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ENDOTHELIN ACTION ON VASCULAR SMOOTH MUSCLE INVOLVES INOSITOL TRISPHOSPHATE AND CALCIUM MOBILIZATION

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SUMMARY Cultured endothelial cells release a potent vasoconstrictor peptide, endothelin. Cumulative addition of synthetic endothelin to isolated rabbit aortic rings elicited a concentration-dependent increase in contractile tension which was endothelium-independent. In cultured rabbit vascular smooth muscle cells loaded with the fluorescent dye fura 2, endothelin induced a concentration-dependent increase in $[Ca^{2+}]_i$ over the range of 0.01 to 100 nM. Moreover, in the absence of extracellular Ca^{2+}, endothelin could still induce an increase in $[Ca^{2+}]_i$. In addition, endothelin stimulated $^{45}Ca^{2+}$ efflux from preloaded vascular smooth muscle cells in the presence and absence of extracellular Ca^{2+}, as well as stimulating $^{45}Ca^{2+}$ influx in a concentration-dependent manner. Measurement of inositol phosphates in [^{3}H]-myoinositol-labelled vascular smooth muscle cells showed that endothelin induced rapid (15 sec), transient formation of inositol trisphosphate. Unlabelled endothelin inhibited [^{125}I]-endothelin binding to cultured rabbit vascular smooth muscle cells in a concentration-dependent manner. Binding was not inhibited by other vasoactive hormones or calcium channel ligands, suggesting cell surface receptors specific for endothelin.

We conclude that one of the initial membrane events in the action of endothelin is to induce phospholipase C-stimulated PIP_2 hydrolysis and that this signalling mechanism is initiated by endothelin/receptor interaction at the plasma membrane.

It is now appreciated that endothelial cells ***in situ*** and ***in vitro*** are capable of releasing vasoactive substances which mediate vasodilatation (1-4), as well as vasoconstriction (5-7). Recently, a novel peptide, termed endothelin, was isolated from conditioned medium of cultured endothelial cells and shown to be an extremely potent agonist for vascular smooth muscle contraction (8). Endothelin is a 21-amino acid peptide with two internal disulfide bridges and is derived from preproendothelin whose mRNA has been detected in porcine aortic and human umbilical vein endothelial cells. Analysis of the human and porcine preproendothelin gene reveals approximately 80% sequence homology in the coding regions and identity of the sequence that codes for mature endothelin (9). Cloning and partial sequencing of preproendothelin from a rat genomic library reveals a 21-amino acid peptide with 15 identical amino acids and 3 conservative residue substitutions compared with mature porcine endothelin (10).

Endothelin shares significant regional homologies with the amino-terminus of alpha-scorpion toxins, peptides which modulate the activation and inactivation of tetrodotoxin-sensitive sodium channels (11). It has been postulated that endothelin may act directly on voltage-dependent

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calcium channels. Here we report that synthetic endothelin induces a dose-dependent increase in cytosolic free calcium ($[Ca^{2+}]_i$) in cultured vascular smooth muscle cells (VSMC) isolated from rabbit aorta. We demonstrate that early signaling events in endothelin action on VSMC involve inositol trisphosphate (IP_3) formation and intracellular calcium mobilization. [^{125}I]-Endothelin binding to vascular smooth muscle demonstrates specificity in that binding is not inhibited by other vasoactive hormones or calcium channel ligands, suggesting cell surface receptors specific for endothelin.

MATERIALS AND METHODS

Aortic Ring Contractile Responses. Contractile responses of rabbit aortic rings were performed as previously described (12) . Additions were made directly to an isolated organ bath perfused with a Krebs-Ringer solution containing (in mM) NaCl 118, KCl 4.5, $NaHCO_3$ 25, NaH_2PO_4 1.0, d-glucose 10, $CaCl_2$ 2.5, $MgCl_2$ 1.0 at pH 7.4 gassed with 95% O_2/5% CO_2 (Buffer A). Endothelial tissue was removed with gentle rubbing. The absence of functional endothelium was assessed by demonstrating the absence of acetylcholine (2 μM)-induced relaxation .

Cell Culture. Rabbit aortic smooth muscle cells were cultured from aortic medial tissue explants and passaged as previously reported (13). Experiments presented utilized cells from subcultures 6 through 12 and used 7 to 14 days after the time of passage.

Fura 2 Microfluorimetry. For microfluorimetry, cultured VSMC grown on glass coverslips (Bellco, Vineland, NJ, #1 thickness) were incubated with 2 μM Fura 2 acetoxymethylester (Molecular Probes, Eugene, OR) for 30 minutes at 37°C, washed and placed in a temperature-controlled perfusion chamber at 37°C mounted on the stage of a Nikon Diaphot microscope (Garden City, NY). A modified Hank's balanced salt solution, containing 1.5 mM $CaCl_2$, 10 mM d-glucose, 10 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.4) and 0.2 mg/ml bovine serum albumin (fraction V RIA grade)(BSA), was used as the perfusate (Buffer B). Cells were perfused at 2.0 ml/min. Fluorescence measurements were made using a Spex (Edison, NJ) Fluorolog II spectrofluorimeter (model CM-1) equipped with a beam splitter and two excitation monochromators allowing alternate excitation of Fura 2 at 340 and 380 nm. A 40x Fluor Nikon objective connected in series with a narrow band pass filter (500 ± 10 nm) and photomultiplier tube was used to collect emission signals from fields containing 5 to 10 cells. Individual coverslips were calibrated with ionomycin (5 μM)/1.5 mM $CaCl_2$ and EGTA (2 mM)/no added Ca^{2+} to obtain maximum and minimum emission signals respectively. Autofluorescence was determined by quenching Fura 2 fluorescence with 2 mM $MnCl_2$ and subtracting 340 and 380 nm values prior to calculating ratios.

Calcium Transport. $^{45}Ca^{2+}$ efflux studies were performed as described with minor modifications (13). Prior to assay, cells were equilibrated for 18 to 24 hours with 1 ml of fresh culture medium containing 2 μCi $^{45}Ca^{2+}$. Replicate plated 35-mm dishes of cultured rabbit VSMC contained 3 to 5 x 10^6 cells/dish; Coulter counter (Coulter Electronics, Hialeah, FA). $^{45}Ca^{2+}$ influx in response to endothelin was also determined as described with minor modifications (14). Buffer B, containing 1.5 mM $CaCl_2$, was supplemented with 2 μCi $^{45}Ca^{2+}$/ml. High K^+ (100 mM) solution was prepared by replacing NaCl with KCl isosmotically. At five minutes $^{45}Ca^{2+}$ influx was terminated by washing the dishes four times with ice-cold calcium- and phosphate-free buffer B containing 10 mM $LaCl_3$.

Inositol Phosphate Determination. Replicate plated 35-mm dishes of cultured rabbit VSMC were prelabelled with [^{3}H]-myoinositol (25 μCi/ml) for 48 hours. Unincorporated isotope was removed by washing the cultures with warm Buffer B. Incubations and aqueous phase extractions of the inositol phosphates were performed as described (15).

[^{125}I]-Endothelin binding to Rabbit VSMC. Measurement of [^{125}I]-endothelin binding was performed on replicate-plated 16-mm cultures of attached confluent rabbit VSMC. Cell counts, determined by Coulter counter, routinely varied by less than 10% between wells. Cells were incubated with 300 μl of Buffer B containing 1 g/dl BSA, 1 mM Bacitracin, 25 mM HEPES (pH 7.4) and 10 μM phenylarsine oxide. Tracer concentrations (30 - 60 pM) of [^{125}I]-endothelin (specific activity 1200 Ci/mmol) were added. At equilibrium (8 hr at 4°C) bound radioactivity was separated from free by four successive washes with ice-cold 150 mM NaCl containing 0.2 g/dl BSA. Specific binding represented total bound radioactivity minus nonspecific binding.

Nonspecific binding was determined in the presence of 200 nM unlabelled endothelin and represented < 1% of total added radioactivity. To determine bound radioactivity cells were solubilized in 0.5% sodium dodecyl sulfate and counted in a LKB CliniGamma counter with 80% efficiency. Studies using photosensitive reagents were light protected.

Materials. The supplies and vendors used in this study were as follows: $^{45}CaCl_2$ (27.2 mCi/g) was obtained from Amersham, [^{3}H]-myoinositol (15 Ci/mmol) from Du Pont-New England Nuclear, endothelin was synthesized by Peninsula Laboratories (Belmont, CA) using Merrifield solid phase methodology and purified by high-pressure liquid chromatography (HPLC). Endothelin was resuspended in 0.2 g/dl BSA to obtain a stock solution of 50 μM. [^{125}I]-endothelin (1200 Ci/mmol) was prepared by the Iodogen method (16) and purified by reverse-phase HPLC. Further materials of the highest available grade were obtained from standard commercial sources as previously described.

RESULTS

Synthetic endothelin induces rabbit aortic ring contractions. Cumulative addition of synthetic endothelin to isolated rabbit aortic rings elicited a concentration-dependent increase in contractile tension (Fig.1A) which was endothelium-independent. Half-maximal responses were estimated from log-logit transformation of the data and averaged 1.01 ± 0.09 nM (mean ± SE, n = 5), and resembled published results for porcine coronary artery strips (8). As compared to phenylephrine the contractile response had a slow onset and was sustained (Fig.1B), findings also similar to those for purified natural endothelin (8) and endothelium-conditioned medium (5-7). Maximal contractile responses elicited by 30 nM endothelin represented 66 ± 3% (mean ± SE, n = 5) of the maximal response to phenylephrine (100 μM). The endothelin response was poorly reversible in that contractile tension of rabbit aortic rings exposed to endothelin fell less than 10% during a 5 minute endothelin-free perfusate wash. By contrast, responses to phenylephrine were

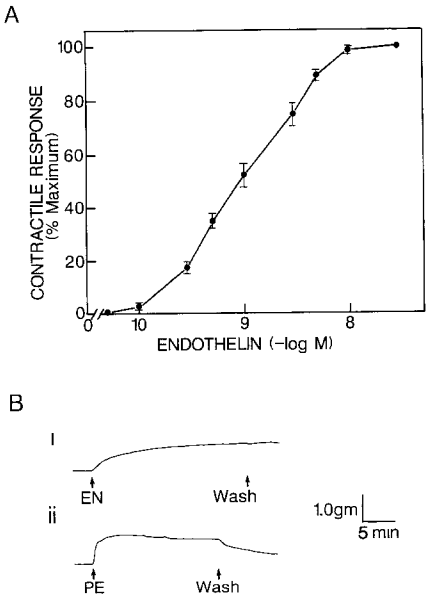

Fig.1. Endothelin-stimulated contractile responses of rabbit aortic rings. (A) Cumulative dose-response curve for endothelin-induced aortic contractile responses. Each experimental value is the mean ± SE of five separate determinations. (B) Contractile response of rabbit aortic rings to (i) 20 nM endothelin (EN) and (ii) 2 μM phenylephrine (PE). A wash with fresh perfusate was performed as indicated.

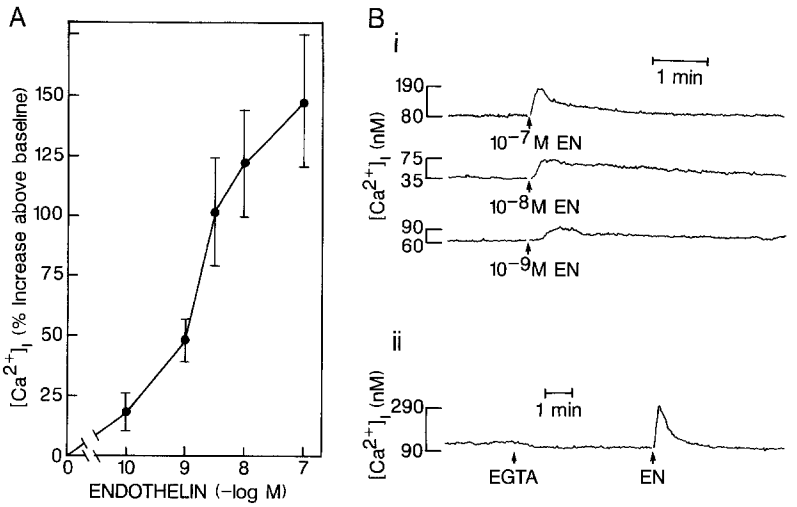

Fig.2. Microfluorimetric determination of cytosolic-free calcium transients in response to endothelin (EN). (A) Concentration-response curve for endothelin-induced increases in $[Ca^{2+}]_i$. Data points (mean ± SE, n = 5 to 8) represent the peak change in calculated $[Ca^{2+}]_i$ expressed as a percent increase above baseline values. (B) Representative fura 2 fluorescence ratios in response to the indicated doses of endothelin (EN) in the presence (i) or absence (ii) of external Ca^{2+}. Perfusion with Ca^{2+}-deplete media (0.5 mM EGTA, no added Ca^{2+}) produced a 7.3 ± 2.2% decrease in baseline $[Ca^{2+}]_i$ after 5 minutes.

promptly reversed using a similar wash-out protocol (Fig.1B). Nifedipine (0.5 μM, 20 min) induced a 30% decrease in tension in endothelin (30 nM)-treated aortic rings. Sodium nitroprusside (10 μM), an activator of soluble guanylate cyclase (17), as well as atrial natriuretic peptide (ANP) (50 nM), known to stimulate VSM particulate guanylate cyclase (18,19) returned endothelin-induced contractile responses in the aortic rings to baseline within 20 minutes, even in the continued presence of endothelin. These studies confirm the biological activity of synthetic porcine/human endothelin on rabbit vascular tissue.

Endothelin mobilizes intracellular Ca^{2+}. To define the effects of endothelin on VSMC calcium (Ca^{2+}) homeostasis we utilized confluent monolayers of cultured rabbit VSMC grown on glass coverslips and loaded with the calcium-sensitive fluorescent dye, fura 2. Changes in intracellular calcium transients were detected by microfluorimetry (Fig.2). Resting cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$, averaged 72.9 ± 6.2 nM (mean ± SE, n = 46) (20). Endothelin induced a prompt, transient increase in $[Ca^{2+}]_i$, followed by a decline to levels that remained slightly above baseline values for 3 to 5 minutes (Fig.2Bi). The magnitude of the peak as well as the time to peak elevation in $[Ca^{2+}]_i$ was concentration-dependent. A 0.1 nM threshold concentration of endothelin was required to elicit a Ca^{2+} transient, while half-maximal responses (ED_{50}) were observed at 1.8 nM (Fig.2A). The time to peak elevation of $[Ca^{2+}]_i$ in response to different endothelin doses were; 23 ± 5 sec. at 100 nM (n = 8), 32 ± 1 sec, at 10 nM (n = 7), 39 ± 7 sec. at 3.2 nM (n = 7), 47 ± 9 sec. at 1 nM (n = 6) and 123 ± 43 sec. at 0.1 nM (n = 6) (mean ± SE). In cultured VSMC which had been exposed to Ca^{2+}-free, EGTA buffer for 5 minutes, endothelin (100 nM) still evoked a 116 ± 29% increase in the $[Ca^{2+}]_i$ (mean ± SE, n = 5) (Fig.2Bii). Although this response was lower than the 147 ± 29% (mean ± SE, n = 8) increase

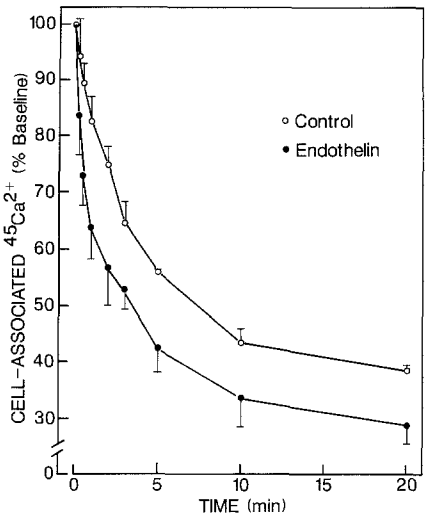

Fig.3. Time course of $^{45}Ca^{2+}$ loss from preloaded cultured rabbit aortic smooth muscle cells expressed as a percent of $^{45}Ca^{2+}$ present at t = 0. The upper and lower curves represent La^{3+}-resistant cell-associated $^{45}Ca^{2+}$ content in control and endothelin-treated (50 nM) cells as a function of time. The bottom curve, representing $^{45}Ca^{2+}$ content in dishes exposed to endothelin, is significantly different (log-transformation of data, SYSTAT program two way analysis of variance, $p < 0.001$).

induced by 100 nM endothelin in Ca^{2+}-replete medium, the magnitude of the responses were not significantly different. These observations suggest that endothelin mobilizes Ca^{2+} from an intracellular compartment. Depletion of extracellular Ca^{2+} reduced the sustained plateau phase of the endothelin $[Ca^{2+}]_i$ increase (measured 5 min. after exposure to endothelin) from a 23 ± 10% increase above basal $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} and endothelin, to a value of 14 ± 4% below the new basal $[Ca^{2+}]_i$ after 5 minutes exposure to Ca^{2+}-free, EGTA buffer. Nifedipine (1 μM) pretreatment for 5 minutes in standard perfusate did not alter the $[Ca^{2+}]_i$ peak in response to endothelin (100 nM) which averaged 141 ± 34% above baseline values (mean ± SE, n = 5) but did reduce the sustained plateau phase of the endothelin $[Ca^{2+}]_i$ increase to a value 10.3 ± 2.9% above baseline levels.

Endothelin increases transmembrane flux of $^{45}Ca^{2+}$. To examine further the cellular mechanisms involved in endothelin action on cultured VSMC, we also tested its ability to modulate transmembrane $^{45}Ca^{2+}$ fluxes. The time-dependent efflux of cell-associated $^{45}Ca^{2+}$ from preloaded cultured VSMC in the presence and absence of endothelin (50 nM) is illustrated in Fig.3. Endothelin significantly stimulated $^{45}Ca^{2+}$ efflux within the first sixty seconds as compared to control ($p < 0.05$). However, the finding that the rate of $^{45}Ca^{2+}$ efflux at time points beyond 1 minute was not significantly different between control and endothelin-treated cells suggests that the predominant effect of endothelin was on the initial rate of efflux. Endothelin also stimulated $^{45}Ca^{2+}$ efflux in Ca^{2+}-free medium (no added Ca^{2+}, 2 mM EGTA). In two separate experiments 50 nM endothelin decreased $^{45}Ca^{2+}$ content at two minutes by 44 ± 1% and 45 ± 4% (mean ± SD, 13 determinations) as compared to a decrease of 32 ± 1% and 25 ± 4% for cells in the absence of endothelin. Thus the demonstration that endothelin stimulates a decrease in cell-associated $^{45}Ca^{2+}$

Table 1. Inositol Phosphate Formation Induced by Endothelin in Cultured Rabbit VSMC.

	$[^3H]IP_1$	$[^3H]IP_2$ (cpm/dish)	$[^3H]IP_3$
Control	33625 ± 1141	1268 ± 83	709 ± 67
Endothelin	46146 ± 997	6289 ± 1404	2758 ± 590

Effect of endothelin (100 nM) on rabbit VSM inositol polyphosphate production at one minute. Values are expressed as mean ± SD of six determinations from a single representative experiment. Similar results were obtained in three separate experiments.

in the absence of external Ca^{2+} provides further evidence that endothelin mobilizes intracellular Ca^{2+} in VSMC.

Endothelin also stimulated $^{45}Ca^{2+}$ influx (measured at five minutes) in a concentration-dependent manner over the range 0.01 to 100 nM with an EC_{50} of 1.2 ± 1.1 nM (mean ± SD, five determinations from two separate experiments). Endothelin (100 nM)- and K^+ (100 mM)-stimulated $^{45}Ca^{2+}$ influx over five minutes averaged 0.67 ± 0.06 and 0.82 ± 0.14 nmol $^{45}Ca^{2+}$/dish respectively, as compared to 0.49 ± 0.05 nmol $^{45}Ca^{2+}$/dish observed in control VSMC (mean ± SE, n = 3 experiments) ($p < 0.05$ experimental compared to control). Brief incubations with nifedipine (0.1 μM, 5 min.) markedly attenuated both endothelin- and K^+-stimulated $^{45}Ca^{2+}$ influx (data not shown).

Endothelin stimulates IP_3 accumulation. The findings that endothelin mobilizes intracellular Ca^{2+} suggested that endothelin may stimulate phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis and inositol trisphosphate (IP_3) formation. Measurement of inositol phosphates (Table 1) in [^{3}H]-myoinositol-labelled VSMC confirmed that endothelin rapidly induced inositol monophosphate (IP_1), inositol diphosphate (IP_2) and IP_3 formation, presumably mediated by activation of phospholipase C. Measurement of inositol phosphates as a function of time demonstrated that endothelin (100 nM) stimulated IP_3 production as early as 15 seconds with a maximal effect at one minute (triplicate determinations, n = 2).

High Affinity [^{125}I]-Endothelin Binding Sites on VSMC. [^{125}I]-Endothelin prepared by the Iodogen method and purified by reverse-phase HPLC was used to determine the nature of rabbit VSMC endothelin binding sites. The concentration-dependent inhibition of ^{125}I-endothelin binding by unlabelled endothelin is illustrated in Fig.4. Log-logit transformation of the data expressed as the fraction of total binding (B/B_0) was used to estimate a value for the 50% inhibition of total binding (B_0) for each competition curve. The observed mean value of 0.40 ± 0.12 nM (mean ± SE, n = 3) is similar to ED_{50} values defined for endothelin-stimulated modulation of cultured rabbit VSMC calcium homeostasis. ^{125}I-Endothelin binding to VSMC demonstrated specificity in that binding was not inhibited by Angiotensin II, arginine vasopressin, bradykinin or histamine. Since it was previously suggested that endothelin may interact directly with voltage-dependent Ca^{2+} channels, the possibility that Ca^{2+} channel agonists and antagonists influence endothelin binding was examined. Binding was not inhibited by the benzothiazepine diltiazem (10 μM), the phenylalkylamine verapamil (10 μM) or the 1,4-dihydropyridines nifedipine (1 μM) and Bay-K 8644 (1 μM) (data not shown).

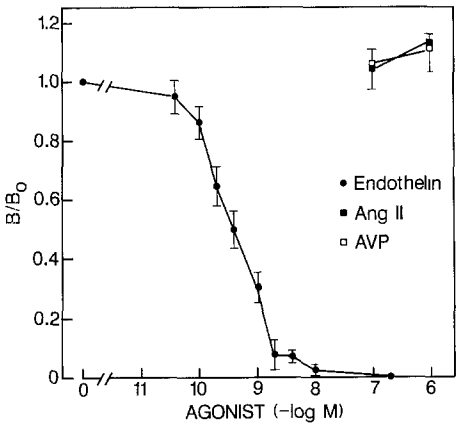

Fig.4. Competitive inhibition of [^{125}I]-endothelin binding to rabbit VSMC. B_0 refers to binding of [^{125}I]-endothelin in the absence of competing substance, and B/B_0 is the fraction of [^{125}I]-endothelin that remains bound in the presence of competing ligand. Data points represent the mean ± SE, for three separate experiments. Determinations for endothelin were done in triplicate and determinations for angiotensin II (Ang II) and arginine vasopressin (AVP) in duplicate. A similar protocol confirmed the failure of bradykinin (1 μM), histamine (10 μM), verapamil (10 μM), diltiazem (10 μM), Bay K-8644 (1 μM) and nifedipine (1 μM) to modulate specific [^{125}I]-endothelin binding (duplicate determinations, three separate experiments).

DISCUSSION

The present study demonstrates that synthetic endothelin, an endothelium-derived vasoconstrictor peptide, is a potent agonist for rabbit aortic smooth muscle contraction. Endothelin potently stimulated elevations of $[Ca^{2+}]_i$ and transmembrane $^{45}Ca^{2+}$ flux in cultured rabbit VSMC, changes that were seen even in the absence of extracellular Ca^{2+}. These results strongly suggest that endothelin-induced alterations of VSMC calcium homeostasis involve, at least in part, the mobilization of intracellular calcium. Moreover, we have found that endothelin stimulates IP_3 formation in rabbit VSMC. Additional studies performed in the A7r5 and A10 cultured vascular smooth muscle cell lines and bovine glomerular mesangial cells have confirmed the ability of this vasoconstrictor peptide to both increase $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} and induce IP_3 formation (manuscripts in preparation). Our data are therefore interpreted to indicate that one of the initial membrane events in the action of endothelin is to induce phospholipase C-stimulated PIP_2 hydrolysis which liberates IP_3, subsequently mobilizing Ca^{2+} from portions of the endoplasmic reticulum (21-25). A recent report by Hirata et al (26) showed that endothelin increased $[Ca^{2+}]_i$ in cultured rat VSMC. However, in contrast to our findings that endothelin increases IP_3 formation, these investigators reported that endothelin did not increase total inositol phosphate formation. The explanation for this discrepency is not clear but may reflect differences in cell lines, culture and assay conditions among others. Sarafotoxins, a group of cardiotoxic 21-amino acid peptides from the venom of the snake ***Atractaspis engaddensis***, exhibit extensive sequence homology with endothelin and also stimulate hydrolysis of phosphoinositides (27). Yanagisawa et al. (8) have hypothesized that endothelin may be a direct activator of voltage-dependent Ca^{2+} channels. Our findings that Ca^{2+} channel ligands do not interfere with [^{125}I]-endothelin binding indicate that endothelin couples to a site distinct from the well-described Ca^{2+} channel binding sites for these ligands. Although nifedipine does not block the initial endothelin-stimulated $[Ca^{2+}]_i$ increase, we

found that it does attenuate endothelin-stimulated $^{45}Ca^{2+}$ influx, thus indicating that endothelin may, indirectly through second messengers, activate a dihydropyridine-sensitive calcium channel in VSMC.

In summary, our observations provide evidence that endothelin shares in common with other vasoactive hormones, such as angiotensin II, norepinephrine and vasopressin, the ability to activate phospholipase C, an initial signaling event which leads to VSMC contraction. It remains to be shown that the differences in the procontractile effect of endothelin on vascular rings, as compared with other constrictor agonists, represents a difference in their cellular mechanisms of action rather than simply differences in the kinetics of endothelin/receptor interaction and processing in intact tissue.

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