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Ionic Requirements of the Endothelin Response in Aorta and Portal Vein

R. Borges, D.V. Carter, H. von Grafenstein, J. Halliday, and D.E. Knight

The vasoconstrictor responses of isolated rat portal vein and aorta to synthetically prepared endothelin are investigated. Both preparations respond to 10^{-9} M levels of the peptide although the aortic response is more sustained than that of the portal vein. Endothelin-evoked contractions, unlike those evoked by scorpion α -toxins (which are homologous to endothelin) or by veratridine, are insensitive to tetrodotoxin or to the removal of sodium ions from the tissue-bathing medium. Contractile responses to endothelin may still be observed in high-potassium depolarizing medium and are not dependent on the presence of extracellular chloride; however, the responses are dependent on the presence of extracellular calcium and are blocked by nitrendipine, nifedipine, or nickel. Endothelin-evoked uptake of ^{45}Ca into aortic tissue is also independent of extracellular sodium or potassium and is blocked by nifedipine. These data strongly suggest that endothelin acts at a site closely coupled to the calcium channel and that depolarization by sodium influx through voltage-dependent channels is not involved in endothelin-induced vasoconstriction. (*Circulation Research* 1989;65:265–271)

Endothelin, a 21-amino acid peptide derived from vascular endothelial cells, has been reported to be the most potent vasoconstrictor known.¹ This endogenous peptide may be involved in the physiological regulation of vascular tone, as well as in the pathophysiology of vascular disorders. A cascade of events initiated at the membrane and resulting in a rise in intracellular free calcium is generally thought to activate the contractile machinery of vascular smooth muscle,^{2–4} but the site at which endothelin acts is unclear. Its 21-amino acid sequence is homologous to scorpion α -toxins,¹ and so one possibility is that, like the α -toxin, endothelin operates by activating sodium channels.⁵ Since sequence data suggest that the sodium channel and the dihydropyridine-sensitive calcium channel belong to the same superfamily of voltage-operated ion channels,⁶ it is possible that endothelin, like Bay K 8644, activates this calcium channel in a more direct way. This paper describes simple whole-tissue experiments that are directed toward testing these two possibilities. The results strongly suggest that endothelin does not act by causing depolarization through activation of sodium channels, but rather acts at the calcium channel itself, or at a receptor closely coupled to the cal-

cium channel to increase calcium influx into vascular smooth muscle cells.

Materials and Methods

Chemicals

Endothelin was synthesized and supplied by Cambridge Research Biochemicals, Cambridge, United Kingdom, and dihydropyridines, Bay K 8644, nitrendipine, and nifedipine were obtained from Bayer, A.G. (Wuppertal, FRG). All other drugs used, including the venom-containing α -toxins from North African scorpion *Leiurus quinquestriatus* (catalogue number V.5251), were obtained from Sigma Chemical Company, St. Louis, Missouri.

Contraction Experiments

Vascular contraction experiments were performed on thoracic aorta and portal vein ring segments from male Sprague-Dawley rats (200–300 g) and, in a limited number of experiments, femoral vein segments from the same rats. The endothelium was mechanically abraded to prevent release of endothelial factors,⁷ and the vascular ring segments (2 mm) were mounted on stirrups in 2.5-ml tissue baths. Resting tensions of vein and aorta were set at 1 g and 2 g, respectively, and the tensions were recorded isometrically (model FT 03 transducer, Grass Instr. Co., Quincy, Massachusetts). Mounted vascular segments were bathed at 37° C in Krebs' solution containing (mM) NaCl 119, KCl 4.4, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25, and glucose 11 (pH 7.4), equilibrated with 5% CO₂-95% O₂.

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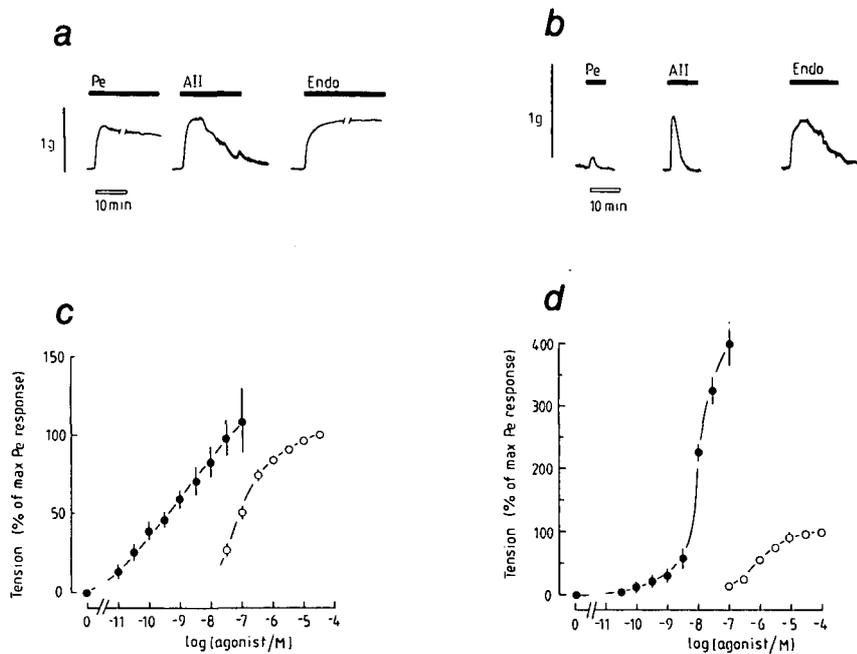


FIGURE 1. Typical tension traces of aorta (panel a) and portal vein (panel b) in response to a series of challenges by 10 μ M phenylephrine, 1 μ M angiotensin II, and 0.01 μ M endothelin. Breaks in tension traces for aorta treated with phenylephrine and endothelin correspond to intervals of 10 and 20 minutes, respectively. Recovery period between challenges was 10–20 minutes for portal vein and aorta. Dose-response curves for endothelin (●) and phenylephrine (○) are shown for aorta (panel c) and portal vein (panel d). Data are normalized to the tension induced by 10 μ M phenylephrine and are means of three determinations on 12 preparations; error bars indicate SEM. Pe, phenylephrine; AII, angiotensin II; Endo, endothelin.

Tissues were depolarized by a potassium-based Krebs' solution in which 119 mM NaCl was replaced by 119 mM KCl. Tissues were also incubated in a sucrose-based solution in which the NaCl and KCl in Krebs' solution were replaced iso-osmotically by sucrose (246.8 mM) and the NaHCO₃ was replaced by Tris buffer (pH 7.3). This solution was bubbled with 100% O₂. Generally, aortic segments responded to endothelin or phenylephrine by developing a sustained tension, whereas portal vein segments gave more transient responses. For this reason, endothelin (and phenylephrine) dose-response curves on vein were performed by challenging the tissue with successive concentrations of agonist separated by a 10-minute recovery period in Krebs' solution. Dose-response curves for aorta were obtained with cumulative doses of agonist. Due to the limited availability and high cost of the peptide, we did not test endothelin concentrations above 10⁻⁷ M. Experiments involving dihydropyridines were performed under sodium lamp illumination.

Renal vascular bed experiments were performed by perfusion of Krebs' solution at 37° C through the renal artery at 3–4 ml/min with an LKB Multiperplex peristaltic pump (model 2115, LKB, Bromma, Sweden), and the inflow perfusion pressure was measured with a Statham pressure transducer (model P23AC, Gould, Cleveland, Ohio). Drugs were injected as a bolus (10 μ l) into the perfusion fluid close to the kidney.

Calcium Flux Measurements

⁴⁵Ca influx experiments were carried out with 2-mm thoracic aortic ring segments mounted on stainless steel hooks and incubated in Krebs' solution at 37° C. Aortic segments were stimulated by transfer to vials containing 10 μ Ci/ml ⁴⁵Ca and 2.5

mM CaCl₂ in either Krebs' solution or a solution in which 119 mM NaCl was replaced by 119 mM KCl. In some cases, endothelin (0.01 μ M) or nifedipine (1 μ M) was added to sodium- or potassium-based solutions. After 10 minutes of incubation, the tissues were washed three times over 30 minutes in ice-cold, calcium-free Krebs' solution (MgCl₂ replacing CaCl₂) containing 2 mM EGTA. Tissues were blotted dry, weighed, and solubilized in NCS tissue solubilizer (Amersham, Arlington Heights, Illinois), and the ⁴⁵Ca was counted. In other experiments, tissues were preincubated with endothelin and/or the potassium solution for 10 minutes before exposure to ⁴⁵Ca for 1.5 minutes. The results are expressed as picomoles of calcium uptake per milligram of tissue.

Results

Figure 1 shows typical tension traces obtained from aortic (a) and portal vein (b) ring segments. Endothelin (0.01 μ M) elicited contraction in both preparations, the extent of which was similar to that obtained by 1 μ M angiotensin II. In artery, the tensions developed were also similar to that induced by a maximal concentration of phenylephrine (10 μ M). In portal vein, the maximal tension induced by phenylephrine was much smaller than the tension that could be induced by endothelin or angiotensin II. Such a difference in the responses to endothelin and phenylephrine was not confined to portal vein, however, since a similar pattern was obtained in femoral vein preparations (four determinations based on two animals). In aorta and portal vein, endothelin appeared to be at least two orders of magnitude more potent than the α_1 -adrenergic receptor agonist (Figures 1c and 1d). The time courses of the endothelin-evoked contrac-

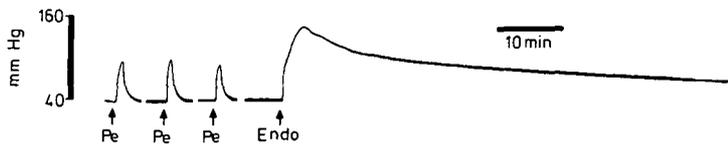


FIGURE 2. Perfusion pressure for constant flow of 4 ml/min through kidney in response to successive bolus injections of 10 nmol phenylephrine and 100 pmol endothelin. Breaks in traces correspond to 10-minute recovery periods. Data show long-lasting effect of a bolus injection of endothelin compared with that of phenylephrine. Pe, phenylephrine; Endo, endothelin.

tions were different, however, in the two preparations; the peptide generally caused a transient response in vein—lasting some 5–10 minutes—but a more maintained response in artery. In the latter case, the tension returned very slowly to baseline levels even when the tissue was washed repeatedly.

The prolonged nature of this increase in tone in arterial vessels was more clearly demonstrated in a perfused kidney preparation in which the perfusion pressure required at a constant flow rate was monitored. Figure 2 shows that while bolus injections of phenylephrine (10 nmol) into the perfusing fluid brought about transient increases in perfusion pressure, a bolus injection of endothelin (0.1 nmol) led to an increase in perfusion pressure that lasted for more than 1 hour. Under some conditions, venous tissue also exhibited a maintained contractile response rather than the more usual transient response. Thus, when sodium in the fluid bathing the tissue was replaced by potassium, the tissue contracted transiently in response to the depolarizing effect of high extracellular potassium. After this initial rise, the tension decreased to near resting levels (Figures 3c and 5). However, a combination of this depolarizing medium and endothelin resulted in a maintained response, and the tension traces of Figure 3 show that this effect occurred if the sodium was replaced by potassium immediately after the tissue was challenged with endothelin (Figure 3a), immediately before the challenge (Figure 3b), or even when the endothelin was presented to the tissue some 20 minutes after the onset of the potassium challenge and at a time when the potassium-induced tension transient had returned to near resting levels (Figure 3c). Such sustained responses to endothelin were inhibited by 10 μ M isoprenaline (data not shown).

Due to the slow reversibility of the endothelin response in aortic tissue, it proved difficult to obtain several consecutive responses for test and control conditions in experiments designed for investigation of the mechanism of action of endothelin. For this reason, most of the results presented here are from portal vein in which the response to endothelin in each test condition could be compared with an earlier control response in the same tissue. However, qualitatively similar results were obtained in aorta when test and control conditions were applied using separate but paired tissues.

Figure 4a shows that the effect of endothelin was unaltered by a cocktail of agents that block gangli-

onic nicotinic receptors, muscarinic receptors, α - and β -adrenergic receptors, H_1 receptors, 5-hydroxytryptamine receptors, angiotensin II receptors, and opioid receptors, applied together with an inhibitor of adrenergic neurone transmitter release and an inhibitor of the cyclooxygenase pathway. Figure 4 describes an experiment in which the preincubation time with the cocktail of antagonists was about 10 minutes. Longer preincubation times (up to 1 hour) were also without effect on the endothelin-induced response.

The alkaloid veratridine and scorpion α -toxin increased the tension in vascular ring preparations,

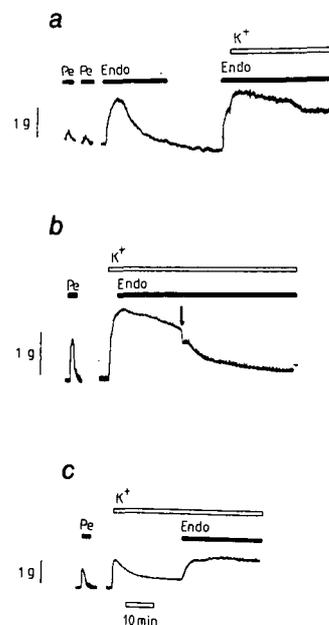


FIGURE 3. Time course of endothelin-induced tension changes in portal vein. Panel a: portal vein was challenged with 10 μ M phenylephrine and then with 0.01 μ M endothelin for a further 20 minutes. After 20 minutes, the tissue was challenged again with 0.01 μ M endothelin and, 2 minutes later, with a high-potassium (120 mM) depolarizing medium (K^+). Panels b and c: Same procedure as in panel a except that potassium challenge precedes endothelin challenge by 3 minutes (b) or by 20 minutes (c). Data show that endothelin-evoked tension traces are better maintained in depolarized tissue. Arrow (panel b) indicates addition of 1 mM nickel to tissue bath. Data are from one experiment but are representative of four other experiments using different preparations. Pe, phenylephrine; Endo, endothelin.

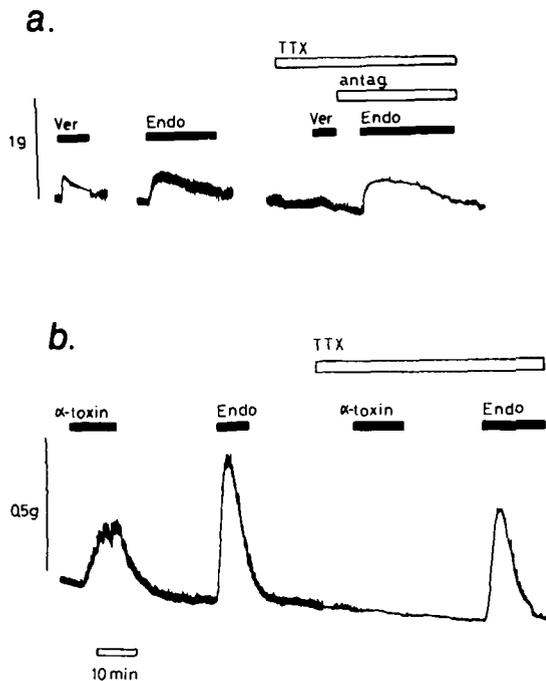


FIGURE 4. Vasoconstrictor response induced by endothelin is not inhibited by a cocktail of antagonists or by tetrodotoxin, unlike responses induced by veratridine (panel a) or scorpion α -toxin (panel b). Portal vein segments were first challenged with 50 μ M veratridine (panel a) and 25 μ g/ml scorpion α -toxin (panel b) and then with 0.01 μ M endothelin. Tetrodotoxin (10 μ M) was then applied, which blocked veratridine and scorpion α -toxin responses but not the rise in tension induced by endothelin. Panel a also shows that endothelin elicits contraction in the presence of a cocktail of antagonists, including (μ M) hexamethonium 10, atropine 1, phentolamine 1, mepyramine 1, ketanserin 1, saralasin 1, propranolol 1, naloxone 1, guanethidine 10, and indomethacin 3. Results were obtained from segments of same portal vein and are representative of four other experiments using tissues from different animals. Ver, veratridine; α -toxin, scorpion α -toxin; TTX, tetrodotoxin; antag., antagonists.

and the finding that both these agents were ineffective on tissues incubated with either tetrodotoxin (10 μ M) or a high-potassium depolarizing medium (Figures 4 and 5) was consistent with the hypothesis that they operate by acting on voltage-sensitive sodium channels. Endothelin, however, elicited contraction in the presence of tetrodotoxin (Figure 4) or when the tissue was incubated in a high-potassium depolarizing medium (Figures 3c, 5, and 6a). These data suggest that the mechanism of action of scorpion α -toxin and veratridine is distinct from that of endothelin. This hypothesis, which suggests that sodium plays little part in the endothelin contraction coupling pathway, is further supported by the finding that the peptide elicited contraction in tissue incubated in solutions devoid of sodium ions (Figure 6b) and containing sucrose to maintain osmolarity. Such an incubation solution might be expected to

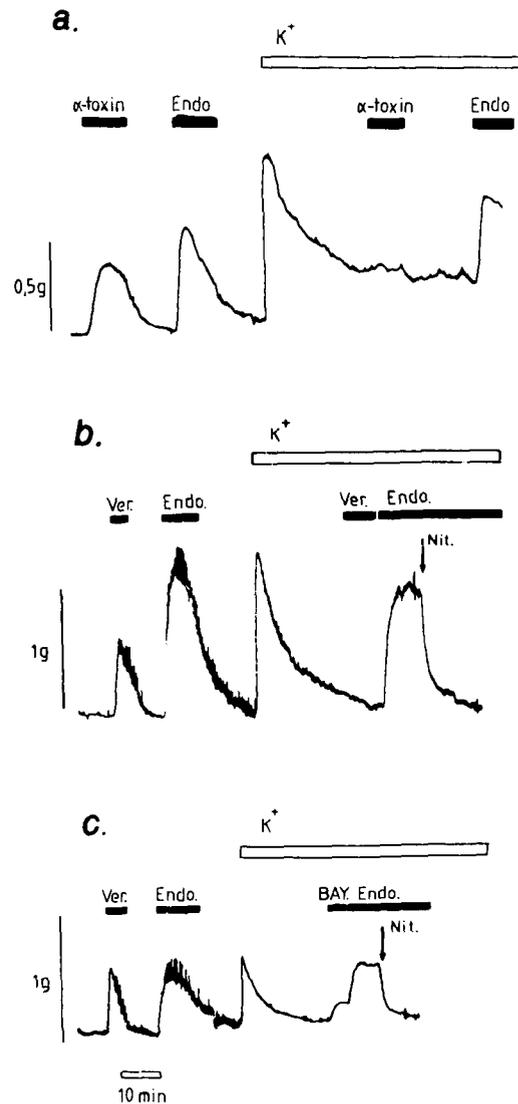


FIGURE 5. Endothelin and calcium-channel agonist Bay K 8644, but not veratridine or scorpion α -toxin, elicit contraction in depolarized portal vein smooth muscle. Scorpion α -toxin (25 μ g/ml) or veratridine (50 μ M) was applied to tissues, followed by endothelin (0.01 μ M). Tissues were then exposed to a depolarizing high-potassium medium (K^+) and, after about 20 minutes, were challenged again with scorpion α -toxin (panel a), veratridine (panel b), or 1 μ M Bay K 8644 (panel c), followed by 0.01 μ M endothelin. Arrows mark addition of calcium-channel antagonist nitrendipine (1 μ M) in panels b and c. Similar data were obtained in three other experiments using different preparations. α -toxin, scorpion α -toxin; Endo., endothelin; Ver., veratridine; BAY, Bay K 8644; Nit., nitrendipine.

have little effect on the resting membrane potential of the tissue and would contain few monovalent ions to perturb this potential. Furthermore, readdition of 100 mM NaCl to tissue bathed in this sucrose-based solution had little effect on the endothelin-induced contractile response (Figure 6). Although the addition of 100 mM NaCl to the

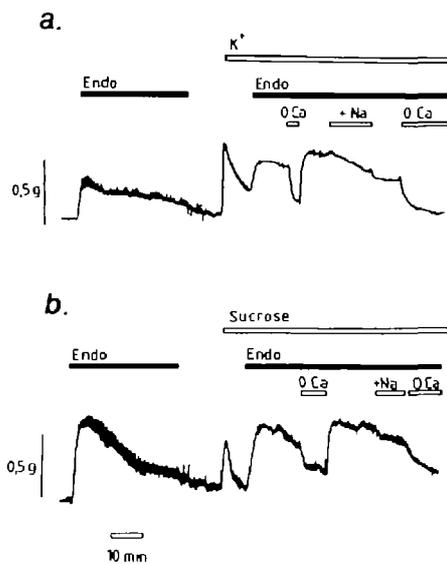


FIGURE 6. Tension induced by endothelin in either fully depolarized tissue or in tissue bathed in a medium lacking monovalent cations is dependent on extracellular calcium and is unaffected by addition of sodium ions to extracellular fluid. After tissues were challenged with $0.01 \mu\text{M}$ endothelin, portal vein segments were immersed in either a depolarizing medium containing 119 mM potassium ions (K^+) in place of 119 mM sodium ions (panel a) or a medium lacking monovalent cations, with iso-osmotic substitution of NaCl and KCl by sucrose and NaHCO_3 by Tris buffer (panel b), and again challenged with $0.01 \mu\text{M}$ endothelin. At times indicated, fluid was changed for solutions lacking calcium (0 Ca), or 100 mM NaCl was added to fluid in tissue chamber ($+\text{Na}$). Results were obtained from segments of same portal vein. Similar results were obtained from two other experiments using tissues from different animals. Endo, endothelin.

sucrose solution would have increased the osmolarity by 200 mosm , control experiments showed that this increase in osmolarity did not modify the response to endothelin (data not shown).

In a separate group of experiments, aorta and portal vein segments were bathed in a medium lacking chloride and then challenged with endothelin. In these media, NaCl was replaced by either sodium thiocyanate or sodium gluconate, KCl by $\text{PO}_4\text{H}_2\text{K}$, and MgCl_2 and CaCl_2 by MgSO_4 and CaSO_4 , respectively. Removal of chloride alone caused a slowly developing increase in tension in aorta and vein; however, endothelin was still able to elicit a full contraction in these chloride-free media (data not shown).

In potassium-depolarized tissues (Figure 5c), vascular tone was increased not only by endothelin but also by the calcium-channel activator Bay K 8644, albeit to a smaller extent. A similar small effect was seen with Bay K 8644 acting on tissue bathed in normal Krebs' solution instead of a high-potassium solution.

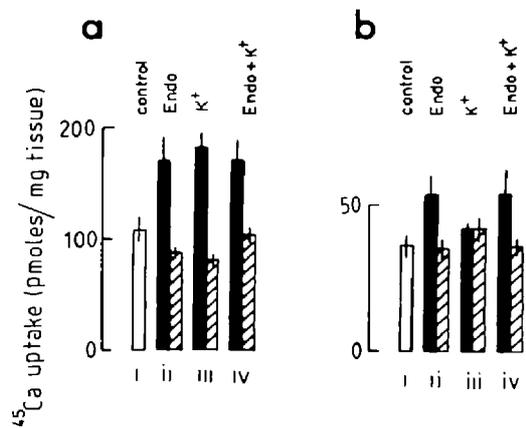


FIGURE 7. Calcium uptake into aorta segments in response to endothelin and potassium challenges. Panel a: Aorta segments were incubated for 10 minutes with ^{45}Ca alone (i), with 0.01 M endothelin (ii), in a high-potassium medium (K^+) (iii), or in the presence of endothelin and high potassium (iv), either with (hatched bars) or without (filled bars) $1 \mu\text{M}$ nifedipine. ^{45}Ca influx for tissue incubated in endothelin, potassium, and endothelin with potassium is significantly greater than that for control tissue ($p < 0.02$). Panel b: Aorta segments were challenged with endothelin and potassium as above for 10 minutes before being exposed to ^{45}Ca for a further 1.5 minutes. Calcium influx for tissues exposed to endothelin (ii) and to endothelin in a high-potassium medium (iv) was significantly higher than that for control tissue (i) ($p < 0.05$). Data are means of four determinations; error bars indicate SEM. Endo, endothelin.

The tension induced by endothelin was inhibited by organic calcium-channel antagonists (e.g., nitrendipine) (Figures 5b and 5c) and inorganic divalent metal ions such as nickel (Figure 3b) and, as shown in Figure 6, could be reversibly abolished by the removal of extracellular calcium. In the absence of extracellular calcium, endothelin did not elicit contraction, suggesting that the peptide acts by increasing the plasma membrane permeability to calcium. This hypothesis is supported by the calcium flux data in Figure 7a, which show a substantial ^{45}Ca influx, measured over 10 minutes, into aortic ring segments in response to endothelin or to depolarization by elevated extracellular potassium. The fluxes induced by maximal levels of these stimuli applied together were not additive, raising the possibility that both endothelin and elevated extracellular potassium stimulate the same population of calcium channels. Figure 7b shows that after 10 minutes of depolarization with high-potassium solution, the calcium influx measured over a 90-second interval was reduced to basal levels (compare Figure 7b, i and iii). At this stage, however, endothelin still increased the calcium influx in depolarized tissue (Figure 7b, iv) to the same level as endothelin acting on normal polarized tissue (Figure 7b, ii). These increases were modest compared with those in Figure 7a, as they were measured over a 90-

second interval only. In both situations, however, the increased calcium influx could be inhibited by the calcium-channel antagonist nifedipine (1 μ M).

Discussion

Endothelin at a concentration of 10 nM evokes contraction in both aortic and portal vein ring segments. Generally, the response in aorta is maintained, but the response in the portal vein starts to decline within minutes, even in the continued presence of endothelin. The relatively transient nature of the endothelin response in portal vein is altered to a more sustained response if the tissue is concurrently depolarized with a high-potassium solution. Clearly, the different nature of the endothelin response in the large artery to that in the vein could be ascribed to differences in specific endothelin receptor-effector coupling mechanisms. However, it is equally possible that the two responses reflect fundamental differences in the properties of these two smooth muscle tissues, with the portal vein generating sporadic waves of contraction and the aorta being capable of sustained tension development.⁸

The ability of bolus injections of endothelin to cause a sustained contraction in kidney vascular bed (Figure 2) contrasts with the transient responses to phenylephrine in this preparation. Because the endothelin effect was sustained for approximately 1 hour even though the kidneys were continuously perfused with fresh solution lacking endothelin, it is likely either that endothelin remains tightly bound to its receptor (or in close proximity to it) or the chain of events linking receptor activation and contraction are long-lasting. This finding is in agreement with other studies¹ that show a sustained effect of endothelin on rat blood pressure. This may be of significance for the possible use of endothelin as a clinical vasoconstrictor agent.

Another clear finding to emerge from this study is that whereas phenylephrine and endothelin are both full agonists in aorta, producing maximum responses of the same order as high-potassium solutions, in portal vein the maximum response to the α_1 -adrenergic receptor agonist phenylephrine is significantly lower than the maximum response to either endothelin or angiotensin II, another endogenous vasoconstrictor peptide. Whether this is of physiological or pathological significance remains to be investigated. The same pattern also was noted in femoral vein preparations.

As to its coupling pathway, endothelin does not seem to activate, directly or indirectly, receptors for a number of other recognized vascular spasmogens including noradrenaline, histamine, 5-hydroxytryptamine, angiotensin II, and cyclooxygenase products.

Yanagisawa et al¹ noted that endothelin has some sequence homology with scorpion α -toxins. These toxins cause excitation by prevention of inactivation of voltage-dependent sodium channels.⁵ The

plant alkaloid veratridine has a similar action but, in addition, shifts the threshold for activation of these channels to more negative potentials.⁵ Both these agents cause contraction of the vascular smooth muscle preparations used in this study, raising the possibility that endothelin, with its similarities in structure to the scorpion α -toxin, might cause vasoconstriction by acting on sodium channels. The findings presented here, however, argue strongly against this possibility. Thus, in contrast with veratridine and scorpion α -toxins that are without effect on the vascular tissues 1) in the presence of tetrodotoxin, 2) in the presence of a high-potassium solution (a medium that would be expected to depolarize the cell and ultimately result in inactivation of voltage-sensitive sodium channels), or 3) in a solution devoid of sodium ions, endothelin still elicits contraction in all these conditions. Indeed, the presence or absence of sodium ions seems not to affect the contractile response evoked by endothelin. This suggests not only absence of any action on voltage-dependent sodium channels but also that the sodium:calcium exchange mechanism in the vascular smooth muscle membrane⁹ does not play a part in endothelin's stimulus-contraction coupling. The finding that endothelin is effective on tissues bathed in either a high-potassium depolarizing medium or a medium lacking chloride argues against a mechanism of action involving a depolarization or hyperpolarization of the cell by a change in the chloride conductance.¹⁰⁻¹²

In contrast with the sodium independence, the contractile responses to endothelin seem to depend absolutely on the presence of extracellular calcium. Thus, the absence of calcium in the external medium prevents endothelin-induced contraction, and removal of calcium causes relaxation from an endothelin-induced contracted state. Inhibition of calcium entry by dihydropyridine calcium-channel antagonists or by the divalent cation nickel has the same effects. Whether activation by endothelin could also bring about the phasic release of small amounts of intracellular calcium was not rigorously tested in the present experiments.

The dependence of endothelin's contractile activity on extracellular calcium is complemented by the ⁴⁵Ca influx data. Conditions in which endothelin evokes contraction in aortic tissues also result in a significant rise in ⁴⁵Ca influx into vascular rings. Both endothelin-mediated contraction and ⁴⁵Ca influx are inhibited by dihydropyridine calcium-channel antagonists. The fact that endothelin can elicit contraction and an increase of calcium influx after 10 minutes in a depolarizing medium at a time when the ⁴⁵Ca influx evoked by the depolarizing medium alone is back to essentially baseline levels has interesting mechanistic implications. Such phenomena, first noted by Evans et al,¹³ have been taken by some as suggestive evidence for calcium entry by receptor-operated channels. Our experiments showing inhibition of ⁴⁵Ca influx by nifedipine and con-

traction of fully depolarized tissue evoked by Bay K 8644 or endothelin suggest either 1) that this putative receptor-operated channel is, like the voltage-operated channel, also sensitive to dihydropyridines or 2) that the flux through voltage-dependent dihydropyridine-sensitive calcium channels can be further increased by endothelin even in depolarizing media.

Taken together, these data strongly suggest that the sodium channel is not the target for endothelin. Whether endothelin binds to the channel itself or to a specific receptor closely coupled to the calcium channel must await patch-clamp and cross-linking experiments.

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KEY WORDS • portal vein • endothelin • scorpion α -toxin • calcium channel • aorta • sodium channels • vasoconstriction • TTK