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Characterization of Ca^{2+} channels involved in endothelin-1-induced mitogenic responses in vascular smooth muscle cells

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Abstract

Ca^{2+} channels involved in the endothelin-1-induced mitogenic response of cultured rat thoracic aorta smooth muscle cells, A7r5 cells, were characterized using the Ca^{2+} channel blockers, LOE 908 and SK&F 96365. Stimulation of A7r5 cells with endothelin-1 induced a mitogenic response as well as a biphasic increase in the intracellular-free Ca^{2+} concentration. Based on the sensitivity to nifedipine, a specific blocker of L-type voltage-operated Ca^{2+} channel (VOCC), Ca^{2+} influx through VOCC has a minor role in endothelin-1-induced mitogenic responses. On the other hand, Ca^{2+} influx through voltage-independent Ca^{2+} channels (VICCs) plays an important part in endothelin-1-induced mitogenesis. Moreover, based on their sensitivity to SK&F 96365 and LOE 908, VICCs consist of two types of Ca^{2+}-permeable nonselective cation channels (designated NSCC-1 and NSCC-2) and a store-operated Ca^{2+} channel (SOCC). Ca^{2+} influx through NSCC-1, NSCC-2 and SOCC contributes to 35%, 30% and 35%, respectively, to the nifedipine-resistant component of the endothelin-1 mitogenic response. © 2001 Published by Elsevier Science B.V.

Keywords: Endothelin-1; Ca^{2+} channel; Cell proliferation

1. Introduction

Endothelin-1 is a potent vasoconstricting peptide with a long duration of action (Yanagisawa et al., 1988). However, later studies have indicated that it also possesses multiple additional biological activities. Of the diverse actions of endothelin-1, its mitogenic properties have attracted much attention because they indicate a possible role for this peptide in the pathogenesis of certain clinical conditions such as hyperlipoproteinemia or atherosclerosis (Haak et al., 1994; Lerman et al., 1991). Endothelin-1 has also been identified as an autocrine/paracrine growth factor for some human cancer cell lines (Shichiri et al., 1991), suggesting that it has a role in oncogenesis or tumor growth. Furthermore, the recent development of transgenic mice with disrupted endothelin-1 gene has revealed a role for this peptide as a growth and/or differentiation factor for certain cell lines in embryogenesis (Eguchi et al., 1992). Some studies have demonstrated the ability of an endothelin type A receptor specific antagonist (BQ123) to block the mitogenic activity of endothelin-1 (Sugawara et al., 1996), suggesting that endothelin-1–endothelin type A receptor interaction is necessary for the mitogenic action of endothelin-1.

Endothelin-1 binds to receptors, predominantly the endothelin type A receptor, on vascular smooth muscle cells (VSMCs) (Eguchi et al., 1992; Hirata et al., 1989), and induces a biphasic increase in the intracellular-free Ca^{2+} concentration ($[Ca^{2+}]_i$) consisting of a transient peak and a subsequent sustained increase. It is generally accepted that the sustained increase in $[Ca^{2+}]_i$ requires the persistent entry of extracellular Ca^{2+}, whereas the transient increase results from mobilization of Ca^{2+} from the intracellular Ca^{2+} store (Gardner, 1989). We have recently shown that the sustained increase in $[Ca^{2+}]_i$ induced by endothelin-1 results from Ca^{2+} entry through three types of voltage-independent Ca^{2+} channel (VICC), as well as through voltage-operated Ca^{2+} channels (VOCCs) in A7r5 cells. The latter include two types of Ca^{2+}-permeable nonselective cation channel (designated NSCC-1 and NSCC-2) and a store-operated Ca^{2+} channel (SOCC) (Iwamuro et al., 1998, 1999). Importantly, we have also shown that these

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channels can be distinguished by their sensitivity to blockers of the receptor-operated Ca^{2+} channel such as SK&F 96365 and LOE 908. Thus, NSCC-1 is sensitive to LOE 908 but resistant to SK&F 96365; NSCC-2 is sensitive to both LOE 908 and SK&F 96365; and SOCC is resistant to LOE 908 but sensitive to SK&F 96365 (Iwamuro et al., 1998, 1999).

Previous reports demonstrated that Ca^{2+} influx plays an important role in mitogenesis (Chung et al., 1994; Fluckiger et al., 1998) induced by several growth factors such as epidermal growth factor (Magni et al., 1991) or angiotensin II (Kojima et al., 1985). However, it is totally unknown whether Ca^{2+} influx is essential for endothelin-1-induced mitogenesis and also what types of Ca^{2+} channels may be involved. This is mainly due to a lack of available specific Ca^{2+} channel blockers. In the present study, we attempted to elucidate which Ca^{2+} entry channels are involved in endothelin-1-induced cell proliferation using two different channel blockers in A7r5 cells.

A7r5 is permanent cell line derived from rat thoracic aorta and showing somewhat different patterns of gene expression and protein regulation compared to primary VSMCs. However, A7r5 cells have been used to assess VSMC function. This cell line was especially important in describing the intracellular Ca^{2+} regulation and Ca^{2+} channels in VSMCs; Ca^{2+} transport systems and intracellular Ca^{2+} stores have been investigated extensively in these cells (Owen, 1985; Giannattasio et al., 1991; Marsault et al., 1997). Several investigators have compared the mechanisms in A7r5 cells to freshly isolated VSMCs and obtained similar results (Owen, 1985; Giannattasio et al., 1991; Solway et al., 1995; Samaha et al., 1996). Therefore, we believe that the database on A7r5 cells justifies their use as a model of VSMCs.

2. Materials and methods

2.1. Cell culture

A7r5 cells (ATCC CRL-1444) were obtained from the American Type Culture Collection. Cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum under a humidified atmosphere of 5% CO_2/95% air.

2.2. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay and [^{3}H]thymidine incorporation

A7r5 cells were seeded into 96-well plates at 5×10^3 cells/well for MTT assay and into 24-well plates at 4×10^4 cells/well for [^{3}H]thymidine incorporation assays. They were incubated overnight in DMEM supplemented with 10% fetal calf serum at 37°C. The cells were deprived of serum for 24 h, washed with phosphate-buffered saline (PBS), and incubated with endothelin-1 for a further 48 h in serum-free DMEM with or without Ca^{2+} channel blockers.

The MTT assay, as a measure of the number of viable cells, was performed as described previously (Sugawara et al., 1996). Briefly, the cells were exposed to 1 mg/ml MTT during the last 4 h of culture with endothelin-1, and then lysed with acidic lysis buffer (20% (w/v) sodium dodecyl sulfate in 50% N, *N*-dimethyl formamide solution; pH 4.7). The absorbance of the lysate at 562 and 630 nm was measured with an EL340 Microtiter Plate Reader (Bio-Tek Instruments, Winooski, VT). The difference between the values at 562 and 630 nm was used as an index of the number of viable cells.

For measurement of [^{3}H]thymidine incorporation, [^{3}H]thymidine (1 mCi/ml) was added during the last half of the 48-h incubation period with endothelin-1. To stop the reaction, the cells were washed three times with ice-cold PBS, incubated with 10% (w/v) trichloroacetic acid at 4°C for 30 min, and subsequently washed three times with ice-cold PBS to remove the trichloroacetic acid-soluble material. The radioactivity incorporated into the trichloroacetic acid-insoluble fraction was recovered in 0.1 N NaOH and counted using a liquid scintillation counter (Aloka, Tokyo, Japan) with the solid scintillator Luma-Cap (Packard, Groningen, Netherland).

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

$[Ca^{2+}]_i$ was measured using the fluorescent probe, fluo-3, as described previously (Enoki et al., 1995). Briefly, A7r5 cells were loaded with fluo-3 by incubating them with 1 mM fluo-3/acetoxymethyl ester for 30 min at 37°C under reduced light. After washing, the cells were suspended at a density of approximately 2×10^7 cells/ml, and 0.5-ml aliquots were used for measurement of fluorescence by a CAF 110 spectrophotometer (JASCO, Tokyo, Japan) with an excitation wavelength of 490 nm and an emission wavelength of 540 nm. At the end of the experiment, Triton X-100, and subsequently, EGTA were added at final concentrations of 0.1% and 5 mM, respectively, to obtain the fluorescence maximum (F_{max}) and the fluorescence 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 0.4 μM (Minta et al., 1989).

2.4. Statistical analysis

All results were expressed as means ± S.E.M. The data were subjected to a two-way analysis of variance, and when a significant F value was encountered, the Newman–Keuls' multiple-range test was used to test for signif-

icant differences between treatment groups. A probability level of $P < 0.05$ was considered statistically significant.

2.5. Chemicals

LOE 908 was kindly provided by Boehringer Ingelheim (Ingelheim, Germany). Other reagents were commercially obtained from the following sources: endothelin-1 from Peptide Institute (Osaka, Japan); SK&F 96365 from Biomol (Plymouth Meeting, PA, USA); fluo-3/AM from Dojindo Laboratories (Kumamoto, Japan); nifedipine and MTT from Sigma (St. Louis, MO, USA).

3. Results

First, we established which subtypes of endothelin receptors were expressed by A7r5 cells using selective blockers of endothelin type A and type B receptors, BQ123 and BQ788, respectively. The effects of these blockers on the increase in $[Ca^{2+}]_i$ induced by 10 nM endothelin-1 were monitored in cells loaded with the Ca^{2+} indicator, fluo-3. The endothelin-1-induced increase in $[Ca^{2+}]_i$ was abolished by 5 μM BQ123 but unaffected by 5 μM BQ788 (data not shown).

After stimulation with endothelin-1, both the number of viable cells as estimated by the MTT assay and mitogenic activity as estimated by [^{3}H]thymidine incorporation increased with time up to 48 h (Fig. 1). Therefore, in subsequent experiments, the stimulation time was set at 48 h.

Endothelin-1 stimulated a mitogenic response in A7r5 cells in a concentration-dependent manner, with EC_{50} values of around 1 nM for both the MTT assay and [^{3}H]thymidine incorporation; the maximal effect was obtained at concentrations of 10 nM (Fig. 2). In the following experiments, endothelin-1 was added to the incubation media at a final concentration of 10 nM to analyze the role of Ca^{2+} channels in endothelin-1 mitogenesis.

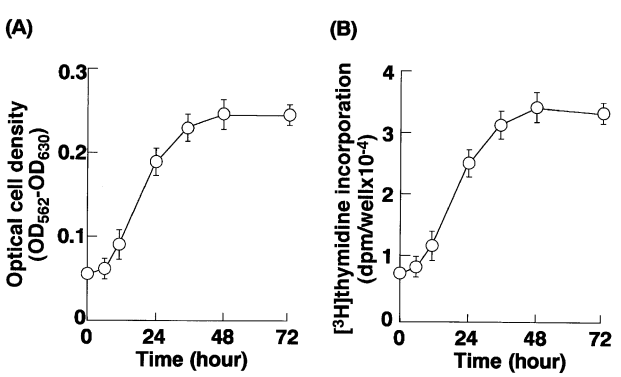

Fig. 1. Time course of the mitogenic response of A7r5 cells following stimulation with endothelin-1. After A7r5 cells had been cultured in serum-free medium for 24 h, they were stimulated with 10 nM endothelin-1 for the indicated time. Four hours or twenty-four hours before the end of the incubation with endothelin-1, either MTT (A) or [^{3}H]thymidine (B) was added to the incubation medium to estimate the number of variable cells and DNA synthesis, respectively. Data are presented as means ± S.E.M. of three determinations, each done in triplicate.

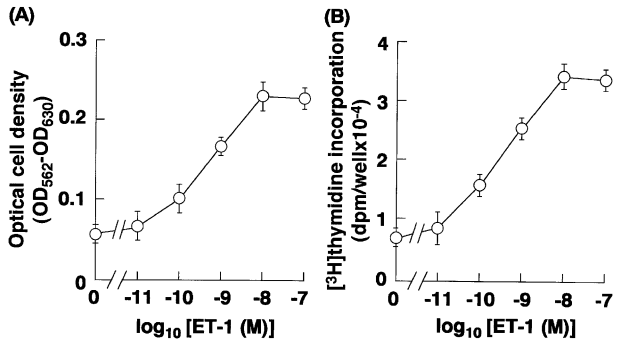

Fig. 2. Effects of various concentrations of endothelin-1 on the number of viable cells (A) and DNA synthesis (B) in A7r5 cells. After A7r5 cells had been deprived of serum for 24 h, they were stimulated with increasing concentrations of endothelin-1 for a further 48 h. The numbers of viable cells (MTT assay) (A) and [^{3}H]thymidine incorporation (B) were determined as described in Materials and methods. Data are presented as mean ± S.E.M. of three determinations, each done in triplicate.

Next, we examined the effects of Ca^{2+} influx through VOCC on the endothelin-1-induced mitogenic response using nifedipine. Nifedipine completely suppressed the high K^+ (45 mM)-induced sustained increase in $[Ca^{2+}]_i$, which causes depolarization of the plasma membrane and subsequent activation of VOCC (Komuro et al., 1997), at concentrations ≥ 1 μM (Fig. 3A). In contrast, it suppressed the endothelin-1-induced sustained increase by a maximum of only about 15% at concentrations ≥ 1 μM (Fig. 3A). The same result was obtained with the mitogenesis assay, namely, in the presence of 1 μM nifedipine, only about 15% inhibition was observed (Fig. 3B).

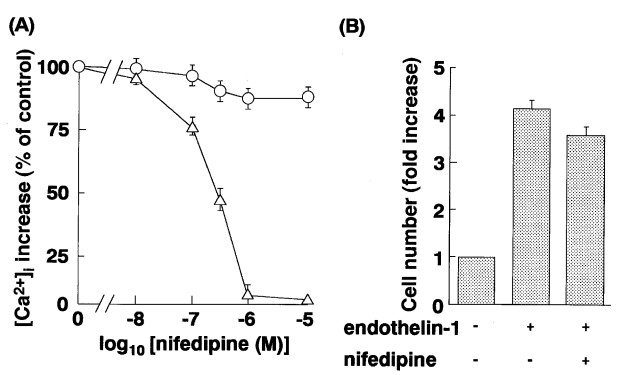

Fig. 3. (A) Effects of various concentrations of nifedipine on the sustained increase in $[Ca^{2+}]_i$ stimulated by 10 nM endothelin-1 or 45 nM KCl. The $[Ca^{2+}]_i$ increase in the presence of nifedipine is expressed as percentage of the $[Ca^{2+}]_i$ increase induced by endothelin-1 (circles) or KCl (triangles) alone. Each point represents the mean ± S.E.M of five experiments. (B) Inhibitory effects of 1 μM nifedipine on the endothelin-1-induced increases in the number of viable cells. The number of viable cells was determined as described in Materials and methods. Data are presented as means ± S.E.M. of three determinations, each done in triplicate.

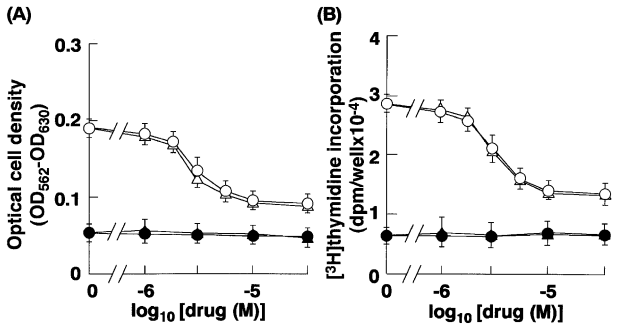

Fig. 4. Inhibitory effects of SK&F 96365 or LOE 908 on endothelin-1-induced increase in the number of viable cells (A) and DNA synthesis (B). Starved A7r5 cells were incubated for 15 min with increasing concentrations of SK&F 96365 (triangles) or LOE 908 (circles) plus 1 μM nifedipine, and then they were stimulated with (open symbols) or without (closed symbols) 10 nM endothelin-1. The numbers of viable cells (MTT assay) and [^{3}H]thymidine incorporation were determined as described in Materials and methods. Data are presented as mean ± S.E.M. of three determinations, each done in triplicate.

Using SK&F 96365 and LOE 908, we then attempted to determine the effects of Ca^{2+} influx through VICCs on the nifedipine-resistant component of the endothelin-1 stimulated mitogenic response. In the following experiments, nifedipine was added to the incubation media at a final concentration of 1 μM to analyze the role of Ca^{2+} channels other than VOCC in endothelin-1 mitogenesis. SK&F 96365 inhibited endothelin-1-induced cell proliferation in a concentration-dependent manner with an IC_{50} value of around 3 μM both in the MTT assay and the [^{3}H]thymidine incorporation assay. Maximal inhibition was observed at concentrations ≥ 10 μM (Fig. 4). The extent of maximal inhibition was around 65% (Fig. 6). Similarly, the IC_{50} values of LOE 908 for inhibition of endothelin-1-induced cell proliferation were around 3 μM for the MTT assay and for [^{3}H]thymidine incorporation, and maximal inhibition was observed at concentrations ≥ 10 μM (Fig. 5). The extent of maximal inhibition was around 65% (Fig. 6). Notably, the endothelin-1-induced mitogenic response was abolished by combined treatment with the maximally effective concentration (10 μM) of LOE 908 and SK&F 96365 (Fig. 6). The extent of maximal inhibition of endothelin-1-induced mitogenic response by SK&F 96365 and/or LOE 908 in the absence of nifedipine was similar to that in its presence (data not shown). In contrast, neither SK&F 96365 nor LOE 908 had any effect at concentrations up to 30 μM on the number of cells in the absence of endothelin-1 (Fig. 4).

In order to confirm that inhibition of the endothelin-1-induced mitogenic response by SK&F 96365 and LOE 908 is due to suppression of Ca^{2+} influx, the effects of these blockers on the endothelin-1-induced increase in $[Ca^{2+}]_i$ were monitored in A7r5 cells loaded with the Ca^{2+} indicator, fluo-3. In the presence of 1 μM nifedipine, the sustained increase in $[Ca^{2+}]_i$ was suppressed by SK&F 96365 or LOE 908 in a concentration-dependent manner with IC_{50} values around 3 μM. Maximal inhibition was observed at concentrations ≥ 10 μM (Fig. 5). The extent of maximal inhibition was around 65% in both cases. Furthermore, the sustained increase in $[Ca^{2+}]_i$ was

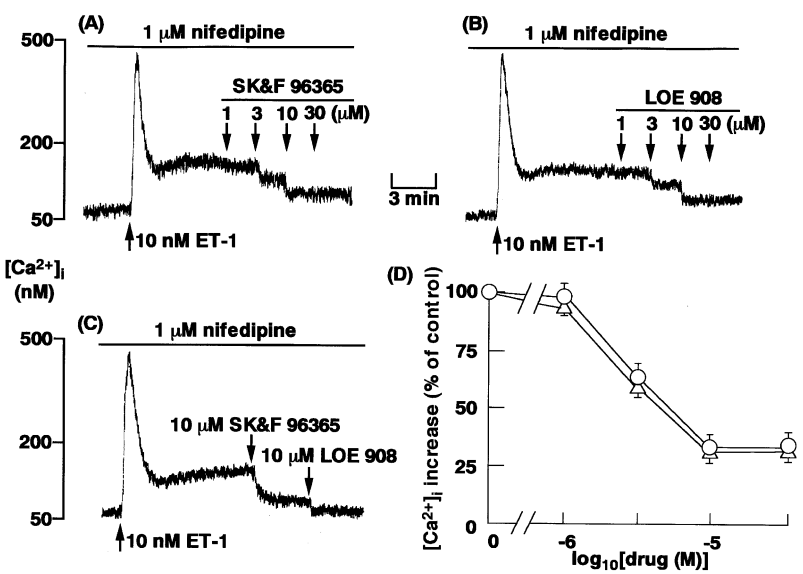

Fig. 5. (A, B, C) Original tracings showing the effects of various concentrations of SK&F 96365 and/or LOE 908 on the increase in $[Ca^{2+}]_i$ in A7r5 cells induced by endothelin-1 in the presence of 1 μM nifedipine. (D) Inhibitory effects of SK&F 96365 or LOE 908 on endothelin-1-induced increases in $[Ca^{2+}]_i$. After A7r5 cells had been cultured in DMEM containing 10% fetal calf serum, they were harvested and loaded with the Ca^{2+} indicator, fluo-3, to monitor $[Ca^{2+}]_i$. When $[Ca^{2+}]_i$ reached a plateau following stimulation with 10nM endothelin-1, nifedipine was added to the incubation medium at the indicated concentrations. $[Ca^{2+}]_i$ increase in the presence of each drug is presented as a percentage of the $[Ca^{2+}]_i$ increase induced by 10 nM endothelin-1 alone. Data are presented as mean ± S.E.M. of three determinations, each done in triplicate.

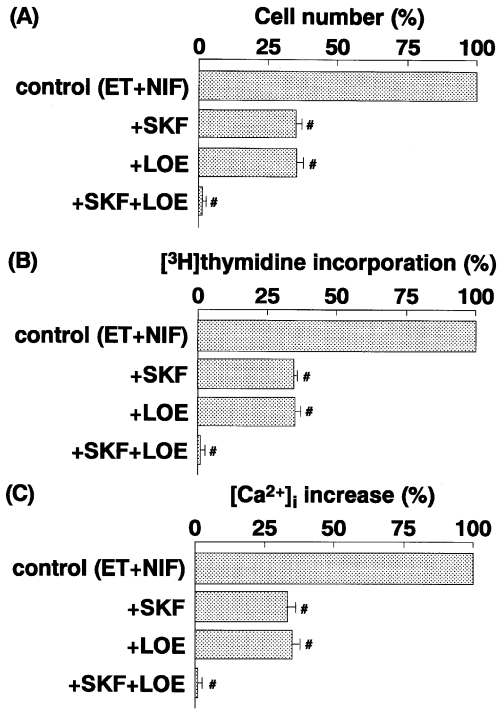

Fig. 6. Inhibitory effects of a maximally effective concentration of SK&F 96365 (10 μM) and/or LOE 908 (10 μM) on the number of viable cells, (A) DNA synthesis (B) and Ca^{2+} influx (C) induced by 10 nM endothelin-1. The MTT assay, [^{3}H]thymidine incorporation and $[Ca^{2+}]_i$ increase were determined as described in Materials and methods. The numbers of viable cells, [^{3}H]thymidine incorporation and $[Ca^{2+}]_i$ increase in the presence of SK&F 96365 and/or LOE 908 are represented as a percentage of values in its absence. Data are presented as mean ± S.E.M. of three experiments. #$P < 0.01$; significantly different from the control values in each experiment.

abolished by combined treatment with 10 μM SK&F 96365 and LOE 908 (Fig. 6).

4. Discussion

We used A7r5 cells derived from rat thoracic aorta smooth muscle cells in the present study. Several researchers have shown that NSCC and SOCC are activated by ET-1 in A7r5 cells and VSMCs from the results using whole-cell patch clamp and $[Ca^{2+}]_i$ monitoring using Ca^{2+} fluorescent probe (Enoki et al., 1995; Minowa et al., 1997; Zhang et al., 1999). There is a possibility that Ca^{2+} channels activated by ET-1 are different between native VSMC and A7r5 cells. Nevertheless, the experiments using A7r5 cells in the present study suggest the importance of Ca^{2+} influx through VICCs for intracellular Ca^{2+} mobilization or cell proliferation induced by ET-1 in VSMCs.

As reported for a variety of cells (Sugawara et al., 1996; Chung et al., 1994; Suzuki et al., 1999; Imokawa et al., 1996; Bogoyevitch et al., 1995; Malarkey et al., 1995), based on the data from the MTT and [^{3}H]thymidine incorporation assays (Figs. 1 and 2), endothelin-1 was also found to act as a mitogen on A7r5 cells via the endothelin type A receptor. A7r5 cells express predominantly endothelin type A receptors. Therefore, the differences of endothelin receptor subtypes between A7r5 cells and VSMCs may be minor.

The Ca^{2+} channels activated by high K^+ stimulation are exclusively VOCCs in A7r5 cells (Fig. 3). Moreover, the high K^+-induced contractions of rat aortic rings also involve Ca^{2+} influx only through VOCC (Zhang et al., 1999). Therefore, at least as far as VOCCs activated by high K^+ stimulation are concerned, A7r5 cells and rat aortic smooth muscle cells may share the same properties. According to the nifedipine sensitivity of endothelin-1-induced mitogenesis, involvement of VOCC in this response was estimated to be minor, at around only 15% (Fig. 3). The endothelin type A receptor is negatively coupled to the L-type VOCC and is positively coupled to receptor-operated Ca^{2+} permeable channels in rabbit cerebral cortex arterioles (Guibert and Beech, 1999). Moreover, vascular contraction and sustained increase in $[Ca^{2+}]_i$ induced by endothelin-1 were resistant to nifedipine in rat aorta smooth muscle cells (Zhang et al., 1999). Therefore, Ca^{2+} channels other than VOCC may play important roles in endothelin-1-induced mitogenic responses in A7r5 cells.

The inhibitory action of SK&F 96365 or LOE 908 on the nifedipine-resistant component of the endothelin-1-induced mitogenic response is considered to be mediated by blockade of Ca^{2+} entry through VICCs for the following reasons. (1) In our recent work using patch-clamp and $[Ca^{2+}]_i$ monitoring, endothelin-1 was found to activate three types of VICCs in A7r5 cells, namely, NSCC-1, NSCC-2 and SOCC. In addition, LOE 908 was found to be a blocker of both NSCC-1 and NSCC-2, whereas SK&F 96365 was found to be a blocker of NSCC-2 and SOCC (Iwamuro et al., 1998, 1999). (2) In the present study, the IC_{50} values of these blockers for the endothelin-1-induced mitogenic response (Figs. 4 and 6) correlated well with those for the endothelin-1-induced $[Ca^{2+}]_i$ response (Figs. 5 and 6). (3) Neither SK&F 96365 nor LOE 908 is considered to exert cytotoxic effects either on the quiescent or endothelin-1-stimulated cells, judging from the data from the MTT and [^{3}H]thymidine incorporation assays (Fig. 4).

Three types of VICC seem to be involved in the nifedipine-resistant component of the endothelin-1-induced mitogenic response in terms of its sensitivity to LOE 908 and SK&F 96365 (Figs. 4 and 6). One type of Ca^{2+} channel is sensitive to LOE 908 but resistant to SK&F 96365, another type is sensitive to both LOE 908 and SK&F 96365, and the third type is resistant to LOE 908 but sensitive to SK&F 96365. Based on pharmacological criteria, these channels are considered to be NSCC-1, NSCC-2 and SOCC, respectively. Moreover, the percent contribution of NSCC-1, and NSCC-2 and SOCC to the nifedipine-resistant endothelin-1-induced mitogenic re-

sponse is calculated to be about 35%, 30% and 35%, respectively (Fig. 6). Thus, it can be concluded that Ca^{2+} influx through NSCC-1, NSCC-2 and SOCC plays an essential role for endothelin-1-induced mitogenesis in A7r5 cells.

Furthermore, it is now widely recognized that extracellular Ca^{2+} influx through VICC is indispensable for cell growth induced by several agonists (Jung et al., 1992; Chattopadhyay et al., 1999). In other words, there is the possibility that blockade of VICCs such as NSCC and SOCC represents a new method for prevention or treatment of vascular diseases such as atherosclerosis or vascular restenosis after balloon injury. We have recently shown that the endothelin-1-induced mitogenic response in Chinese hamster ovary cells expressing recombinant endothelin type A receptors involves a mitogen-activated protein kinase cascade, the activation of which is dependent on both protein kinase C and phosphatidylinositol 3-kinase (Sugawara et al., 1996). However, it is not known whether the same signaling pathways operate in A7r5 cells. It remains to be determined which signalling pathways are involved in the endothelin-1-induced mitogenic response and which step (s) of the intracellular signaling pathways requires Ca^{2+}.

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