L-type voltage-dependent Ca$^{2+}$ channels in cerebral microvascular endothelial cells and ET-1 biosynthesis

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Yakubu, Momoh A., and Charles W. Leffler. L-type voltage-dependent Ca$^{2+}$ channels in cerebral microvascular endothelial cells and ET-1 biosynthesis. Am J Physiol Cell Physiol 283: C1687–C1695, 2002.—We investigated the role of intracellular calcium concentration ([Ca$^{2+}$]) in endothelial-1 (ET-1) production, the effects of potential vasospastic agents on [Ca$^{2+}$], and the presence of L-type voltage-dependent Ca$^{2+}$ channels in cerebral microvascular endothelial cells. Primary cultures of endothelial cells isolated from piglet cerebral microvessels were used. Confluent cells were exposed to either the thromboxane receptor agonist U-46619 (1 μM), 5-hydroxytryptamine (5-HT; 0.1 mM), or lysophosphatidic acid (LPA; 1 μM) alone or after pretreatment with the Ca$^{2+}$-chelating agent EDTA (100 mM), the L-type Ca$^{2+}$ channel blocker verapamil (10 μM), or the antagonist of receptor-operated Ca$^{2+}$ channel SKF-96365 HCl (10 μM) for 15 min. ET-1 production increased from 1.2 (control) to 8.2 fold in endothelial cells exposed to 5-HT. However, pretreatment with EDTA (100 mM) or nifedipine (4 μM) diminished increases in [Ca$^{2+}$], induced by the vasoactive agents. These results indicate that 1) elevated [Ca$^{2+}$] signals are involved in ET-1 biosynthesis induced by specific spasmogenic agents, 2) the increases in [Ca$^{2+}$] induced by the vasoactive agents tested involve receptor as well as L-type voltage-dependent Ca$^{2+}$ channels, and 3) primary cultures of cerebral microvascular endothelial cells express L-type voltage-dependent Ca$^{2+}$ channels.

CEREBRAL VASOSPASM is the most frequent serious complication (7–10, 14, 17, 22, 40) and cause of cerebral ischemia and death after aneurysm, brain trauma, and subarachnoid hemorrhage (SAH) (8, 10, 14, 17, 22, 40). However, the mechanisms behind the development of cerebral vasospasm are still not fully understood. Pathological studies of the subarachnoid space after SAH have indicated that the most prominent process taking place in the cerebrospinal fluid (CSF) is the hemolysis of blood clots (8, 17, 22). After hemolysis, vasoactive agents accumulate in the CSF, leading to increased levels of 5-hydroxytryptamine (5-HT), lysophosphatidic acid (LPA), thromboxane, oxyhemoglobin, etc. (14, 17, 22, 28, 40). These substances that are released by clotting blood and during hemolysis of blood clots are known to have contractile properties and can stimulate the production of other potent constrictor agents (8, 10, 15, 22, 30, 33–37, 40). Vasoactive agents can interact with cerebral microvessels to produce structural changes along with alteration of cerebral microvascular responses to dilator and constrictor stimuli (9, 10, 15, 23, 24). The changes in cerebral microvascular responses and metabolism induced by breakdown products of blood clots may be mediated via the regulation of second messenger systems which include protein kinase C (PKC), intracellular calcium concentration ([Ca$^{2+}$]), inositol trisphosphate (IP$_3$), and diacylglycerol (DAG).

Intracellular Ca$^{2+}$ signals have been implicated in the regulation of cellular functions such as production and release of many vasoactive factors (e.g., ET-1) under physiological and pathological conditions. Ca$^{2+}$ signals may be triggered via Ca$^{2+}$ channels such as receptor-activated, store-operated, capacitative Ca$^{2+}$ entry or voltage-operated Ca$^{2+}$ channels (20). Of these channels, voltage-operated channels are designated as the most important in the regulation of cellular functions, especially in the synthesis and release of vasoactive factors (4, 20). However, expressions of voltage-operated Ca$^{2+}$ channels in endothelial cells have been a matter of controversy and debate (19), and the role of voltage-operated Ca$^{2+}$ channels and intracellular Ca$^{2+}$ in the biosynthesis and release of ET-1 from cerebral microvascular endothelial cells is not clear.

ET-1 is the most potent naturally occurring vasoconstrictor agent known (13, 28, 33–39) and has been implicated in the consequences of hemorrhage-induced alteration of cerebral microcirculation and the development of cerebral vasospasm (8, 15, 28, 33, 36). Vasoactive factors released from blood clotting and hemo-

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lysate have been reported to stimulate ET-1 production from vascular cells (8, 14, 17, 30, 35), and the mechanism(s) behind ET-1 production is not well understood. After hemorrhage, increases in [Ca$^{2+}$]$_i$ have been observed in major cerebral arteries, along with other vasoactive agents (8, 10, 17, 15, 40). Accumulations of [Ca$^{2+}$]$_i$ in cerebral arteries after hemorrhage may play significant roles in the pathogenesis of hemorrhage-induced alterations of cerebral microcirculation, including ET-1 production and microvascular constriction, but the role of Ca$^{2+}$ in ET-1 biosynthesis is not known. In the present study, we investigated the hypothesis that breakdown products of blood stimulate influx of Ca$^{2+}$ into cerebral microvascular endothelial cells via voltage-dependent Ca$^{2+}$ channels and that this influx plays a role in increasing ET-1 biosynthesis.

METHODS

Primary Culture of Cerebral Microvascular Endothelial Cells

Primary cultures of cerebral microvascular endothelial cells from newborn pig brain cortex were established as previously described (12, 23, 24, 35). Briefly, cerebral cortical microvessels (60–300 μm) were isolated by differential filtration of cerebral cortex homogenate, first through 300-μm and then through 60-μm nylon mesh screens. The isolated microvessels were incubated in collagenase-dispase solution (1 mg/ml) for 2 h at 37°C. At the end of the incubation, the dispersed microvascular endothelial cells were separated by using Percoll density gradient centrifugation. Endothelial cells were resuspended in culture medium consisting of 20% fetal bovine serum (20% FBS), 2 mg/ml sodium bicarbonate, 1 U/ml heparin, 30 mg/ml endothelial cell growth supplement, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2.5 mg/ml amphotericin B. Endothelial cells were plated on 12-well Costar plates coated with Matrigel. Endothelial cell cultures were maintained in a 5% CO$_2$/air incubator at 37°C. The culture medium was changed frequently until the cells attained confluence (after 5–7 days of cultivation). At confluence, the cells were serum deprived by decreasing the medium concentration of fetal calf serum to 0.5% for at least 24 h before they were used for the experiments.

We showed previously that our primary cultures of cerebral microvascular endothelial cells were more than 95% endothelial cells with typical endothelial cell characteristics (12, 23, 24). The endothelial cells we used display density-dependent inhibition of proliferation, form strict monolayers, and require endothelial growth supplement for survival. The cells express factor VIII and retain the elongated phenotype characteristic of microvascular endothelial cells. They are polarized directionally to specific transport. The cells maintain physiological responsiveness to stimuli and increase prostaglandin production in response to hypercapnia and bradykinin (12, 23, 24).

Determination of the Role of Extracellular Ca$^{2+}$ in ET-1 Production by Endothelial Cells

ET-1 production by confluent endothelial cells was measured after 4 h of treatment with medium (control), thomboxane analog, U-46619 (1 μM), 5-HT (0.1 mM), or LPA (1 μM). At the end of the incubation, the medium and cells scraped from the plate were collected and stored at −70°C. To investigate the role of extracellular Ca$^{2+}$ in the regulation of ET-1 production by these potential vasoconstrictor agents, endothelial cells were pretreated for 15 min with either Ca$^{2+}$-chelating agent EDTA (100 μM), the L-type Ca$^{2+}$ channel blocker verapamil (10 μM), or 10 μM SKF-96365 HCl [1-(b-[3-(4-methoxyphenyl) propoxy]-4-methoxyphenethyl]-1H-imidazole HCl], a receptor-operated Ca$^{2+}$ channel antagonist. In the continued presence of EDTA, verapamil, or SKF-96365, the cells were then exposed to U-46619, 5-HT, or LPA for 4 h. At the end of the incubation, medium and cells were collected and stored at −70°C until assayed.

ET-1 Assay

ET-1 was measured in the collected medium by using radioimmunoassay (RIA) kit; Amersham Life Sciences, Arlington Heights, IL, according to the manufacturer’s instructions. The system utilizes a high specific activity of $^{125}$I-labeled ET-1 synthetic tracer, together with a highly specific and sensitive antisera. Separation of the bound antibody from the free fraction was achieved with the addition of an Amerlex-M second antibody preparation to the reaction mixture (unknown, antibody, and $^{125}$I-ET-1) which was incubated for 16–24 h at 2–8°C (overnight delay addition protocol). The mixture was centrifuged at 3,000 rpm at 16°C for 15 min. The supernatant was then removed by vacuum suction, and radioactivity in the pellet was determined in a gamma counter. The concentration of the unlabelled ET-1 in the sample was determined by interpolation from the standard curve (0.25–32 fmol/tube). The sensitivity, as determined by 50% displacement of tracer, was 4.5 fmol.

Measurement of Cytosolic Ca$^{2+}$

Cerebral microvascular endothelial cells were planted on Matrigel-coated plastic Aclar cover slips (Allied Engineered Plastics) and grown to confluence. At confluence, the cells were serum deprived by decreasing the medium concentration of fetal calf serum to 0.5% for at least 24 h. Cells were loaded with 4 mM fura 2-AM for 30 min at 37°C in serum-free medium. At the end of the incubation, the coverslips were inserted diagonally into the quartz cuvettes and perfused with Krebs buffer (in mM): 120 NaCl, 5 KCl, 0.62 MgSO$_4$, 1.8 CaCl$_2$, 10 HEPES, and 6 glucose, pH 7.4, at 37°C. [Ca$^{2+}$]$_i$ was determined by measuring fluorescence of Ca$^{2+}$/fura 2 with a Perkin Elmer luminescence spectrometer (model LS 50B) at excitation wavelengths of 340 and 380 nm, and emission was monitored at 510 nm.

Statistics

All experiments for ET-1 production were conducted in triplicate, and the means were used as data points. There was minimal variation in protein concentration among wells, and the results were corrected for protein concentration and expressed as femtomoles per microgram of protein. Values for ET-1 production are presented as means ± SE. The results were subjected to two-way analysis of variance (ANOVA) for repeated measures with Fisher PLSD to isolate differences between groups. A level of $P < 0.05$ was considered significant. The results presented for the measurement of [Ca$^{2+}$]$_i$ signal are representative of at least three similar results.

Reagents

EDTA, 5-HT, verapamil, nifedipine, and bradykinin were purchased from Sigma Chemical (St. Louis, MO), LPA was purchased from Avanti Polar Lipids (Alabaster, AL), and
SKF-963365 was purchased from Calbiochem (San Diego, CA). ET-1 RIA kits were purchased from Amersham Life Sciences. Cell culture reagents were obtained from Life Technologies (Gaithersburg, MD) and Sigma. Matrigel (growth factor reduced) was purchased from Becton Dickinson (Bedford, MA). Fetal bovine serum was purchased from Hyclone.

RESULTS

Effects of Regulation of Ca2+ on ET-1 Production

Incubation of cerebral microvascular endothelial cells in culture with U-46619 (1 μM), 5-HT (0.1 mM), or LPA (1 μM) for 4 h significantly increased ET-1 production compared with PBS control. The increased production of ET-1 in response to all of these agents was significantly attenuated in the presence of the Ca2+-chelating agent EDTA (0.1 M; Fig. 1). Similarly, 15 min of pretreatment of cells with the L-type Ca2+ channel blocker verapamil (10 μM) attenuated elevations of ET-1 production (Fig. 2). In addition, we investigated the effects of blocking receptor-operated Ca2+ channels on U-46619- (1 μM), 5-HT- (0.1 mM), or LPA-(1 μM) induced elevations of ET-1 biosynthesis. Treatment of confluent endothelial cells with SKF-96365 HCl (10 μM), a receptor-operated Ca2+ channel blocker, prevented the elevations of ET-1 production (Fig. 3).

Regulation of [Ca2+], in Cultured Cerebral Microvascular Endothelial Cells

Effects of U-46619, LPA, and 5-HT on [Ca2+]. Figure 4 shows effects of LPA (1 μM), U-46619 (1 μM), or 5-HT (0.1 mM) on [Ca2+]-signaling responses in cultured cerebral microvascular endothelial cells. Applications of U-46619, LPA, or 5-HT to the bathing solution of the cultured endothelial cells induced elevation of [Ca2+], measured as the fluorescence ratio (F340/380). [Ca2+]i returned to the resting state levels after washout. The results presented are representative tracings of at least three such experiments.

In another experiment, we determined whether U-46619 might activate Ca2+ release from intracellular stores in addition to opening both voltage- and receptor-gated channels. Endothelial cells were perfused with thromboxane analog U-46619 (1 μM) in Krebs buffer (1.8 mM Ca2+). After the responses returned to baseline, the cells were then perfused with U-46619 in Ca2+-free (0 mM Ca2+) Krebs buffer containing (1.0 mM) EGTA [MgCl2 (1.8 mM) was substituted for CaCl2 (1.8 mM)]. Perfusion of endothelial cells with U-46619 (1 μM) in Ca2+-free Krebs buffer increased intracellular Ca2+. However, Ca2+ signal induced by U-46619 in 0 mM Ca2+ was markedly reduced compared with that observed in the presence of Ca2+.
of 1.8 mM Ca$^{2+}$ (Fig. 5). This observation indicates that U-46619 can increase [Ca$^{2+}$], through Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores, as well as through Ca$^{2+}$ channels.

Roles of voltage-dependent channels in vasoactive agent-induced [Ca$^{2+}$], signals. Verapamil, a voltage-dependent Ca$^{2+}$ channel blocker, and SKF-96365, a receptor-operated Ca$^{2+}$ channel blocker, attenuate ET-1 production by endothelial cells (see Figs. 1 and 2). These results suggest that both voltage- and receptor-operated Ca$^{2+}$ channels may be involved in the regulation of [Ca$^{2+}$], and ET-1 production by endothelial cells. We therefore investigated the effects of membrane depolarization with high K$^+$ (60 mM) on increases in [Ca$^{2+}$], induced by vasoactive agents. Effects of depolarization on vasoactive agent-induced increases in [Ca$^{2+}$], were determined in confluent cells after perfusion of cells with high K$^+$. Depolarization of confluent endothelial cells by high K$^+$ diminished increases in [Ca$^{2+}$], by 5-HT (0.1 mM) or bradykinin (1 μM). After perfusion of endothelial cells with high K$^+$, increased basal intracellular [Ca$^{2+}$], was observed (Fig. 6, A and B). The results presented (Fig. 6, A and B) suggest that [Ca$^{2+}$], signals induced by vasoactive agents in cerebral microvascular endothelial cells involve voltage-operated Ca$^{2+}$ channels. However, in the presence of membrane depolarization, Ca$^{2+}$ responses to 5-HT or bradykinin applications were not completely abolished. Persistence of agonist-induced increases in cytosolic Ca$^{2+}$ observed (Fig. 6) in the presence of high K$^+$ may be due to either Ca$^{2+}$ release or Ca$^{2+}$ influx via channels other than voltage-operated channels.

Next, we investigated the effects of blocking L-type voltage-operated Ca$^{2+}$ channels pharmacologically on the [Ca$^{2+}$], responses to these vasoactive agents. Confluent endothelial cells were pretreated with specific L-type voltage-operated Ca$^{2+}$ channel blocker nifedipine (4 μM), and the effects of the pretreatment were
detected on the 5-HT and bradykinin-induced elevation of $[\text{Ca}^{2+}]_i$. (Fig. 7, A and B). Pretreatment of endothelial cells with nifedipine diminished bradykinin and 5-HT-induced elevations of $[\text{Ca}^{2+}]_i$. This result further shows that the mechanism mediating the vasoactive agents’ induced increase in endothelial cell $[\text{Ca}^{2+}]_i$ involves the L-type voltage-operated, as well as receptor-operated, $\text{Ca}^{2+}$ channels. These data also suggest that voltage-operated $\text{Ca}^{2+}$ channels are expressed in primary culture of cerebral microvascular endothelial cells. In the presence of nifedipine, $\text{Ca}^{2+}$ signals in response to bradykinin but not 5-HT application were not completely blocked. This could be due to $\text{Ca}^{2+}$ entry via channels other than voltage-operated and/or effects of nifedipine causing $\text{Ca}^{2+}$ release from an internal store (25). The latter is unlikely because the same was not observed after application of 5-HT. The $\text{Ca}^{2+}$ signals observed may be due to activation of other bradykinin-sensitive (nifedipine-insensitive) ion channels present in endothelial cells (20).

**Effects of repeated applications of vasoactive agents on $[\text{Ca}^{2+}]_i$ signal.** To determine whether increases in $[\text{Ca}^{2+}]_i$ in response to vasoactive agents are reproducible overtime, cells were stimulated twice with bradykinin (1 μM) or 5-HT (0.1 mM). The second application of bradykinin or 5-HT produced $\text{Ca}^{2+}$ signals that were similar to those observed after the first application (Fig. 8, A and B). In another experiment, we determined the effects of exposure of endothelial cells to high K$^+$ on $\text{Ca}^{2+}$-signaling responses to application of vasoactive agents. After the effect of high K$^+$ on the $\text{Ca}^{2+}$-signaling responses to bradykinin or 5-HT were determined, the endothelial cells were perfused with normal Krebs buffer to wash out the high K$^+$. Then effects of the prior exposure to high K$^+$ on $\text{Ca}^{2+}$-signaling responses to repeated applications of bradykinin or 5-HT were determined. The repeated applications of bradykinin or 5-HT resulted in increased $[\text{Ca}^{2+}]_i$-signaling responses which were greater than those observed in the presence of high K$^+$ and comparable to responses elicited under control conditions. These observations showed reversibility of the effects of depolarization on $\text{Ca}^{2+}$-signaling responses (Fig. 8, C and D). In addition, repeated applications of bradykinin did not result in any significant attenuation of responses. These results showed that repeated application of vasoactive agents did not result in significant desensitization of the $\text{Ca}^{2+}$-signaling responses and

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**Fig. 6.** Effects of membrane depolarization by high K$^+$ (60 mM) on the increases in $[\text{Ca}^{2+}]_i$, induced by 5-HT (0.1 mM; A) or bradykinin (1 μM; B). Responses were recorded before and after the perfusion of endothelial cells with high K$^+$. Confluent cell monolayers, serum deprived for 24 h, were preincubated with fura 2-AM for 30 min and superfused with Krebs buffer along with vasoactive agents. Changes in $[\text{Ca}^{2+}]_i$, were recorded as the ratio $(F_{340}/F_{380})$ of fura 2 fluorescence at excitation wavelengths of 340 and 380. The traces are representative of 3 such results.
that the attenuation of responses observed after high 

**DISCUSSION**

The novelty of our present findings are that 1) cerebral microvascular endothelial cells in primary culture express receptor-operated and L-type voltage-dependent Ca\(^{2+}\) channels, 2) breakdown products of blood induce elevation of [Ca\(^{2+}\)]\(_i\) in endothelial cells via activation of both receptor- and voltage-operated Ca\(^{2+}\) channels, and 3) increases in ET-1 production from cerebral microvascular endothelial cells caused by structurally dissimilar vasoactive agents found in blood hemolysates are attenuated by Ca\(^{2+}\)-free medium, L-type voltage-dependent, and receptor-operated Ca\(^{2+}\) channel blockade.

One of the important findings of the present study among others is the expression of L-type voltage-gated Ca\(^{2+}\) channel in cerebral microvascular endothelial cells. The presence of voltage-gated Ca\(^{2+}\) channel in endothelial cell is very important for many reasons and has wide implications. Endothelial cells form a unique signal transduction surface in the vascular system, as well as provide a pathway for delivery of oxygen from blood to tissue and production and release of vasoactive factors, e.g., nitric oxide, prostacyclin, and endothelin. Ca\(^{2+}\) channels have been implicated in the modulation of endothelial function and may be involved in the trafficking of macromolecules by endocytosis, the biosynthetic secretory pathway, and exocytosis. Ion channels are also implicated in controlling endothelial proliferation and angiogenesis. These functions have been shown to be triggered via ion channels which either provide Ca\(^{2+}\) entry pathways or provide the driving force for Ca\(^{2+}\) influx through these pathways. Endothelial cells modulate the tone of vascular smooth muscle cells via release of vasoactive agents, which in turn control blood pressure and blood flow (20). But the presence of voltage-gated ion channels in endothelial cells (a nonexcitable cell) has been difficult to reconcile with the slow and often small changes in membrane potential. However, incidences of low-current voltage-gated ion channels in both freshly isolated and cultured endothelial cells (a nonexcitable cell) has been difficult to reconcile with the slow and often small changes in membrane potential.
Thus the presence of voltage-gated \( \text{Ca}^{2+} \) channels in endothelial cells may be important for endothelial functions and have wide implications because the channels could play a significant role in the physiology and pathology of vascular systems. However, the presence of L-type voltage-gated \( \text{Ca}^{2+} \) channels in endothelial cells has been a source of controversy. Although lack of voltage-gated \( \text{Ca}^{2+} \) channels has been reported in endothelial cells isolated from pig coronary artery (32) or rabbit and rat aorta (19), the presence of voltage-gated \( \text{Ca}^{2+} \) channels has been demonstrated in freshly isolated capillary endothelial cells from bovine adrenal glands (5, 6). The differences reported in the expression of voltage-gated \( \text{Ca}^{2+} \) channels in endothelial cells could be due to the methods used in cell isolation, the origin of cells used (isolation from microvessels or macrovessels), or even the species. The origin of the endothelial cells could significantly influence the types of ion channels expressed by the endothelial cells. Microvessels and macrovessels play different roles in the vascular system and, as such, may vary considerably in their expression of ion channels. The functions of conductance and resistance vessels in the vascular systems are different and could influence the channels that are expressed in the endothelial cells. Hence, the expression of voltage-gated \( \text{Ca}^{2+} \) channels in endothelial cells could be vessel and function specific. The resistance vessels play important roles in the regulation of systemic blood flow and perfusion of end organs. The functional roles of the voltage-dependent \( \text{Ca}^{2+} \) channels involve the production and release of many vasoactive endothelial factors that regulate vascular tone, as well as control of macromolecular trafficking such as endocytosis, exocytosis, biosynthetic secretory pathway, and transcytosis (20). Voltage-gated \( \text{Ca}^{2+} \) channels are responsible for the long-lasting increases in free \( \text{Ca}^{2+} \), during different stimuli and provide the signals for maintaining endothelial functions (2, 4, 20). In the present study, we have shown results from confluent, highly homogenous, and fully differentiated primary culture of cerebral microvascular endothelial cells that are consistent with the presence of L-type voltage-gated \( \text{Ca}^{2+} \) channels. Cells in culture can behave differently from those under in vivo conditions, depending on the condition of culture (3–6, 20, 32), so we used young primary cultures of endothelial cells that we had shown to retain
their endothelial characteristics to minimize such differences (23, 24).

ET-1 is one of the most important vascular endothelial factors produced, and its biosynthesis could be controlled by intracellular Ca\(^{2+}\) signals, especially after cerebral hemorrhage. Intracellular Ca\(^{2+}\) signals result from stimulation of cells by vasoactive agents. Such signals can result from opening of plasma membrane Ca\(^{2+}\) channels or Ca\(^{2+}\) release from intracellular stores or both. Interactions of vasoactive agents with their receptors can directly activate Ca\(^{2+}\) channels, as well as initiate Ca\(^{2+}\) release from intracellular stores. Activation of plasma membrane receptors can lead to the generation of IP\(_3\), a known cytosolic messenger that can directly activate Ca\(^{2+}\) channels, as well as initiate Ca\(^{2+}\) release from intracellular stores. The role of elevated calcium ions in the stimulation of ET-1 production from cerebral microvascular endothelial cells is not yet clear. We have shown that regulation of [Ca\(^{2+}\)], affects ET-1 biosynthesis from endothelial cells by vasoactive agents and that these vasoactive agents are capable of elevating [Ca\(^{2+}\)], via both receptor- and voltage-operated Ca\(^{2+}\) channels, but the mechanism by which such elevation in [Ca\(^{2+}\)], regulates ET-1 synthesis is still not known. ET-1 is known to be synthesized de novo, with production being regulated at the mRNA transcriptional and translation level (28, 38, 39).

Endothelin gene transcriptions are modulated by various factors, such as thrombin, angiotensin II, phorbol ester, shear stress, and calcium ionophore (13, 21, 28, 38, 39). It is probable that elevated intracellular Ca\(^{2+}\) induced by blood clot-derived agents can modulate ET-1 gene transcription at the mRNA level. ET-1 is produced from a 203 amino acid protein precursor, preproendothelin-1, which is converted to big ET-1, a 39 amino acid precursor peptide, by endopeptidase. The big ET-1 is converted to the mature ET-1 by a novel protease, endothelin-converting enzyme (ECE). Processing of big ET-1 to ET-1 is essential for full expression of biological activity (13, 21, 28, 38, 39). Therefore, the step involving ECE is a rate-limiting step for ET-1 production and could be a target for regulation by the blood clot-derived vasoactive agents via elevated cytosolic Ca\(^{2+}\). Whether or not ECE is a Ca\(^{2+}\)-dependent enzyme is uncertain. Elevated Ca\(^{2+}\) has been shown to activate specific transcription factors (4, 29), but whether there is a particular transcription factor for ET-1 production susceptible to activation by elevated intracellular Ca\(^{2+}\) is not known. Certainly, our results are consistent with studies that suggest that Ca\(^{2+}\) entry is required for ET-1 release from human coronary endothelial cultures (10).

The route of Ca\(^{2+}\) entry into the cell can determine the particular transcription factors that are activated (4, 29). Bading et al. (1) have demonstrated that Ca\(^{2+}\) entry through the voltage-dependent L-type Ca\(^{2+}\) channels and N-methyl-d-aspartate (NMDA) receptors initiate gene transcription through distinct DNA regulatory elements. This might be the case in the present study in which we have shown that elevated Ca\(^{2+}\) observed in cerebral microvascular endothelial cells is mediated via receptor- as well as voltage-dependent Ca\(^{2+}\) channels. Hence, the increase in ET-1 biosynthesis induced by the clot-derived vasoactive agents via elevated intracellular Ca\(^{2+}\) may be mediated through activation of a specific transcription factor for ET-1 biosynthesis (possibly through activation of the gene(s) for the production of the numerous enzymes involved in ET-1 synthesis).

In the present study, we used the specific L-type voltage-gated Ca\(^{2+}\) channel blocker nifedipine, and, consistent with the presence of the L-type Ca\(^{2+}\) channel, nifedipine diminished the vasoactive agent-induced Ca\(^{2+}\) signal in endothelial cells. However, others have reported that nifedipine can increase [Ca\(^{2+}\)], by activation of Ca\(^{2+}\) release from intracellular store in smooth muscle cells and extracellular Ca\(^{2+}\) influx in coronary endothelial cells (3, 25). The intracellular effects of nifedipine reported may be due to high concentration (10 μM) or to the cell type used in the study. Although Raicu and Florea (25) used smooth muscle cells and 10 μM nifedipine, Berkels et al. (3) used coronary endothelial cells. Hence, such effects of nifedipine could be cell selective and may induce effects other than the selective blockade of the L-type Ca\(^{2+}\) channel. In the present study, we did not observe any increase in [Ca\(^{2+}\)]i upon nifedipine treatment.

In conclusion, elevated intracellular Ca\(^{2+}\) plays a significant role in the increased production of ET-1 caused by specific spasmogenic agents. Ca\(^{2+}\) signaling and ET-1 production could be involved in hemorrhage-induced alteration of cerebral microvascular reactivities and development of vasospasm. The presence of voltage-gated calcium channels in endothelial cells has wide implications. Pharmacological manipulation of the L-type voltage-gated calcium channel in many cases is readily accomplished and is also of therapeutic significance. L-type channels are very accessible to pharmacological modification and could be manipulated to influence release of mediators of endothelium-dependent relaxing and contracting factors.

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