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Inhibition of a store-operated Ca^{2+} entry pathway in human endothelial cells by the isoquinoline derivative LOE 908

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1 The novel cation channel blocker, LOE 908, was tested for its effects on Ca^{2+} entry and membrane currents activated by depletion of intracellular Ca^{2+} stores in human endothelial cells.

2 LOE 908 inhibited store-operated Ca^{2+} entry induced by direct depletion of Ca^{2+} stores with 100 nM thapsigargin or 100 nM ionomycin with an EC_{50} of 2 μM and 4 μM, respectively.

3 LOE 908 did not affect thapsigargin- or ionomycin-induced Ca^{2+} release from intracellular stores up to concentrations of 3 μM.

4 LOE 908 reversibly suppressed thapsigargin- as well as ionomycin-induced whole-cell membrane currents.

5 The LOE 908-sensitive membrane conductance corresponded to a cation permeability of 5.5 and 6.9 fold selectivity for Ca^{2+} over K^+ in the presence of thapsigargin and ionomycin, respectively.

6 Our results suggest that the isoquinoline, LOE 908 is a novel, potent inhibitor of the store-operated (capacitive) Ca^{2+} entry pathway in endothelial cells.

Keywords: Endothelial cells; Ca^{2+} stores; Ca^{2+} entry; membrane currents; LOE 908

Introduction

Depletion of intracellular Ca^{2+} stores serves as an important cellular signal which initiates Ca^{2+} entry into vascular endothelial cells (Schilling *et al.*, 1992; Dolor *et al.*, 1992). According to the model of store-dependent (capacitive) Ca^{2+} entry (Putney, 1990), the filling state of rapidly exchanging Ca^{2+} stores determines Ca^{2+} entry in a variety of tissues. Distinctly different Ca^{2+} transport systems appear to be involved in store-dependent Ca^{2+} entry into different cell types. In mast cells, store-dependent Ca^{2+} entry has been found to involve activation of a highly selective Ca^{2+} permeability (Hoth & Penner, 1993; Reinsprecht *et al.*, 1995). In contrast, store-dependent Ca^{2+} entry into endothelial cells was found to be based on membrane conductances with low or moderate Ca^{2+} selectivity (Vaca & Kunze, 1994). As yet, pharmacological tools capable of dissecting these distinct store-dependent Ca^{2+} permeabilities have not been identified. The isoquinoline derivative, LOE 908, has been reported to block nonselective, vasopressin-activated Ca^{2+} channels in vascular smooth muscle (Krautwurst *et al.*, 1994) but not Ca^{2+} currents initiated by store-depletion in mast cells (Franzius *et al.*, 1994). With the present study, we demonstrate that this compound is a blocker of an endothelial Ca^{2+} conductance activated by depletion of intracellular Ca^{2+} stores.

Methods

Cell culture

Endothelial cells were isolated and cultured from human umbilical cords as described by Groschner *et al.* (1994). Primary cultures and subcultured cells (passage 1) were used for experimentation.

Measurement of intracellular free Ca^{2+}

Endothelial cells were loaded with Fura-2/AM as described previously (Groschner *et al.*, 1994). In brief, endothelial cells were harvested and resuspended in HEPES buffer (in mM): NaCl 145, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1, HEPES 10, pH 7.4 containing Fura-2/AM 2 μM. After a 45 min incubation at 37°C, the cells were washed and resuspended at a final concentration of 1.25×10^6 cells ml^{-1} in nominally Ca^{2+}-free HEPES buffer, supplemented with 0.1 mM EGTA. The free Ca^{2+} concentration of this nominally Ca^{2+}-free solution was below 1 μM as measured with a Ca^{2+}-sensitive electrode. Fura-2 fluorescence was monitored with the ratio-fluorescence-spectroscopy technique using a dual wavelength-spectrofluorimeter (Hitachi F-2000, Tokyo, Japan). The fluorescent Ca^{2+} indicator was excited alternately at 340 nm and 380 nm and emission was collected at 510 nm. Changes in the intracellular free Ca^{2+} (Ca_i) were recorded as changes in the Ca^{2+}-sensitive fluorescence ratio (340:380).

Ca^{2+} release was induced in nominally Ca^{2+}-free solution, and Ca^{2+} entry was monitored as the increment in Ca_i induced by addition of 2 mM extracellular Ca^{2+} after a stable Ca_i value was obtained subsequent to Ca^{2+} release. For quantification of Ca^{2+} release, the increment in Ca^{2+}-sensitive fluorescence ratio induced by thapsigargin or ionomycin in Ca^{2+}-free solution was used. All experiments were corrected for the fluorescence produced by LOE 908 itself. Since the fluorescence of LOE 908 was found to be dependent on the presence of cells, we determined autofluorescence under conditions identical to those of Fura-2 experiments. We found that the level of autofluorescence did not change during treatment of cells with ionomycin or thapsigargin, as well as during elevation of extracellular Ca^{2+}.

Measurement of membrane currents

Whole-cell currents were recorded with the amphotericin B-perforated-patch technique as described by Groschner *et al.* (1994). Experiments were performed at room temperature. For voltage clamp and current amplification a List EPC/7 patch-clamp amplifier (List, Darmstadt, Germany) was used. Effects on membrane conductance were studied by holding the cells at −60 mV and applying voltage ramps (0.1 mV ms^{-1}; 0.2 Hz). The bath solution contained (mM): $CaCl_2$ 10, $MgCl_2$ 1, N-methyl-D-glucamine (NMDG) 68, aspartic acid 132.5, tetra-

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ethylammonium (TEA) hydroxide 64.5, 4,4'-diisothiocyanostilben 2,2'-disulphonic acid (DIDS) 0.3, HEPES 5, glucose 6. The pipette solution contained: K gluconate 135, KCl 10, $MgCl_2$ 5, HEPES 15. pH of all solutions was adjusted to 7.4 with NMDG. Amphothericin B (240 $\mu g\ ml^{-1}$; Sigma, Munich, Germany) was added to the pipette solution immediately prior to use. Reversal potentials of thapsigargin- and ionomycin-induced membrane currents were determined by subtraction of control currents (ramp-responses) measured in the absence of drugs. Similarly, reversal potentials of LOE 908-sensitive currents were determined by subtraction of currents recorded in the presence of LOE 908 from those recorded in the absence of the blocker.

Statistics

Averaged data are given as mean ± s.e.mean from the indicated number of experiments. Statistical analysis was performed using Student's *t* test. Differences were considered statistically significant at $P<0.05$.

Materials

(R,S)-(3,4-dihydro 6,7-dimethoxy-isoquinoline-1-yl)-2-phenyl-N,N-di-[2-(2,3,4trimethoxyphenyl)ethyl]-acetamide (LOE 908) was kindly provided by Drs N. Mayer and A. Walland, Boehringer-Ingelheim (Ingelheim, Germany). All tissue culture media were from Gibco BRL, Paisley, U.K. and ingredients from Flow Laboratories (Meckenheim, Germany). All other enzymes, drugs and chemicals were purchased from Sigma (Munich, Germany).

Results

In a first set of experiments we investigated the effects of LOE 908 on thapsigargin- and ionomycin-induced intracellular Ca^{2+} signals. In nominally Ca^{2+}-free solution ($Ca^{2+}<1\ \mu M$), 100 nM thapsigargin evoked a transient rise in intracellular free Ca^{2+} (Ca_i) due to discharge of intracellular Ca^{2+} stores (Figure 1a). Similarly, ionomycin depleted intracellular Ca^{2+} stores at sub-μmolar concentrations (0.1 μM) as evident from induction of a transient increase in Ca_i (Figure 1b). Re-establishment of a physiological Ca^{2+} gradient subsequent to store depletion by either thapsigargin or ionomycin produced a large increase in Ca_i, indicating activation of a Ca^{2+} permeability in store-depleted cells. Rises in Ca_i induced by elevation of extracellular Ca^{2+} were taken as a measure of store depletion-induced Ca^{2+} entry. Ca^{2+} entry induced by either thapsigargin or ionomycin were similarly blocked by La^{3+} (50 μM, $n=3$). Addition of 100 nM iomomycin to thapsigargin (1 μM)-treated cells failed to produce a significant increment in Ca^{2+} entry ($n=3$, not shown) indicating that direct ionophore-mediated Ca^{2+} entry is negligible at this concentration of ionomycin. In the presence of 3 μM LOE 908, Ca^{2+} release evoked by thapsigargin or ionomycin was only slightly reduced (to 88.2 ± 6.0% and 87.5 ± 4.2%, respectively; $n=4$), whereas Ca^{2+} entry was clearly suppressed (Figure 1). Figure 2 shows the concentration-dependence of the inhibitory effects of LOE 908 on thapsigargin- and ionomycin-induced Ca^{2+} entry. At a concentration of 3 μM, LOE 908 inhibited thapsigargin-induced Ca^{2+} entry to 49.1 ± 5.3%, and ionomycin-induced Ca^{2+} entry to 68.2% ± 5.0% ($n=4$). The potency of LOE 908 as an inhibitor of Ca^{2+} entry was slightly higher in the presence of thapsigargin ($EC_{50}=2\ \mu M$) than in the presence of ionomycin ($EC_{50}=4\ \mu M$). At concentrations higher than these EC_{50} values for inhibition of Ca^{2+} entry, LOE 908 exerted in addition a moderate inhibitory effect on Ca^{2+} release. In the presence of 10 μM LOE 908, Ca^{2+} release evoked by either thapsigargin or ionomycin was inhibited to 67 ± 4% and 62.5 ± 3.4% of control, respectively, while Ca^{2+} entry was reduced to 29.1 ± 2.0% and 37.0 ± 9.5%, respectively ($n=4$). Since these results suggest that LOE 908 is a potent blocker of the store-operated Ca^{2+} entry pathway, we aimed to demonstrate blockade of store-operated Ca^{2+} currents.

The effects of LOE 908 on thapsigargin- and ionomycin-induced membrane currents were studied using an extracellular solution containing 10 mM Ca^{2+} as the main charge carrying cation in the extracellular solution as well as TEA and DIDS

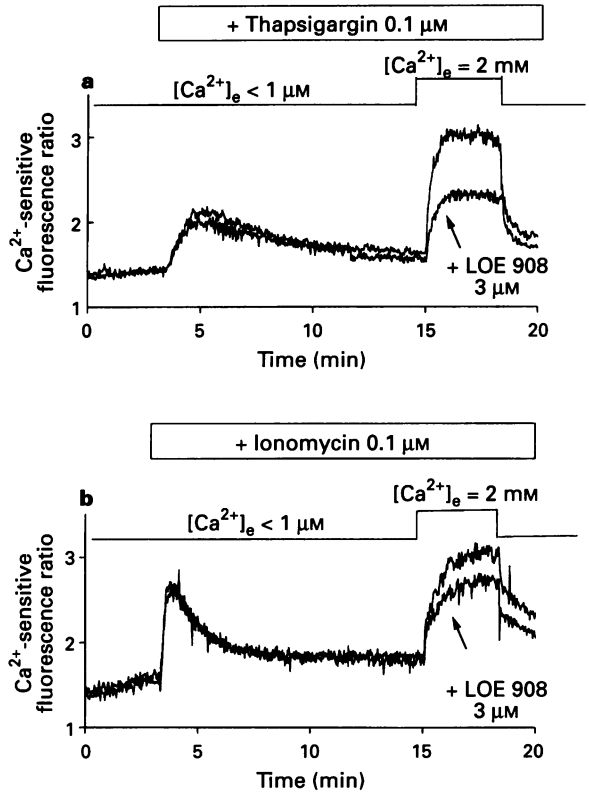

Figure 1 LOE 908 inhibits Ca^{2+} entry induced by depletion of intracellular Ca^{2+} stores. Modulation of thapsigargin (a) and ionomycin (b) -induced changes in intracellular free Ca^{2+} (Ca_i) by LOE 908 in nominally Ca^{2+}-free solution, and during elevation of extracellular Ca^{2+} to 2 mM. Time courses of Ca_i are given as Ca^{2+}-sensitive fluorescence ratios. Application of thapsigargin and ionomycin as well as changes in the extracellular Ca^{2+} concentration are indicated.

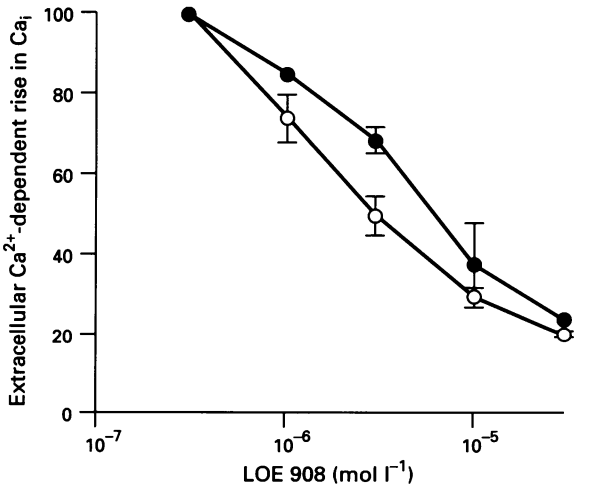

Figure 2 Concentration-dependent inhibition of thapsigargin (○)- and ionomycin (●)-induced Ca^{2+} entry by LOE 908. As a measure of Ca^{2+} entry, extracellular Ca^{2+}-dependent increases in Ca_i are given, expressed as % of control. Mean values ± s.e.mean ($n=4$).

to block, or at least minimize voltage-dependent K^+ and Cl^- currents. Thapsigargin induced a membrane current which reversed close to neutral potential (0.3 ± 4 mV, $n=13$) as calculated for the maximum current responses. In most experiments, the store-depletion activated current exhibited a moderate run-down. Since evaluation of drug effects is difficult under such conditions, we tried to confirm the effects of LOE 908 by demonstrating reversibility of the changes in membrane current observed during administration of the Ca^{2+} entry blocker. LOE 908, 10 μM, inhibited the thapsigargin-induced inward current at -80 mV within 2 min significantly to $46.1 \pm 5.8\%$ ($n=7$) of the initial level. A further increase in the concentration of LOE 908 to 30 μM resulted in almost complete suppression of the inward current as illustrated in Figure 3a and b. Upon washout of LOE 908 in the continuous presence of thapsigargin the inward current at -80 mV recovered quickly. A similar partial recovery of the inward current during washout of LOE 908 was observed in all of 4 experiments. The LOE 908-sensitive membrane current reversed at -3.5 ± 7 mV ($n=9$), as estimated from the intersection of the current-voltage curves obtained in the absence and presence of 10 μM LOE 908. Applying constant field theory (Fatt & Ginsborg, 1958; Lewis, 1979), and assuming an intracellular free Ca^{2+} concentration of 30 nM, a permeability ratio (P_{Ca}/P_K) of 5.5 was calculated. LOE 908 by itself did not affect membrane currents in non-stimulated cells as illustrated in Figure 3c. Pretreatment of cells with 10 μM LOE 908 prevented the current response to thapsigargin. In the presence of

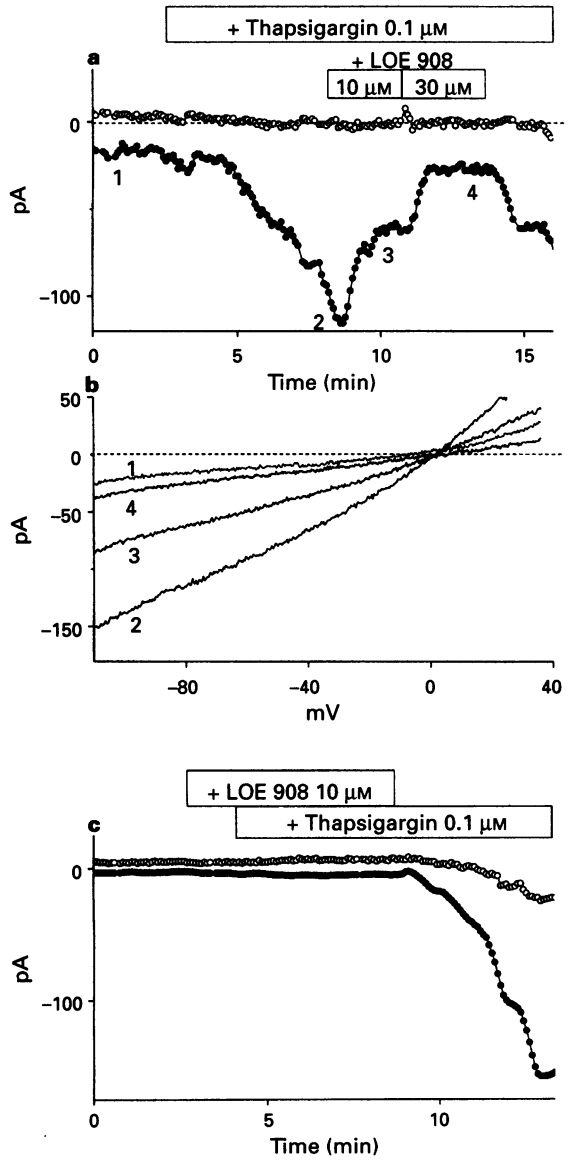

Figure 3 LOE 908 inhibits thapsigargin-induced membrane currents. (a) Effects of 10 μM and 30 μM LOE 908 on thapsigargin-induced membrane currents. Time course of membrane currents at 0 mV (○) and -80 mV (●) as determined from current responses to depolarizing voltage ramps (-120 to $+40$ mV) are shown. Application of thapsigargin and LOE 908 is indicated. (b) Individual current responses to voltage ramps corresponding to time points indicated in (a). Zero current level is indicated by dashed line. (c) Prevention of thapsigargin-induced membrane currents by pretreatment with 10 μM LOE 908. Time course of membrane currents at 0 mV (○) and -80 mV (●) are shown. Application of thapsigargin and LOE 908 is indicated.

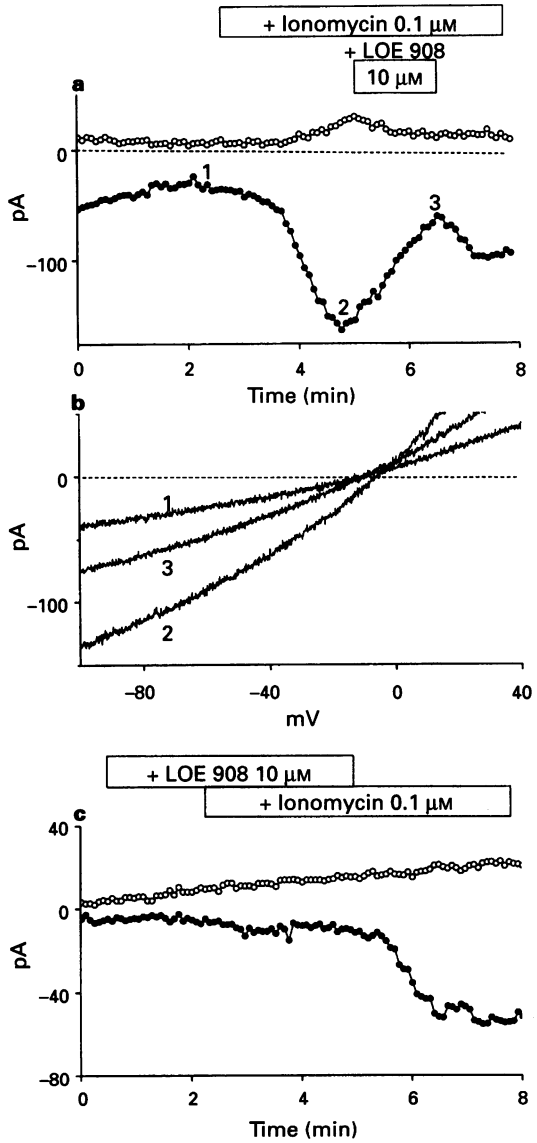

Figure 4 LOE 908 inhibits ionomycin-induced membrane currents. (a) Effects of 10 μM LOE 908 on ionomycin-induced membrane currents. Time course of membrane currents at 0 mV (○) and -80 mV (●) as determined from current responses to depolarizing voltage ramps (-120 to $+40$ mV) are shown. Application of ionomycin and LOE 908 is indicated. (b) Individual current responses to voltage ramps corresponding to time points indicated in (a). Zero current level is indicated by dashed line. (c) Suppression of ionomycin-induced membrane currents by pretreatment with 10 μM LOE 908. Time course of membrane currents at 0 mV (○) and -80 mV (●) are shown. Application of ionomycin and LOE 908 is indicated.

10 μM LOE 908, the inward current measured at −80 mV barely increased and subsequent washout of LOE 908 in the continuous presence of thapsigargin resulted in a marked rise in inward current as shown in Figure 3c. The maximum inward current evoked by thapsigargin in the presence of LOE 908 amounted to 5.9±2.9% of the current which developed within 2 min of washout of LOE 908 in the presence of thapsigargin ($n=3$). Ionomycin (100 nM) induced a membrane current which reversed at −4.5±3 mV ($n=22$). As illustrated in Figure 4a and b, the ionomycin-induced current was reversibly inhibited by LOE 908. At 10 μM LOE 908, the inward current measured at −80 mV was significantly reduced to 40.2±5.4% ($n=5$). The LOE-sensitive membrane current reversed at 0.3±2.2 mV ($n=4$). This value was not significantly different from that calculated in the presence of thapsigargin and corresponds to a permeability ratio (P_{Ca}/P_K) of 6.9 when assuming an intracellular free Ca^{2+} concentration of 30 nM. Pretreatment of cells with 10 μM LOE 908 significantly suppressed the ionomycin-induced current, which in turn recovered quickly upon washout of LOE 908 (Figure 4c). The maximum inward current evoked by ionomycin in the presence of LOE 908 was 8.5±0.3% of the current which developed within 2 min of washout of LOE 908 in the presence of ionomycin ($n=3$). These results demonstrate that LOE 908 is able to inhibit the store-operated membrane conductance in endothelial cells.

Discussion

With the present study we demonstrate that the isoqinoline derivative, LOE 908, inhibits store-dependent Ca^{2+} entry into vascular endothelial cells in the low μmolar range. Only a few pharmacological tools which modulate store-dependent Ca^{2+} entry pathways have as yet been identified. Most Ca^{2+} entry blockers such as the imidazole derivative, SK&F 96365, were found to block a variety of ion channels including various voltage-gated channels (Merrit *et al.*, 1990; Groschner *et al.*, 1994). Recently, the isoquinoline derivative, LOE 908, has been reported to block nonselective cation channels with appreciably higher potency than voltage-gated L-type channels (Krautwurst *et al.*, 1994) or the highly Ca^{2+}-selective store-operated channels in mast cells (Franzius *et al.*, 1994). Since Ca^{2+} entry into endothelial cells involves store-operated channels of low or moderate Ca^{2+} selectivity (Gericke *et al.*, 1994; Groschner *et al.*, 1994; Vaca & Kunze, 1995), it was of interest to test whether this type of store-operated ion channel is a target of LOE 908. Endothelial Ca^{2+} stores were depleted without activation of membrane receptors using two different tools, i.e. thapsigargin and ionomycin. Thapsigargin is well known to cause depletion of Ca^{2+} stores via inhibition of the stores' Ca^{2+}ATPase (Dolor *et al.*, 1992), and ionomycin has recently been demonstrated to initiate Ca^{2+} entry into endothelial cells at sub-μmolar concentrations mainly via store depletion, due to its preferential effects on the intracellular Ca^{2+} store (Morgan & Jacob, 1994). LOE 908 inhibited thapsigargin- and ionomycin-induced Ca^{2+} entry with similar potency in the low μmolar range. As direct ionomycin-mediated Ca^{2+} transport across the plasma membrane was not detectable when testing for additional effects of ionomycin in thapsigargin-depleted cells, we cannot exclude the possibility that the slightly lower potency of LOE 908 in the presence of ionomycin reflects a small LOE 908-resistant direct ionophore action. Nonetheless, our results demonstrate clearly that LOE 908 is able to suppress Ca^{2+} entry into store-depleted endothelial cells.

The target of the blocking effect of LOE 908 may be either the Ca^{2+} store, the signalling mechanisms which links the Ca^{2+} store with the plasma membrane Ca^{2+} channel, or the store-operated Ca^{2+} channel itself. At high concentrations (>10 μM) the isoquinoline derivative, LOE 908, inhibited the Ca^{2+} release-induced rise in Ca_i. Thus, at concentrations of 10 μM and higher, part of the LOE 908-induced inhibition of Ca^{2+} entry is likely to be due to reduced store depletion, and the concentration-response relationship of LOE 908-induced inhibition of Ca^{2+} entry as shown in Figure 2 may be somewhat distorted in terms of over-estimation of the maximum effect of LOE 908 on the Ca^{2+} entry pathway. Nonetheless, at lower concentrations, LOE 908 inhibited selectively the Ca^{2+} entry without affecting the Ca^{2+} release-induced rise in Ca_i. Moreover, thapsigargin-induced membrane currents were inhibited not only when cells were pretreated with LOE 908, but also when LOE 908 was administered subsequently to store depletion. These results clearly suggest that LOE 908 is able to inhibit store-operated Ca^{2+} entry and membrane conductance independently of effects on store depletion. It remains to be clarified whether the site of action of LOE 908 resides at the ion channel protein or rather within the cascade of signal transduction between the store and the plasmamembrane ion channel.

Store depletion has been reported to activate a nonselective cation conductance in endothelial cells (Gericke *et al.*, 1994). In this study we have observed thapsigargin- and ionomycin-induced membrane currents which reversed close to neutral potential at approximately physiological K^+ and Ca^{2+} gradients. The LOE 908-sensitive membrane current components reversed consistently close to neutral potential. According to constant field theory (Fatt & Ginsborg, 1958; Lewis, 1979) these membrane currents correspond to a membrane conductance of moderate selectivity for Ca^{2+} over K^+ ($P_{Ca^{2+}}/P_{K^+}=5.5-6.9$). We have previously reported on a similar membrane conductance which is activated in endothelial cells upon stimulation of histamine receptors (Groschner *et al.*, 1994), and recently a store-dependent ion channel with about 10 fold selectivity for Ca^{2+} over K^+ has been described in bovine aortic endothelium (Vaca & Kunze, 1994). Our results suggest that the isoquinoline, LOE 908, is a potent inhibitor of this specific type of cation channel. Since LOE 908 has been reported to be a weak inhibitor of the highly selective store-operated Ca^{2+} channels (Franzius *et al.*, 1994), this compound may allow for a pharmacological discrimination between distinct classes of store-operated channels. Thus, LOE 908 represents a novel type of Ca^{2+} entry blocker with a unique pharmacological profile.

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