

Endothelin-induced Increases in Vascular Smooth Muscle Ca^{2+} Do Not Depend on Dihydropyridine-sensitive Ca^{2+} Channels

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Abstract

Endothelin is a potent mammalian vasoconstrictive peptide with structural homology to cation channel-binding insect toxins. We tested the proposal that this peptide directly activates dihydropyridine-sensitive Ca^{2+} channels in cultured vascular smooth muscle (VSM) cells. First, we found that cell Ca^{2+} can be altered in VSM by activation of voltage-operated Ca^{2+} channels. KCl-induced depolarization and the dihydropyridine Ca^{2+} channel agonist (–) Bay K 8644 (10 μM) both raised cell Ca^{2+} more than twofold; the effect of KCl was blocked by the inhibitory enantiomer, (+) Bay K 8644 (40 μM). Similar responses were observed in Chinese hamster ovary (CHO) cells. Synthetic endothelin (4 $\times 10^{-8}$ M) raised Ca^{2+} in VSM but not CHO cells from 100 ± 17 to 561 ± 34 nM within 12 s. Ca^{2+} subsequently fell to basal levels after 30 min. Half maximal Ca^{2+} response was at 4×10^{-9} M endothelin. Unlike endothelin, thrombin raised Ca^{2+} in both VSM and CHO cells. The Ca^{2+} responses to endothelin and thrombin were not affected by nicardipine (1 μM), (+) Bay K 8644, or Ca^{2+} -free solutions. Lastly, both hormones caused release of inositol phosphates in VSM cells. However, the response to thrombin was more than 10-fold larger and was more rapid than the response to endothelin; the thrombin response was sensitive to pertussis toxin, while the response to endothelin was not. Thus endothelin, like thrombin, raises cell Ca^{2+} in VSM by mobilization of intracellular stores and not by activation of dihydropyridine-sensitive Ca^{2+} channels. However, their receptors are distinct and they exhibit important differences in signal transduction.

Introduction

Endothelin (1) is a mammalian hormone recently added to a growing list of vasoactive or growth-promoting substances that are produced by vascular endothelial cells and presumably act on the subjacent vascular smooth muscle (VSM)¹ cells to alter

vascular tone and/or cell proliferation (for reviews, see references 2–4). Endothelin is a potent vasoconstrictor both in vitro and in vivo, capable of producing prolonged hypertension in the rat (1). More recently, endothelin has been shown to induce the release of atrial natriuretic factor from cultured rat atrial myocytes (5).

Among the known hormones produced by mammalian organisms, the structure of endothelin is unique: a 21 amino acid peptide with two disulfide bridges. Endothelin is almost identical to the asp venom, sarafotoxin 6B (6), and exhibits striking homology to α -scorpion toxin, one of a family of toxins known to bind and activate the voltage-operated Na^+ channel (7). The Na^+ channel has several features in common with voltage-operated Ca^{2+} channels (8).

Dihydropyridine-sensitive, voltage-operated Ca^{2+} channels are found in excitable cells (8) and are believed to be responsible for the inward Ca^{2+} current that follows membrane depolarization. This Ca^{2+} current could activate the contractile machinery via an increase in cell Ca^{2+} . VSM contains this type of channel, but its role in contractile function is incompletely understood. Many agents cause contraction without altering the membrane potential (9, 10), and Ca^{2+} channel blockade blunts the contractile response to agents that do not cause depolarization (11). Some of these phenomena could be explained if there were biological agents that opened Ca^{2+} channels without membrane depolarization. Bay K 8644 is such an agent (12), but it is a synthetic substance not found in biological systems.

Several lines of evidence suggest, and indeed Yanagisawa et al. proposed (1), that endothelin exerts its effects by activating voltage-operated Ca^{2+} channels in VSM. First, both contraction of vascular strips (1) and release of atrial natriuretic peptide (5) were blocked by nicardipine, a Ca^{2+} channel antagonist. Second, the contractile activity was also blocked by removal of extracellular Ca^{2+} (1). Lastly, endothelin has been shown to elevate Ca^{2+} in cultured aortic smooth muscle cells and this action is partially blocked by nicardipine (13). We therefore tested the proposal that endothelin acts on voltage-operated Ca^{2+} channels. We find in cultured VSM muscle cells that the initial increase in cellular Ca^{2+} induced by endothelin is not mediated by activation of Ca^{2+} channels, but by mobilization of intracellular Ca^{2+} (Ca^{2+}_i) stores.

Methods

Materials. [³H]Myoinositol was purchased from New England Nuclear (Boston, MA), fura-2 and 4-bromo A23187 from Molecular Probes Inc. (Junction City, OR), Dowex AG1X8 resin from Bio-Rad Laboratories (Richmond, CA), pertussis toxin from List Biochemicals (Belmont, CA), synthetic endothelin from Peptide Institute (Osaka, Japan), and [³H]myoinositol from New England Nuclear. Highly purified human thrombin was generously supplied by John W. Fenton II

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1. *Abbreviations used in this paper:* Ca^{2+}_i , free intracellular Ca^{2+} ; CHO, Chinese hamster ovary; IP, inositol monophosphate; IP₂, inositol bisphosphate; IP₃, inositol trisphosphate; VSM, vascular smooth muscle.

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(Albany, NY). (+) and (-) Bay K 8644 was the kind gift of Sandoz Ltd. (Basel, Switzerland). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture. Neonatal rat VSM cells (strain R22, clone D) were obtained from Peter Jones (University of Southern California) (14) and used for the present studies at passages 17–29. The cells were maintained in Eagle's MEM with 10% fetal bovine serum, 2% tryptose phosphate broth, penicillin (50 U/ml), and streptomycin (50 U/ml) in a humidified atmosphere of 5% CO₂, 95% air at 37°C. Cells were harvested with 3 ml trypsin-versene and 3 ml 0.2% pancreatin and subcultured at a 1:10 dilution weekly. Culture medium was changed every other day until the cells were confluent. For biochemical studies, confluent monolayers of cells were grown in 35-mm, 6-well plates (2 ml medium). For Ca²⁺_i measurements cells were grown in Leighton tubes (Costar, Cambridge, MA).

Measurement of Ca²⁺_i. Cells growing on the 1 × 3-cm plastic strips from the Leighton tubes were removed and mounted at a 60° angle in 1 × 1-cm acrylic fluorescence cuvettes (Sarstedt, Inc., Princeton, NJ) with a magnetic stirrer below the cell strip. The cell strips were washed three times with 2 ml of assay medium consisting of 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM Na₂HPO₄, 25 mM glucose, 25 mM Hepes/NaOH, and 0.5 mg/ml BSA. Cells were then incubated with 2 μM fura-2 for 40 min at 37°C and washed two times with assay medium to remove extracellular fura-2. Fluorescence (excitation wavelength = 340 or 380 nm, emission wavelength = 510 nm) was measured with a spectrofluorometer (model 8000; SLM Instruments, Inc., Urbana, IL). Excitation light was alternated between 340 and 380 nm (6-s dwell at each wavelength) with a computer controlled excitation monochromator. Background light scattering and autofluorescence were < 10% of the fluorescence signal and were subtracted during data analysis. At the end of each experiment, intracellular fura-2 was calibrated by measuring fluorescence after addition of 20 μM 4-bromo A23187; it was then measured again after addition of 16 mM EGTA. [Ca²⁺]_i was calculated using the previously described formulae (15).

Measurement of inositol polyphosphate production. VSM cells were grown to confluence in 35-mm dishes with 2 ml of medium. To ensure a maximal response to ligands, studies were performed 3–4 d after the last change of medium. Phosphoinositide hydrolysis was monitored by measuring [³H]inositol phosphate accumulation with a modification (16) of the procedure originally described by Berridge et al. (17). Briefly, the cells were labeled by incubation with [³H]myo-inositol (5 μCi/dish) in culture medium for 24 h. When used, pertussis toxin (100 ng/ml) was added to the medium 4 h before the end of incubation. The radioactive medium was aspirated and the cells were washed three times with Dulbecco PBS, 0.2% BSA (assay medium). Cells were incubated with this assay medium for 1 h at 37°C to remove unincorporated radioactivity. After an additional wash with assay medium, cells were preincubated with 1 ml assay medium containing 10 mM LiCl for 15 min at 37°C. Ligands were then added and incubated for the indicated times. Incubations were terminated with aspiration of medium and addition of 1 ml ice-cold 15% TCA. Cells were extracted with TCA at 4°C for 30 min. The extract was then transferred to glass tubes and the TCA was removed by five washes with 5 vol of water-saturated diethyl ether. The samples were then neutralized and separated by anion-exchange chromatography with Dowex AG1X8, formate form (Bio-Rad Laboratories). The columns were prepared in Pasteur capillary pipettes packed with 5 cm of resin. Free inositol, glycerophosphoinositol, and inositol mono-, bis-, and triphosphates (IP₁, IP₂, and IP₃) were successively eluted with (a) 8 ml of water, (b) 60 mM ammonium formate/5 mM sodium tetraborate, (c) 200 mM ammonium formate/100 mM formic acid, (d) 450 mM ammonium formate/100 mM formic acid, and (e) 1 M ammonium formate/100 mM formic acid. Radioactivity was counted in a scintillation counter. IP₃, as defined by this assay, actually consists of inositols 1,3,4-Tris, 1,4,5-Tris, and 1,3,4,5-tetrakisphosphate (18).

Data presentation and statistics. Results are presented as mean ± SE for experiments with three or more observations. All comparisons were based on statistical analysis of experiments performed on the same day

using the same batch of cells. Comparisons were made using the unpaired *t* test.

Results

To demonstrate that our preparation of cultured neonatal rat VSM cells exhibits Ca²⁺ channel activity, we determined the Ca²⁺_i response to membrane depolarization and to the two enantiomeric forms of Bay K 8644, (+) and (-) Bay K 8644 (12).² When cells were rapidly depolarized with medium containing 140 mM KCl, Ca²⁺_i immediately rose from 100 ± 23 to 197 ± 31 nM (Fig. 1 A). This increase was blocked by the inhibitory enantiomer of Bay K 8644, (+) Bay K 8644 (40 μM), by nifedipine (1 μM), and by a Ca²⁺-free medium (not shown). In normal assay solution (Ca²⁺ = 2 mM, K⁺ = 4 mM), the Ca²⁺ channel agonist, (-) Bay K 8644 (10 μM), also raised Ca²⁺_i from 100 ± 23 to 220 ± 11 nM (Fig. 1 B). Subsequent administration of (+) Bay K 8644 caused a rapid fall in Ca²⁺_i to 165 ± 14 nM. Furthermore, the response to (-) Bay K 8644 was virtually obliterated by prior administration of (+) Bay K 8644 (Fig. 1 B). In separate experiments (not shown), similar findings were made with Chinese hamster ovary (CHO) cells. These results provide strong evidence for the presence of dihydropyridine-sensitive Ca²⁺ channels in both cultured cell systems.

In Ca²⁺-containing solutions, synthetic endothelin (4 × 10⁻⁸ M) raised VSM cell Ca²⁺ from 100 ± 17 to 561 ± 34 nM within 12 s (Fig. 2). Ca²⁺_i then fell to basal levels over the following 30 min. The minimum dose of endothelin that gave a reproducibly detectable Ca²⁺ response was 1 nM (Fig. 3 A); the half maximal response was 4 nM (see below). The response of VSM cell Ca²⁺ to maximal doses of thrombin (0.4 U/ml) was almost identical to that induced by 4 × 10⁻⁸ M endothelin (Fig. 2). CHO cells exhibited a similar response to thrombin but, unlike VSM cells, exhibited no response to endothelin (Fig. 2). Thus, CHO cells which exhibit dihydropyridine-sensitive Ca²⁺ channel activity do not respond to endothelin. Furthermore, neither (+) Bay K 8644 (40 μM, Fig. 3 A) nor nifedipine (1 μM, not shown) altered the peak Ca²⁺ response to endothelin or the time course for recovery of Ca²⁺_i (Fig. 3 A).

In Ca²⁺-free solutions the peak Ca²⁺ response to both low (10⁻⁹ M) and high (10⁻⁷ M) concentrations of endothelin was unaltered (Fig. 3 B). However, the time course for the Ca²⁺ elevation was significantly shortened at both concentrations, with Ca²⁺ returning to basal levels in 5 min, compared with 30 min in Ca²⁺-containing solutions. Similar findings were made for thrombin (not shown). Thus, the initial Ca²⁺ spike response to both endothelin and thrombin appears to derive from Ca²⁺_i stores and not from activation of Ca²⁺ channels. This is true over the full effective dose range of endothelin. Furthermore, the maintenance of increased Ca²⁺_i observed after prolonged incubation with endothelin in Ca²⁺-containing solutions is not altered by Ca²⁺ channel blockade and is therefore also unlikely to derive from channel activity.

In many systems, mobilization of Ca²⁺ from intracellular stores is correlated with, and presumably caused by, the release of inositol 1,4,5-trisphosphate from phosphatidyl IP₂. We

2. While racemic Bay K 8644 is a dihydropyridine Ca²⁺ channel agonist, its optical isomers exert opposite effects on the Ca²⁺ channel, the (-) form being an agonist and the (+) form being an antagonist (12).

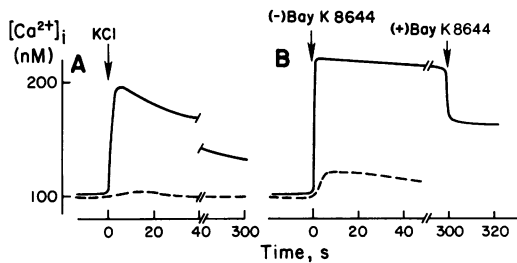


Figure 1. Evidence for voltage-operated Ca^{2+} channels in cultured VSM cells. Ca^{2+}_i was measured in confluent monolayers of VSM cells bathed in a physiologic solution (solution A, see Methods). In *A*, the bathing solution was rapidly changed to a similar solution containing 140 mM K^+ , 4 mM Na^+ either in the absence (solid line) or presence (dashed line) of (-) Bay K 8644 (40 μM). In *B*, (-) Bay K 8644 (10 μM) was added to cells bathed in regular assay solution (solid line). 5 min later (+) Bay K 8644 (40 μM) was added. In a separate experiment (dashed line) (+) Bay K 8644 (40 μM) was added 5 min before (-) Bay K 8644. Data shown are representative of four similar experiments.

therefore compared the Ca^{2+} response with the release of IP_3 (see Methods) in VSM cells at various doses of thrombin and endothelin. Thrombin increased Ca^{2+} over the range of 0.04 to 0.4 U/ml, while endothelin raised Ca^{2+} over the range of 4×10^{-10} to 10^{-8} M (Fig. 4). Both agents induced the release of IP_3 , but for both there was a dissociation between doses causing a Ca^{2+} response and doses causing release of IP_3 . However, all doses of thrombin that produced a measurable increase in Ca^{2+} also produced a measurable increase in IP_3 (for example, 0.04 U/ml of thrombin raised Ca^{2+} from 114 to 166 nM and IP_3 to 150% of basal). On the other hand, even 10^{-8} M endothelin, a dose which caused a nearly maximal increase in Ca^{2+} , produced no measurable increase of IP_3 . 10^{-8} - 10^{-7} M endothelin did produce statistically significant increases in IP_3 , but they were far lower than produced by comparable doses of thrombin. In a separate experiment, 10^{-6} M endothelin, two orders of magnitude higher than the dose producing a maximal Ca^{2+} increase, raised IP_3 to only $160 \pm 10\%$ of the basal level.

Because of the difference in levels of IP_3 produced by thrombin and endothelin, we examined the production of IP_3 , IP_2 , and IP (see Methods) induced by endothelin (4×10^{-8} M) and thrombin (0.4 U/ml) over a time course of 60 s. For thrombin, the peak level of IP_3 occurred either simultaneously with or before the peak Ca^{2+} (15 s). IP_2 and IP appeared later,

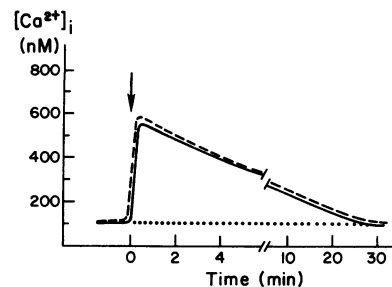


Figure 2. Ca^{2+} response to endothelin and thrombin. Ca^{2+}_i was measured in cultured VSM cells bathed in assay solution A (see Fig. 1). Endothelin (4×10^{-8} M) was added to VSM (dashed line) or CHO cells (dotted line) at the arrow. Thrombin (0.4 U/ml) gave identical responses in both cell types (solid line). Data shown are representative of four similar experiments.

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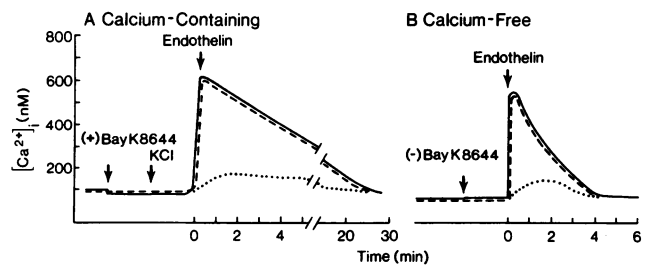


Figure 3. Effect of Ca^{2+} removal and of Ca^{2+} channel antagonists on the response to endothelin. Ca^{2+}_i was measured in VSM cells as in Fig. 1. In *A*, the bathing solution contained 2 mM Ca^{2+} . The calcium channel antagonist, (+) Bay K 8644, blocked the response to 140 mM KCl replacement (solid line) as shown in Fig. 1 *A*. Under this condition of Ca^{2+} channel blockade, the response to endothelin (10^{-7} M, solid line), was identical to the response without channel blockers (dashed line). 10^{-9} M endothelin gave a small but reproducible response which was also not affected by (+) Bay K 8644 (two identical tracings shown as one dotted line). In *B*, the bathing solution contained 16 mM EGTA and no added extracellular Ca^{2+} . Under these conditions, (-) Bay K 8644 (10 μM) failed to raise Ca^{2+} (solid line). Endothelin (10^{-7} M), when added alone (dashed line) or after (-) Bay K 8644 (solid line) caused a peak Ca^{2+} increase of similar magnitude to that observed with Ca^{2+} -containing solutions (Figs. 2 and 3 *A*). The response to endothelin was, however, of shorter duration in Ca^{2+} -free solutions. The peak response to lower doses of endothelin (1×10^{-9} M) was also not affected by extracellular EGTA (compare dotted lines in *A* and *B*). Data shown are representative of three similar experiments.

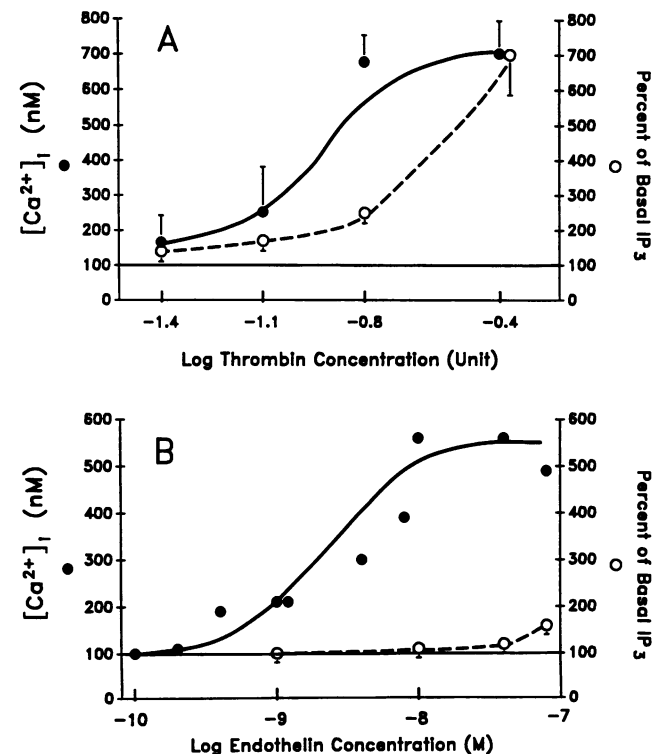


Figure 4. Comparison of dose responses for thrombin- and endothelin-induced increases in Ca^{2+} and IP_3 . Cells were incubated with thrombin (*A*) or endothelin (*B*) at the indicated concentrations. In separate experiments, Ca^{2+} (closed circles, solid lines) or IP_3 (open circles, dashed lines) were measured as described in Methods. Data shown are peak values achieved for Ca^{2+} or IP_3 . (See Figs. 2 and 5 for detailed time courses for Ca^{2+} and IP_3 , respectively.)

as was predicted. In contrast, the peak IP₃ induced by endothelin occurred after the peak Ca²⁺ (Ca²⁺, 12 s; IP₃, 30 s). Thus, inositol phosphate generation by endothelin appears to be temporally dissociated from the Ca²⁺ response.

Many agonists that activate phospholipase C to release soluble inositol phosphates do so via the intermediate action of a pertussis toxin-sensitive GTP binding protein, G_p (19, 20). As we previously found for thrombin (16), inositol phosphate release was blocked by 4 h exposure to pertussis toxin (Fig. 5). However, endothelin-induced release of inositol phosphate was not significantly altered by pertussis toxin treatment. Thus, the mechanism by which the endothelin receptor induces inositol phosphate release, and perhaps mobilization of Ca²⁺, seems to be different from the mechanism used by the thrombin receptor.

Discussion

Considerable attention has recently been focused on the role of the endothelium in the control of vascular function. The endothelium appears to participate in the local control of vascular tone and in the response to vascular injury, and it may play a role in the pathogenesis of vascular diseases (2). The endothelium exerts its effects on VSM by producing vasoactive agents and growth factors (2). Endothelin is the most recent of these substances to be described (1).

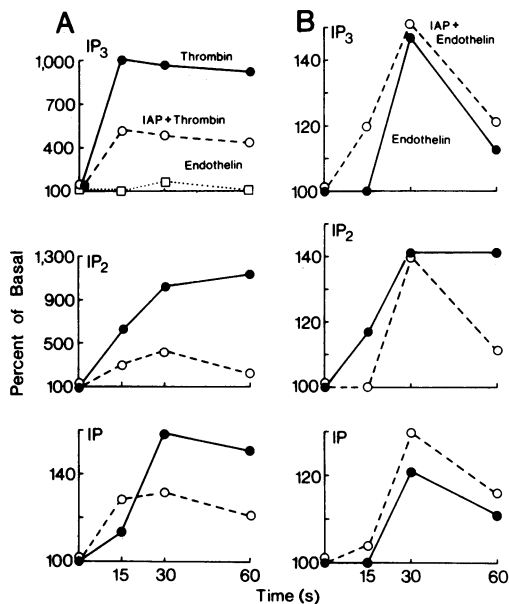


Figure 5. Inositol phosphate release in response to thrombin and endothelin. In *A*, [³H]myoinositol labeled cells were incubated for the indicated times with thrombin (0.4 U/ml) in the absence (*solid lines*), or the presence (*dashed lines*) of pertussis toxin (100 ng/ml, 4 h incubation). Endothelin (4×10^{-8} M, *dotted line*) caused considerably less IP₃ production than thrombin. Doses of endothelin as high as 10^{-7} M yielded no higher levels of IP₃ and 10^{-9} M endothelin yielded no measurable inositol phosphate production (not shown). In *B*, labeled cells were incubated with endothelin (4×10^{-8} M) either in the absence (*solid lines*) or presence (*dashed lines*) of pertussis toxin (*IAP*; 100 ng/ml, 4 h incubation). Note difference in scale between *A* and *B*. Basal inositol phosphate release was: IP₃, 168 ± 22 cpm/dish; IP₂, 99 ± 18 cpm/dish; IP, 820 ± 18 cpm/dish. Data shown are representative of three similar experiments.

Endothelin is a small peptide with two disulfide bridges (1). This structure is analogous to scorpion toxins which act directly on ion channels. In light of the unique structure of endothelin, we were initially intrigued by the possibility that it might be a naturally occurring agonist of the voltage-operated Ca²⁺ channel in VSM.

The findings of this paper indicate that the initial increase in Ca²⁺ induced by endothelin is not due to activation of dihydropyridine-sensitive Ca²⁺ channels. Unlike (–) BAY K 8644, endothelin was able to elevate cell Ca²⁺ normally in the absence of extracellular Ca²⁺, and endothelin's action was not blocked by demonstrably effective Ca²⁺ channel antagonists. Furthermore, endothelin failed to exhibit any effect on cell Ca²⁺ in a second cell line (CHO) that was shown to contain Ca²⁺ channels. These data argue against a direct action of endothelin on the Ca²⁺ channel. In this way endothelin is different from its structural homologue, scorpion toxin, which binds to and directly activates voltage-operated Na⁺ channels (7). How then do we explain the blocking effects of nifedipine and of Ca²⁺-free media on endothelin's physiological effects? It is possible that endothelin's actions may lead to secondary opening of Ca²⁺ channels after the initial Ca²⁺ peak. However, such channels would have to be dihydropyridine insensitive, since dihydropyridine blockers did not alter the time course of the Ca²⁺_i response to endothelin. Alternatively, VSM cells in situ may have properties different from those of the cells studied here.

As an initial event, endothelin appears to mobilize Ca²⁺_i stores like another well-studied agonist, thrombin. Like thrombin, endothelin induces the release of soluble inositol phosphates. However, the details of the endothelin response differed significantly from those observed with thrombin. First, the magnitude of the inositol phosphate release by a maximal dose of endothelin (10^{-6} M) was dramatically smaller than that released by thrombin. 10^{-8} M endothelin, sufficient to raise intracellular Ca²⁺ to its maximal level in our system, failed to induce any detectable increase in inositol phosphates. Second, even at high concentrations of endothelin (10^{-7} – 10^{-6} M), the peak concentration of IP₃ occurred after 30-s exposure to hormone, well after the peak level of Ca²⁺ had occurred (12 s). By contrast, with thrombin both events occurred at approximately the same time (10–15 s). Lastly, unlike thrombin, endothelin-induced inositol phosphate release was not sensitive to pertussis toxin. This suggests a significant difference in signal transduction for the two hormones. Since the thrombin response was only partially sensitive to pertussis toxin, it is also conceivable that thrombin uses two separate signal transduction systems.

Dissociation between the Ca²⁺ and inositol phosphate responses to various agents have been observed in other systems (21), but the difference between the level and time course of inositol phosphate generation by thrombin and endothelin was particularly striking, especially in view of the similarity of their Ca²⁺ responses. Because the assay system we used does not separate inositols 1,4,5-tris, 1,3,4-tris, and 1,3,4,5-tetrakisphosphate, it is conceivable that one of these, or another isomer might correlate more closely with the Ca²⁺ response. If so, the relevant isomer would have to be a minor species, because total inositol phosphates (IP₃ + IP₂ + IP in our assay system) were significantly lower with maximal doses of endothelin than with maximal doses of thrombin.

It should also be noted that increased Ca^{2+} can activate phospholipase C and thereby increase inositol phosphate levels (22, 23). In HL-60 cells (a human promyelocytic cell line), the Ca^{2+} ionophore, ionomycin, gave rise to increases in IP_3 similar in magnitude (23) to those we observed with endothelin in VSM. Thus, the possibility should be considered that endothelin-induced increases in inositol phosphates are the consequence rather than the cause of increased Ca^{2+} by endothelin. In any event, the relationship between release of IP_3 and intracellular stores of Ca^{2+} does not appear to be simple. Because of the striking dissociation between IP_3 production and Ca^{2+} mobilization by endothelin, this agent may be useful in further studies on the mechanisms of Ca^{2+} mobilization by peptide hormones.

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