 96365 suppressed the contractions in a concentration-dependent manner with an IC_{50} value of 10.2 ± 1.2 μM ($n=8$), and complete inhibition was observed at concentrations higher than 30 μM.

Effects of removal of extracellular Ca^{2+} and nifedipine on ET-1-induced contractions

In the presence of extracellular Ca^{2+}, ET-1 produced contractions of the aortic rings in a concentration-dependent manner with an EC_{50} value of 1.0 ± 0.2 nM ($n=8$), and the maximal response was obtained at concentrations higher than 10 nM (Figure 4a). In the absence of extracellular Ca^{2+}, the ET-1-induced contractions were reduced to negligible levels (Figure 4a; Table 1).

Nifedipine suppressed the contractions induced by 45 mM KCl in a concentration-dependent manner with an IC_{50} value of 1.0 ± 0.3 nM ($n=8$), and complete inhibition was observed at concentrations higher than 1 μM (Figure 4b). In contrast, nifedipine up to 10 μM had no effect on the contractions induced by 10 nM ET-1 (Figure 4b).

Effects of varying concentrations of LOE 908 and SK&F 96365 on the contractions induced by lower or higher concentrations of ET-1

As shown in Figure 5, LOE 908 suppressed the contractions induced by 0.1, 1.0 and 10 nM ET-1 in a concentration-

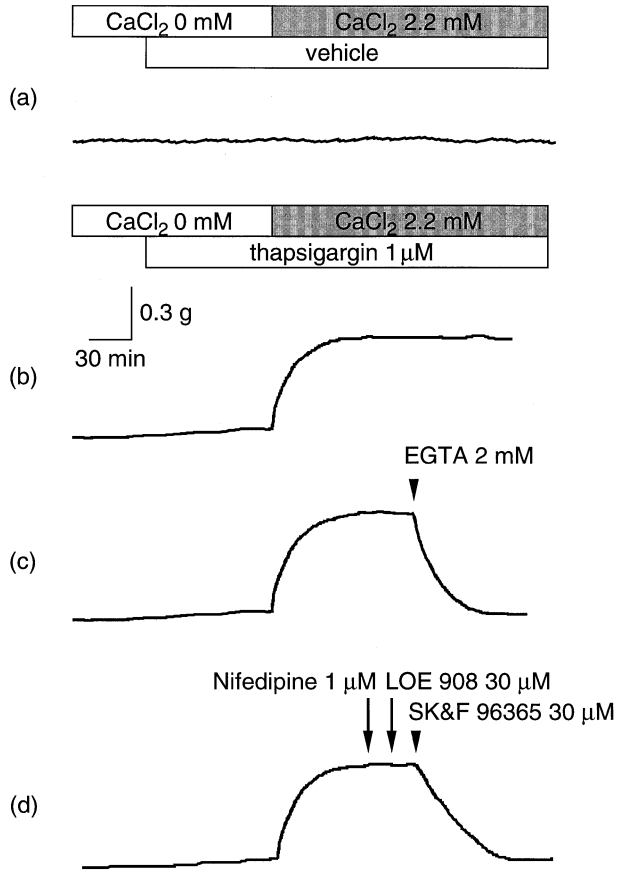

Figure 1 Typical tracings showing the effects of removal of extracellular Ca^{2+}, nifedipine, LOE 908 or SK&F 96365 on the Ca^{2+}-induced contractions of rat thoracic aortic rings without endothelium pretreated with thapsigargin. After the aortic rings had been preincubated for 90 min in Ca^{2+}-free Krebs solution with vehicle alone (0.1% dimethyl sulphoxide) (a) or with 1 μM thapsigargin (b, c and d), the bath solution was switched to normal Krebs solution containing 2.2 mM Ca^{2+}. During the plateau phase of the contractions, 2 mM EGTA was added to the bath solution (c): in panel (d), 1 μM nifedipine, 30 μM LOE 908 and 30 μM SK&F 96365 were sequentially added.

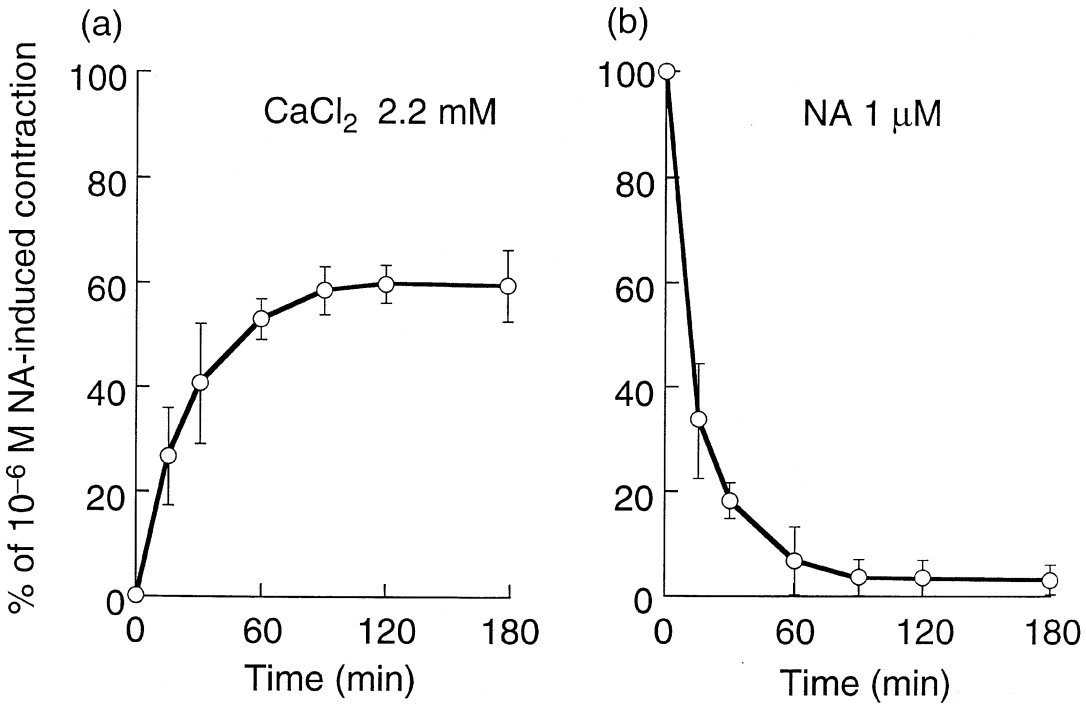

Figure 2 Changes in the amplitude of Ca^{2+}-induced or NA-induced contractions of rat thoracic aortic rings without endothelium in Ca^{2+}-free solution following treatment with thapsigargin. After the aortic rings had been preincubated with 1 μM thapsigargin in Ca^{2+}-free Krebs solution for varying time intervals, the bath solution was switched to normal Krebs solution containing 1 μM thapsigargin or NA was added to the bath solution at the final concentration of 1 μM. The contractions induced by Ca^{2+} or NA were plotted against the time following the beginning of thapsigargin treatment. The contractions by Ca^{2+} or NA were represented as percentages of those by 1 μM NA at the beginning of the experiment. Each point represents mean value$\pm$s.e.mean of six experiments.

Table 1 Effects of removal of extracellular Ca^{2+}, LOE 908 and SK&F 96365 on the contractions of rat thoracic aortic rings induced by varying concentrations of ET-1

	Contractions after various treatments (%)		
	ET-1 (0.1 nM)	*ET-1* (1 nM)	*ET-1* (10 nM)
ET-1 alone	100.0$\pm$8.5	100.0$\pm$7.7	100.0$\pm$11.4
$-Ca^{2+}$ (+2 mM EGTA)	2.2$\pm$2.0*	2.8$\pm$2.0*	2.8$\pm$2.1*
+LOE 908 (30 μM)	4.0$\pm$1.0*	34.4$\pm$2.2*†	44.4$\pm$3.9*†§
+SK&F 96365 (30 μM)	4.2$\pm$1.3*	10.3$\pm$3.3*#	10.9$\pm$3.0*#
+LOE 908 (30 μM)+SK&F 96365 (30 μM)	3.9$\pm$1.1*	4.1$\pm$1.1*#‡	4.0$\pm$1.0*#‡

Developed tension was expressed as percentages of the tension induced by ET-1 alone. Values were expressed as the means$\pm$s.e.mean of eight experiments. *$P<0.01$, significantly different from ET-1 alone; †$P<0.01$ and §$P<0.01$, significantly different from 0.1 and 1 nM ET-1, respectively; #$P<0.01$ and ‡$P<0.01$, significantly different from LOE 908 and SK&F 96365, respectively.

dependent manner with IC_{50} values of 4.3$\pm$0.3 μM ($n=8$), 9.4$\pm$1.2 μM ($n=8$) and 10.6$\pm$1.3 μM ($n=8$), respectively: the value for 0.1 nM ET-1 was significantly smaller than the values for 1.0 and 10 nM ET-1. The maximal inhibition was obtained at concentrations higher than 30 μM, although the extent of inhibition was smaller with higher concentrations of ET-1. That is, the contractions at 0.1 nM ET-1 were completely suppressed, but about 34.4$\pm$2.2% ($n=8$) and 44.4$\pm$3.9% ($n=8$) were left unsuppressed at 1.0 and 10 nM ET-1, respectively (Figure 5; Table 1).

SK&F 96365 also suppressed the contractions induced by these concentrations of ET-1 in a concentration-dependent manner with IC_{50} values of 4.3$\pm$0.2 μM ($n=8$), 12.4$\pm$1.7 μM ($n=8$) and 12.6$\pm$1.9 μM ($n=8$), respectively: the value for 0.1 nM ET-1 was significantly smaller than the values for 1.0 and 10 nM ET-1. The maximal inhibition amounting to 90% or greater was obtained at concentrations higher than 30 μM, respectively. As in LOE 908, the maximal inhibition by SK&F 96365 became slightly smaller with higher concentrations of ET-1: the SK&F 96365-resistant parts were 4.0$\pm$1.0% ($n=8$), 10.3$\pm$3.3% ($n=8$) and 10.9$\pm$3.0% ($n=8$) for 0.1, 1.0 and 10 nM ET-1, respectively (the value for 0.1 nM ET-1 was significantly smaller than the values for 1.0 and 10 nM ET-1).

Effects of varying concentrations of SK&F 96365 on the contractions induced by higher (1.0 and 10 nM) concentrations of ET-1 in the presence of a maximally effective concentration of LOE 908

To pharmacologically characterize the ET-1-induced contractions which are resistant to LOE 908, we examined the effect of varying concentrations of SK&F 96365 on the contractions induced by ET-1 in the presence of a maximally effective concentration (30 μM) of LOE 908. As shown in Figure 6, SK&F 96365 inhibited the LOE 908-resistant contractions induced by 1 and 10 nM ET-1 in a concentration-dependent

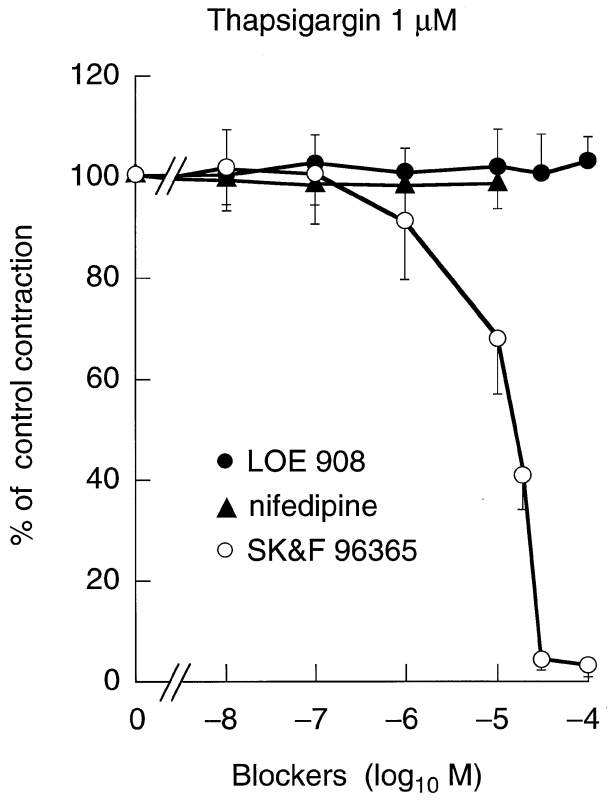

Figure 3 Effects of varying concentrations of nifedipine, LOE 908 or SK&F 96365 on the Ca^{2+}-induced contractions of rat thoracic aortic rings without endothelium pretreated with thapsigargin. Experimental protocols were the same as those in Figure 1, except that nifedipine, LOE 908 or SK&F 96365 was added to the bath solution 60 min before stimulation with 2.2 mM Ca^{2+}. The contractions were represented as percentages of the tension in the absence of the drugs. Each point represents the mean value±s.e.mean of eight experiments.

manner with IC_{50} values of 9.9±2.1 μM ($n=8$) and 10.4±1.9 μM ($n=8$), respectively: the IC_{50} values were not significantly different from the IC_{50} values against the Ca^{2+}-induced contractions in the thapsigargin-pretreated aortic rings. Complete inhibition was obtained at concentrations higher than 30 μM.

Measurement of $[Ca^{2+}]_i$ in cultured VSMCs

To confirm whether LOE 908 and SK&F 96365 actually suppress Ca^{2+} entry, we examined the effects of these drugs on the increase in $[Ca^{2+}]_i$ in cultured VSMCs induced by thapsigargin or ET-1.

In cultured VSMCs which had been incubated in Ca^{2+}-free solution, addition of thapsigargin alone induced a transient increase in $[Ca^{2+}]_i$ (Figure 7). When 2.2 mM Ca^{2+} was added after return of $[Ca^{2+}]_i$ to basal levels, it induced a sustained increase in $[Ca^{2+}]_i$ amounting to 300 μM (Figure 7). The increase was abolished by addition of 2 mM EGTA (to 3.2±0.4% of control responses, $n=28$; Figure 7a), but it was unaffected by LOE 908 up to 30 μM (Figures 7b and 8). SK&F 96365 suppressed the increase in a concentration-dependent manner with an IC_{50} value of 0.9±0.1 μM ($n=30$; Figures 7b and 8): the IC_{50} value in cultured VSMCs was significantly smaller than the value against the contractions of aortic rings (Figure 3). Complete inhibition was obtained at concentrations higher than 10 μM (Figures 7b and 8).

Both lower (0.1 nM) and higher (10 nM) concentrations of ET-1 induced an increase in $[Ca^{2+}]_i$ in cultured VSMCs (Figures 9 and 11). The increase in $[Ca^{2+}]_i$ induced by 0.1 nM ET-1 was abolished by removal of extracellular Ca^{2+} (Figure 9b; Table 2), whereas only the sustained increase induced by 10 nM ET-1 was abolished with an initial transient increase being unaffected (Figure 11b; Table 2). Again nifedipine had no effect on the increase in $[Ca^{2+}]_i$ in cultured VSMCs induced by either concentration of ET-1 (Figure 11c; Table 2).

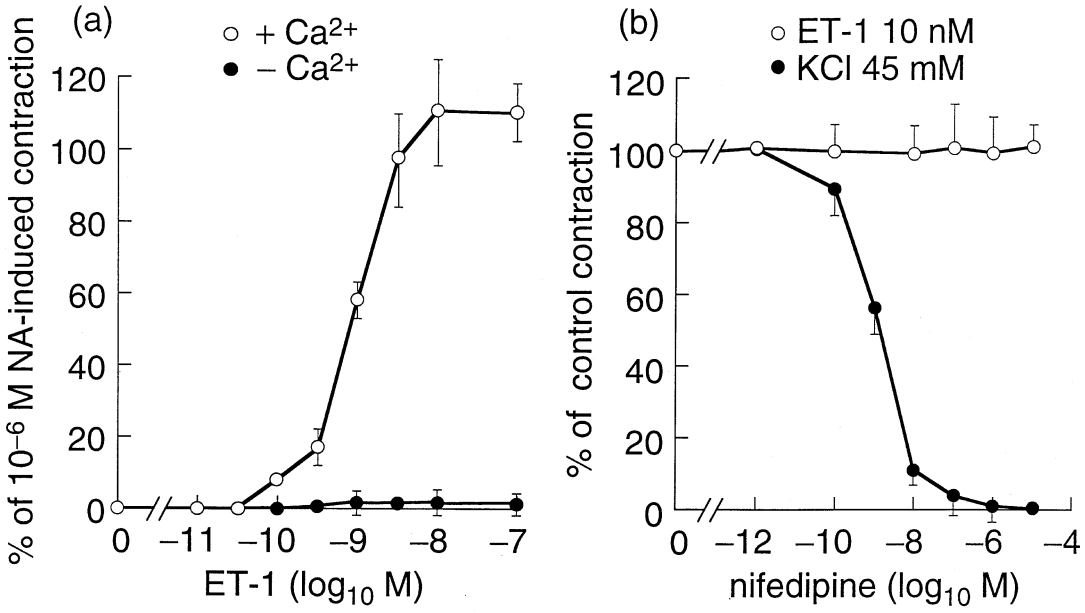

Figure 4 Effects of removal of extracellular Ca^{2+} on the contractions of rat thoracic aortic rings without endothelium induced by varying concentrations of ET-1 (a) and of varying concentrations of nifedipine on the contractions induced by 10 nM ET-1 or 45 mM KCl (b). (a) The contractile responses to varying concentrations of ET-1 were examined in the absence or presence of extracellular Ca^{2+} and represented as percentages of the tension induced by 1 μM NA in each preparation. (b) Varying concentrations of nifedipine were added 60 min before stimulation with 10 nM ET-1 or 45 mM KCl. The contractions in the presence of nifedipine were represented as percentages of the tension induced by ET-1 or KCl alone. Each point represents mean value±s.e.mean of eight experiments.

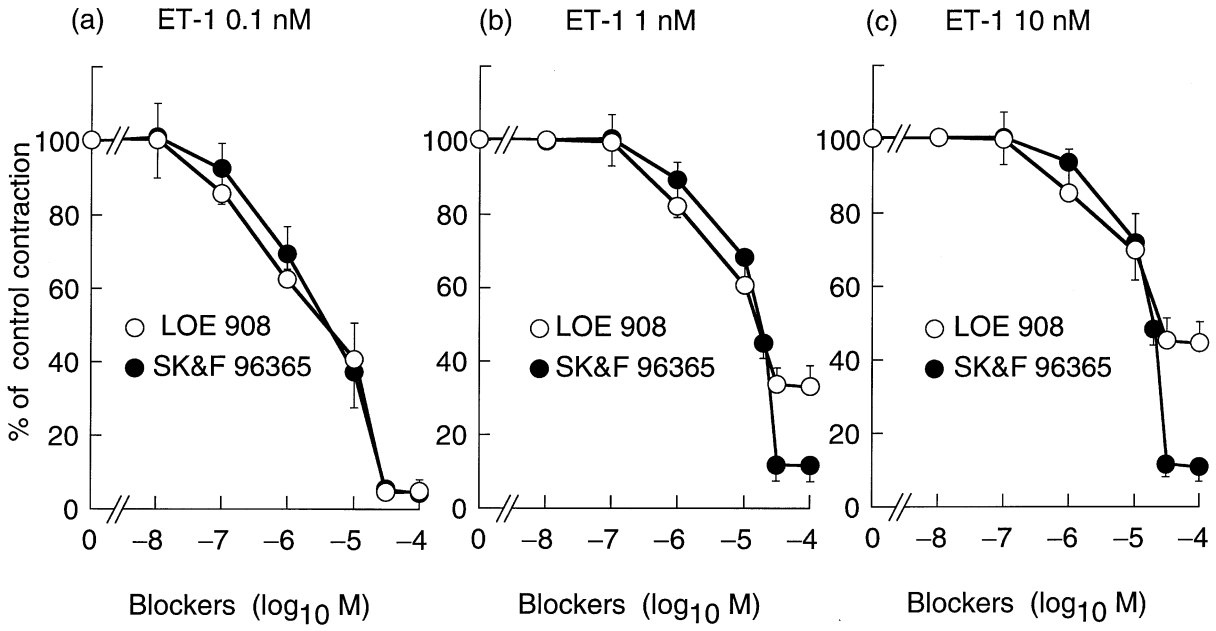

Figure 5 Effects of varying concentrations of LOE 908 or SK&F 96365 on the contractions of rat thoracic aortic rings without endothelium induced by 0.1, 1 and 10 nM ET-1. Varying concentrations of LOE 908 and SK&F 96365 were added 60 min before stimulation with ET-1. The contractions in the presence of drugs were represented as percentages of the tension induced by ET-1 alone. Each point represents mean value ± s.e.mean of eight experiments.

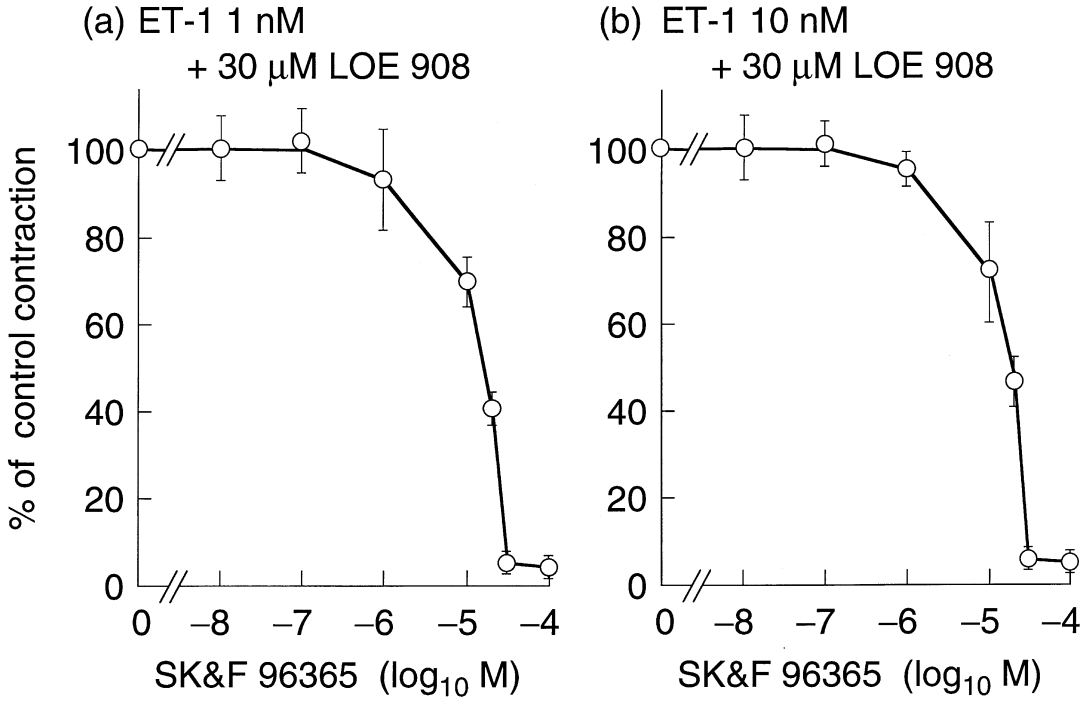

Figure 6 Effects of varying concentrations of SK&F 96365 on the LOE 908-resistant contractions of rat thoracic aortic rings without endothelium induced by higher concentrations of ET-1. Varying concentrations of SK&F 96365 along with a maximally effective concentration (30 μM) of LOE 908 were added 60 min before stimulation with 1 or 10 nM ET-1. The contractions were represented as percentages of the tension in the absence of SK&F 96365. Each point represents mean value ± s.e.mean of eight experiments.

To obtain maximally effective concentrations of LOE 908 and SK&F 96365 in cultured VSMCs, we reexamined concentration-response curves for the increase in $[Ca^{2+}]_i$ induced by the highest concentration (10 nM) of ET-1. The increase in $[Ca^{2+}]_i$ was suppressed by either LOE 908 or SK&F 96365 in a concentration-dependent manner with IC_{50} values of 0.12 ± 0.02 μM ($n = 30$) and 0.72 ± 0.15 μM ($n = 30$), respectively. The maximal inhibition amounting to about 60 and 90% was obtained at concentrations higher than 1 μM LOE 908 and 10 μM SK&F 96365, respectively (Figure 10; Table 2).

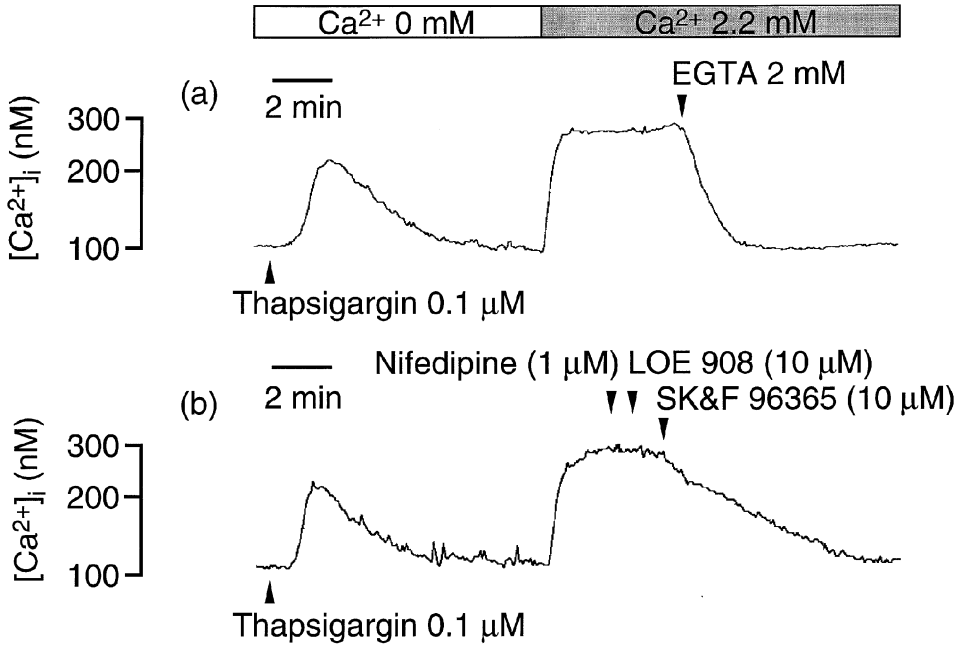

Figure 7 Typical tracings showing the effects of removal of extracellular Ca^{2+} (a) and Ca^{2+} channel blockers (b) on Ca^{2+} entry induced by depletion of intracellular Ca^{2+} stores in cultured VSMCs prepared from the rat thoracic aorta. The VSMCs were enzymatically dispersed from the rat thoracic aorta and cultured in 35-mm glass-bottomed plastic dishes as described in the Methods section. The cultured VSMCs were loaded with a Ca^{2+} indicator fluo-3, and changes in $[Ca^{2+}]_i$ were measured by monitoring the fluo-3 fluorescence. After the fluo-3-loaded cells were preincubated in Ca^{2+}-free Krebs-HEPES solution containing 0.1 mM EGTA and 0.1 μM thapsigargin for 10 min, the medium was switched to normal Krebs-HEPES solution containing 2.2 mM Ca^{2+}. During the plateau phase of $[Ca^{2+}]_i$ increase induced by Ca^{2+}, EGTA (a) or Ca^{2+} channel blockers such as nifedipine, LOE 908 and SK&F 96365 (b) were added to the medium as indicated by arrowheads.

Table 2 Summary of effects of removal of extracellular Ca^{2+}, nifedipine, LOE 908 and SK&F 96365 on increases in $[Ca^{2+}]_i$ in single VSMCs induced by varying concentrations of ET-1

	ET-1-induced increases in $[Ca^{2+}]_i$ (%)					
	Et-1 (0.1 nM)	n	*ET-1* (1 nM)	n	*ET-1* (10 nM)	n
ET-1 alone	100.0±9.5	15	100.0±9.2	15	100.0±9.4	20
$-Ca^{2+}$	2.5±2.1*	10	2.8±2.2*	10	2.2±2.5*	20
+Nifedipine (1 μM)	98.0±5.1	11	97.2±3.4	15	97.5±7.0	28
+LOE 908 (10 μM)	3.7±2.1*	25	28.1±3.5*†	28	46.4±5.1*†§	30
+SK&F 96365 (10 μM)	3.9±2.0*	20	11.0±3.3*#	25	10.7±3.0*#	30
+LOE 908 (10 μM)+SK&F 96365 (10 μM)	4.0±1.9*	10	6.2±4.0*#	10	6.5±3.5*#	28

The increase in $[Ca^{2+}]_i$ was represented as percentages of the increase in $[Ca^{2+}]_i$ induced by each concentration of ET-1. *$P<0.01$, significantly different from ET-1 alone; †$P<0.01$ and ‡$P<0.01$, significantly different from 0.1 and 1 nM Et-1, respectively; #$P<0.01$, significantly different from LOE 908.

Using the maximally effective concentrations of LOE 908 (10 μM) and SK&F 96365 (10 μM), we examined the contributions of Ca^{2+} entry through LOE 908- or SK&F 96365-sensitive channels to the increase in $[Ca^{2+}]_i$ induced by 0.1, 1 and 10 nM ET-1.

The increase in $[Ca^{2+}]_i$ induced by 0.1 nM ET-1 was abolished by either LOE 908 or SK&F 96365 (Figure 9c and d; Table 2). In contrast, the increase in $[Ca^{2+}]_i$ induced by higher concentrations of ET-1 became resistant to LOE 908: 28.1±3.5% at 1.0 nM ET-1 ($n=28$) and 46.4±5.1% at 10 nM ET-1 ($n=30$) were left unsuppressed (Figure 11d; Table 2). SK&F 96365 more markedly suppressed the increase in $[Ca^{2+}]_i$ induced by 1.0 or 10 nM ET-1, namely to 11.0±2.3% ($n=25$) and 10.7±1.0% ($n=30$), respectively (Figure 11e; Table 2). SK&F 96365 in combination with LOE 908 tended to further suppress the increase but the suppression was not statistically significant (Figure 11f; Table 2).

Discussion

Properties of Ca^{2+}-induced contractions of rat aorta and increases in $[Ca^{2+}]_i$ in cultured VSMCs pretreated with thapsigargin or ionomycin

In the rat thoracic aorta and cultured VSMCs pretreated with thapsigargin or ionomycin, addition of Ca^{2+} induced contractions and increases in $[Ca^{2+}]_i$, respectively. The contractions and increases in $[Ca^{2+}]_i$ are considered to result from Ca^{2+} influx through SOCC for the following reasons. First, thapsigargin and ionomycin share the common

pharmacological action of depleting the intracellular Ca^{2+} store, although their interactive molecules are different:

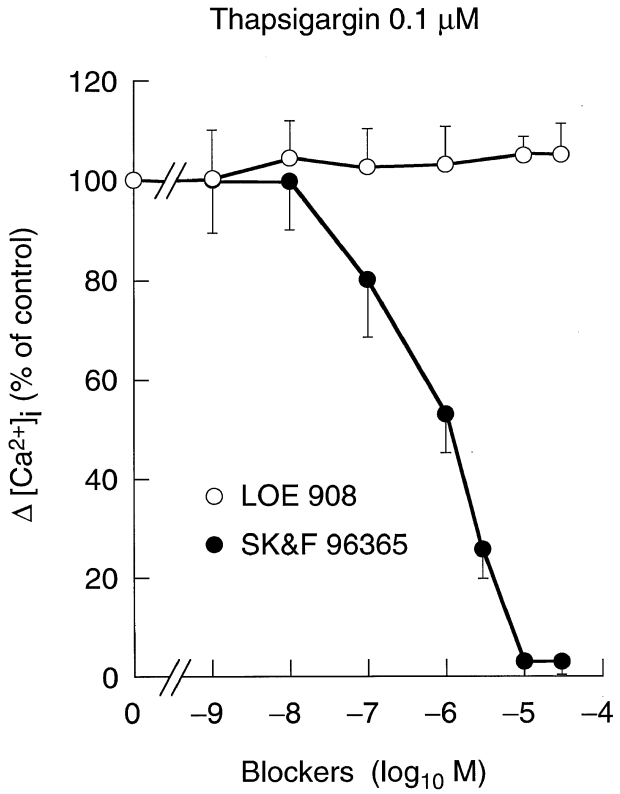

Figure 8 Effects of varying concentrations of LOE 908 or SK&F 96365 on the thapsigargin/Ca^{2+}-induced increase in $[Ca^{2+}]_i$ of the cultured VSMCs. The fluo-3-loaded VSMCs were stimulated by 2.2 mM Ca^{2+} as described in the legend for Figure 7. During the plateau phase of $[Ca^{2+}]_i$ increase, varying concentrations of Ca^{2+} channel blockers such as LOE 908 and SK&F 96365 were added to the medium. The increase in $[Ca^{2+}]_i$ were represented as percentages of the value in the absence of the blockers. Each point represents mean value ± s.e.mean of 30 experiments.

thapsigargin is a specific blocker of a Ca^{2+}-pump on the membrane of sarcoplasmic reticulum (SR) (Thastrup *et al.*, 1990) and ionomycin at lower concentrations interacts mainly with the SR membrane to make it permeable to Ca^{2+} (Morgan & Jacob, 1994). The resulting store depletion is considered to lead to activation of SOCC (Takemura *et al.*, 1989; Thastrup *et al.*, 1990; Morgan & Jacob, 1994). Secondly, the contractions and increases in $[Ca^{2+}]_i$ depend totally on influx of extracellular Ca^{2+} but are resistant to an inhibitor of VOCC nifedipine.

Effects of inhibitors of voltage-independent Ca^{2+} channels such as SK&F 96365 and LOE 908 on Ca^{2+}-induced contractions of rat aorta and increases in $[Ca^{2+}]_i$ in cultured VSMCs pretreated with thapsigargin or ionomycin

The present study indicates that SK&F 96365 is a potent inhibitor of Ca^{2+} influx through SOCC, judging from abolition of Ca^{2+}-induced contractions of rat thoracic aorta and Ca^{2+}-induced increases in $[Ca^{2+}]_i$ in cultured VSMCs which had been pretreated with thapsigargin or ionomycin. This result is essentially consistent with previous reports that the drug abolishes the thapsigargin-induced increases in whole-cell Ca^{2+} currents in mast cells (Franzius *et al.*, 1994) and single smooth muscle cells of the mouse anococcygeus (Wayman *et al.*, 1996) and in $[Ca^{2+}]_i$ in HL-60 cells (Koch *et al.*, 1994) and A7r5 cells (Iwamuro *et al.*, 1999) as an index of Ca^{2+} entry through SOCC.

In contrast, LOE 908 was found to possess no inhibitory effect on SOCC, based on no inhibition of the Ca^{2+}-induced contractions and Ca^{2+}-induced increases in $[Ca^{2+}]_i$ by this drug. The present results are in good agreement with our recent data that the drug produces no inhibitory effect on the Ca^{2+}-induced increase in $[Ca^{2+}]_i$ in A7r5 cells pretreated with thapsigargin (Iwamuro *et al.*, 1999). However, the data are discrepant with another report (Encabo *et al.*, 1996), where this drug inhibits the Ca^{2+}-induced increase in $[Ca^{2+}]_i$ in human endothelial cells. The

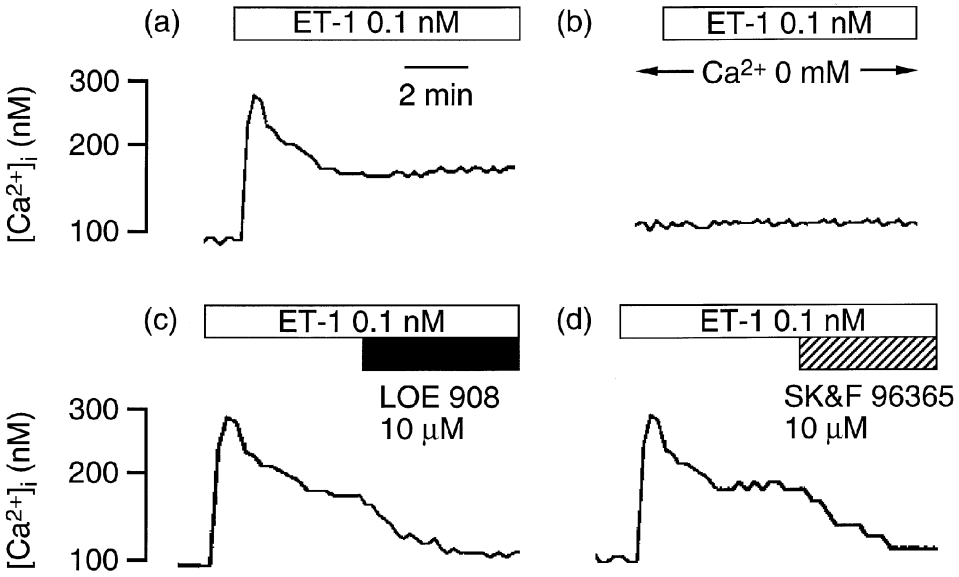

Figure 9 Typical tracings showing the effects of removal of extracellular Ca^{2+} (b), LOE 908 (c) or SK&F 96365 (d) on the increase in $[Ca^{2+}]_i$ in cultured VSMCs induced by a lower concentration (0.1 nM) of ET-1. Preparation of cultured VSMCs and monitoring of $[Ca^{2+}]_i$ were performed as described in the legend to Figure 7. The experiments were performed in normal Krebs-HEPES (a, c and d) or Ca^{2+}-free Krebs-HEPES solution containing 0.1 mM EGTA (b). At the beginning of each bar, ET-1 or Ca^{2+} channel blockers were added to the medium at the indicated concentrations.

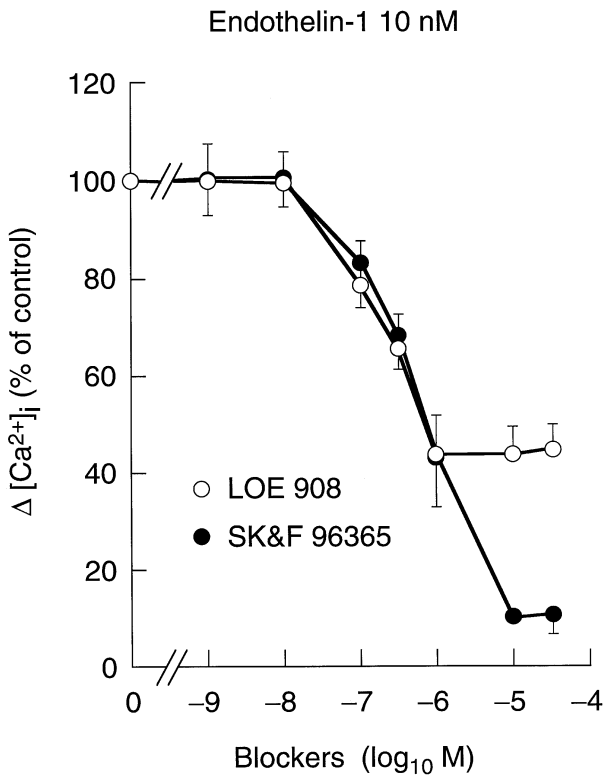

Figure 10 Effects of varying concentrations of LOE 908 or SK&F 96365 on the ET-1-induced increase in $[Ca^{2+}]_i$ of the cultured VSMCs. The fluo-3-loaded VSMCs were stimulated by 10 nM ET-1 in normal Krebs-HEPES solution. Preparation of cultured VSMCs and monitoring of $[Ca^{2+}]_i$ were performed as described in the legend to Figure 7. During the plateau phase of $[Ca^{2+}]_i$ increase, varying concentrations of Ca^{2+} channel blockers such as LOE 908 and SK&F 96365 were added to the medium. The increase in $[Ca^{2+}]_i$ were represented as percentages of the value in the absence of the blockers. Each point represents mean value ± s.e.mean of 30 experiments.

reason for this discrepancy is at present unknown but one possibility is that different types of SOCC in terms of the sensitivity to the drug are expressed in endothelial cells.

Effects of Ca^{2+} removal, nifedipine, SK&F 96365 or LOE 908 on contractions and increases in $[Ca^{2+}]_i$ induced by ET-1

The contractions and increases in $[Ca^{2+}]_i$ induced by lower ($\leqslant$0.1 nM) or higher ($\geqslant$1 nM) concentrations of ET-1 depend totally on influx of extracellular Ca^{2+}, as evidenced by abolition of the contractions and increases in $[Ca^{2+}]_i$ after removal of extracellular Ca^{2+}. However, VOCC does not seem to be involved in the contractions and increases in $[Ca^{2+}]_i$ induced by ET-1, judging from insensitivity of these parameters to nifedipine. These results are consistent with previous reports (Kasuya *et al*., 1989; Sakata *et al*., 1989; Chabrier *et al*., 1989; D'Orleans-Juste *et al*., 1989; Turner *et al*., 1989; Nakajima *et al*., 1996; Zhang *et al*., 1998).

The contractions and increases in $[Ca^{2+}]_i$ induced by lower concentrations of ET-1 ($\leqslant$0.1 nM) seem to result from Ca^{2+} entry through the channel which is sensitive to both LOE 908 and SK&F 96365, because the responses are abolished by either of the drugs alone.

Based on the sensitivities of the contractions and increases in $[Ca^{2+}]_i$ to LOE 908 and SK&F 96365, the major portion of the contraction at higher concentrations of ET-1 ($\geqslant$1 nM) seems to result from Ca^{2+} entry through two types of Ca^{2+} entry channel. One channel is sensitive to both LOE 908 and SK&F 96365, whereas the other is resistant to LOE 908 but sensitive to SK&F 96365: the sensitivity of the latter channel to SK&F 96365 is similar to that of the channel activated by thapsigargin (SOCC), judging from the IC_{50} values for the contractions induced by thapsigargin/Ca^{2+} and for LOE 908-resistant contractions induced by ET-1. Furthermore, only in the tension study, there was a small but significant contraction which is sensitive to LOE 908 but resistant to SK&F 96365.

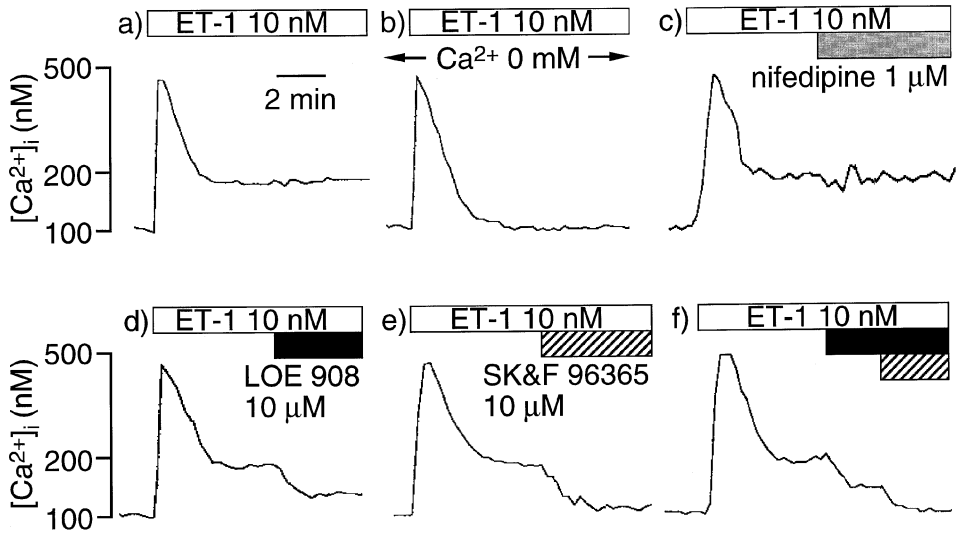

Figure 11 Typical tracings showing the effects of removal of extracellular Ca^{2+} (b), nifedipine (c), LOE 908 (d), SK&F 96365 (e) or LOE 908 plus SK&F 96365 (f) on the increase in $[Ca^{2+}]_i$ in cultured VSMCs induced by a higher concentration (10 nM) of ET-1. Preparation of cultured VSMCs and monitoring of $[Ca^{2+}]_i$ were performed as described in the legend to Figure 7. The experiments were performed in normal Krebs-HEPES (a, c, d, e and f) or Ca^{2+}-free Krebs-HEPES solution containing 0.1 mM EGTA (b). At the beginning of each bar, ET-1 or Ca^{2+} channel blockers were added to the medium at the indicated concentrations.

According to our definition based on the sensitivities to LOE 908 and SK&F 96365 (see Introduction), the Ca^{2+} entry channel which is involved in the contractions at lower concentrations of ET-1 is considered to be NSCC-2 (sensitive to both LOE 908 and SK&F 96365). On the other hand, the channels which are involved in the major portion of the contractions at higher concentrations of ET-1 seem to be NSCC-2 and SOCC (resistant to LOE 908 but sensitive to SK&F 96365), with a minor contribution from NSCC-1 (sensitive to LOE 908 but resistant to SK&F 96365).

Notably, the contribution of SOCC to the contractions and increases in $[Ca^{2+}]_i$ induced by ET-1 apparently increases with increases in the concentrations of ET-1, judging from the increase in the proportion of the contractions and increases in $[Ca^{2+}]_i$ resistant to LOE 908. This result is consistent with the notion that formation of IP_3 occurs at higher concentrations of ET-1 than contractions (Kasuya *et al*., 1989) and our data that a transient increase in $[Ca^{2+}]_i$ resulting from Ca^{2+} mobilization from SR *via* increased formation of IP_3 occurs at higher concentrations of ET-1.

Ca^{2+} entry channels activated by ET-1 seem to be different between native VSMCs of rat aorta and A7r5 cells (Iwamuro *et al*., 1999). First, NSCC-1 which is characterized by sensitivity to LOE 908 and insensitivity to SK&F 96365 is activated by both lower and higher concentrations of ET-1 in A7r5 cells, whereas its activation can be noted only at higher concentrations of ET-1 in the native cells: instead, NSCC-2 is activated by these concentrations of ET-1 in the native cells. The mechanisms for replacement of NSCC-1 with NSCC-2 in the native cells are at present unknown and remains to be determined.

The IC_{50} values for LOE 908 or SK&F 96365 in cultured VSMCs were smaller than those in the aortic rings. The reason for this discrepancy is at present unknown but one possibility is that infiltration of the drugs through the whole tissue is not so efficient. Alternatively, there might be some metabolizing enzymes or uptake mechanism for these drugs in the cells other than VSMCs.

In summary, the present study combined with our recent findings clearly showed that the contractions of rat thoracic aorta by lower concentrations of ET-1 involve a Ca^{2+} entry channel sensitive to both LOE 908 and SK&F 96365 (NSCC-2), whereas the contractions by higher concentrations of ET-1 involve a Ca^{2+} entry channel which is resistant to LOE 908 but sensitive to SK&F 96365 (SOCC) in addition to NSCC-2. Additionally, NSCC-1 seems to contribute to a small portion of the contractions by higher concentrations of ET-1.

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