Endothelin-1 activates phospholipases and channels at similar concentrations in porcine coronary arteries

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Jones, Allan W., Lawrence Magliola, Carrie B. Waters, and Leona J. Rubin. Endothelin-1 activates phospholipases and channels at similar concentrations in porcine coronary arteries. Am. J. Physiol. 274 (Cell Physiol. 43): C1583-C1591, 1998.—Sensitivity of endothelin-1 (ET-1)-ion channel interactions has been proposed to exceed that of ET-1-phospholipase activation in vascular smooth muscle. We wanted to determine whether short-circuiting ion channels with staphylococcal α-toxin pores would shift the ET-1-force relation to the right as predicted from the above proposal. Medium size porcine coronary arteries (outer diameter 0.7–1.5 mm) were mounted on isometric force transducers. ET-1 concentration response curves were compared between intact rings and those subjected to α-toxin treatment with Ca buffered at 0.1 µM. The EC50 for treated rings (1.5 ± 1.0 nM, n = 5 pigs) was similar to that for intact rings (1.9 ± 0.4 nM). The Ca sensitivity of the α-toxin-treated rings (EC50 = 0.43 ± 0.08 µM) was similar to that reported by other laboratories for intact and α-toxin-treated arteries and was shifted eightfold to the left by a high concentration of ET-1 (10 nM). Measurements of [32P]phosphatidic acid ([32P]PA) levels were used to evaluate phospholipase activity in intact arteries. The time courses for [32P]PA production and contraction were similar in response to high (100 nM) and to low (1 nM) ET-1. Significant increases in both steady-state contraction and [32P]PA occurred over a wide range of ET-1 concentrations (0.3–100 nM). Our findings support the concept that ET-1-phospholipase coupling is functional over physiologically relevant concentrations.

THE POTENT AGONIST endothelin-1 (ET-1) activates vascular smooth muscle by a combination of increased cytosolic Ca activity [cytosolic Ca concentration ([Ca]i)] and sensitization of the contractile system to [Ca]i (35, 46). Much evidence supports the concept that ET receptors are coupled to mechanisms that increase Ca entry. ET-1 stimulates Ca release from sarcoplasmic reticulum (SR), and shifts the Ca-force relation curves to the left in intact vascular smooth muscle cells (23, 27, 61). It has been proposed that ET receptors interact via G protein linkages with 1) membrane ion channels and 2) membrane phospholipases such as phospholipase C (PLC) and phospholipase D (PLD) (35, 46). The first process would stimulate Ca entry via voltage-sensitive mechanisms and would be inhibited by Ca channel antagonists or stimulated by membrane depolarization (24). The second process would stimulate the production of second messengers that are associated with Ca release from SR and increased sensitivity of the Ca-force relation (12, 35, 40, 46).

It has been proposed that the sensitivity of ET-1-channel interactions significantly exceeds that of phospholipase activation (9, 24, 35, 40). For instance, contractions caused by low concentrations of ET are sensitive to Ca channel blockers that shifted the ET-force relation curves to the right (i.e., EC50 from 1 to 10 nM) (24, 61). Also, elevated extracellular K concentration shifted the response to the left (EC50 = 0.1 nM) (24). By inference, ET-force relation in the presence of maximal Ca channel blockade would represent the dependence of force development on the coupling between ET-1 and phospholipase activity leading to SR Ca release and sensitization. In the presence of an intact sarcolemma, however, the control of [Ca]i is problematic. Also, Ca channel blockers were shown to depress SR Ca release in vascular smooth muscle made permeable with staphylococcal α-toxin (26). Because SR Ca release relied on PLC activation, the results derived from Ca channel blockers may not be specific for membrane Ca channel effects.

An alternative approach to the use of Ca channel blockers is to permeabilize the surface membrane with α-toxin, thereby circumventing problems associated with sarcolemmal Ca control. After treatment with α-toxin, receptor-second messenger signaling mechanisms (referred to as pharmacomechanical coupling) remain functional, thus allowing Ca sensitization to occur, while [Ca]i, can be controlled by a Ca-buffer solution (26, 42). Importantly, the pores formed by α-toxin short-circuit the contribution of Ca channels and thus obviate the use of Ca channel blockers. Under these conditions, ET-1 was shown to increase the Ca sensitivity of the contractile system; however, only high concentrations of ET-1 were used (> 10 nM) (14, 42, 57). A similar shift in Ca sensitivity also was observed in arteries permeabilized with β-escin and exposed to a high concentration of ET (10 nM) (63). It was our objective to determine whether short-circuiting Ca channels with α-toxin pores would shift the ET-force curves to the right as predicted from the proposal that phospholipase coupling mechanisms are less sensitive to ET-1 than are channel mechanisms (9, 12, 24, 35). We hypothesized that no shift in this relation would occur after treatment with α-toxin because ET-1 stimulation of phospholipase products in intact cells often exhibited sensitivities similar to those of ET-1-force relations (31, 44, 48). Hence, ET-phospholipase coupling is functional over physiologically relevant concentrations.
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MATERIALS AND METHODS

Tissues. Branches (outer diameter 0.7–1.5 mm, measured by a scale in the dissection microscope) of the major coronary arteries from pigs were trimmed of loose connective tissue, nerves, and vasa vasorum in a dissection solution at room temperature. This was followed by incubation at 35°C in a physiological salt solution (PSS) gassed with air. The hearts (maintained in ice-cold dissection solution during transit) were obtained from slaughterhouse pigs and from anesthetized female Yucatan pigs at the University of Missouri. The contractile sensitivity to ET-1 was similar in arteries from these two sources, as well as in vessels used after overnight cold storage (unpublished observations). For contractile studies, rings (outer diameter 0.7–1.1 mm, wall thickness ~0.10–0.14 mm, and length 0.3–1.0 mm) were denuded of endothelium by rotating them over silk suture. They were mounted on a force transducer (Grass) with an attached micrometer drive and were stretched to 1.4 times the resting length. All rings were tested with a high-K solution (80 mM K, substituted for Na) to establish a reference contractile. Siliconized cups were used to make solutions that contained ET-1. Arteries used for phosphatidic acid (PA) assay were mounted on wires to facilitate handling.

Reagents and solutions. Normal PSS had a composition (in mM) of 132 NaCl, 5 KCl, 1.5 CaCl2, 1.2 MgCl2, 1.2 NaH2PO4, 10 Na-HEPES (pH 7.4), and 11.2 glucose. The dissection and the PO4-free solutions were the same, except that the former contained low Ca (0.2 mM) and that PO4 was omitted from the latter. The relaxing solution (R2) used for the permeabilization study was composed of (in mM) 130 potassium propionate, 4 MgCl2, 20 K-PIPES (pH 7.0), 2 K-ETG, 4 Na2ATP, 20 creatine phosphate, 0.001 FCCP (a mitochondrial inhibitor), and 11.4 glucose. The desired free Ca was achieved by adding calcium propionate on the basis of calculations using a computer program provided by Dr. R. Moreland (37). ET-1 was purchased from Peninsula Laboratory and diluted in 10 mM acetic acid. Staphylococcal α-toxin was obtained from LIST and was diluted in deionized water. Other biochemical compounds were purchased from Sigma.

Permeabilization protocol. After the intact rings recovered from K depolarization, they were placed into 5 ml R2 (−0 Ca) for 30 min at 35°C. A transient contraction occurred, which may reflect the action of ATP on purinergic receptors that eventually desensitize (39). This procedure also reduced Ca stores, since control experiments showed that maximal ET-1 produced no contracture after 30 min in R3 (unpublished observation). Rings then were transferred for 30 min into a similar solution (300 µl) containing α-toxin (30–50 µg/ml) and 10 mM sodium azide to inhibit ecto-ATPase activity (58). We did not use 5 mM DIDS as recommended (58) because control experiments showed that DIDS inhibited greatly the contractile responses of permeabilized preparations (unpublished observations). The rings were transferred to fresh R2, and the addition of experimental agents was commenced after 30 min. The concentration of ATP in the R3 solution (analyzed by the chromatography core laboratory at Dalton Cardiovascular Research Center) was 4.0 mM after 30 min of the permeabilization protocol, compared with 4.3 mM in an unused sample. During the experiments, we kept the volume of R2 high (5 ml) compared with the tissue size (0.5 µl) to ensure constant ATP in the solutions.

Another methodological consideration was the choice of EGTA concentration. High concentrations (e.g., 10 mM) appear to sensitize smooth muscle to Ca (25, 54). Because low concentrations (e.g., 0.2 mM) may not provide adequate buffering, we used the concentration 2 mM, which was recommended following a systematic evaluation (25). Our observations also were limited to the steady-state tonic responses to ET-1. Despite efforts to optimize the permeabilization protocol, considerable variability in the contractile response was observed among pigs. On exposure to 10 µM Ca, the permeabilized rings had to generate at least 80% of the response to K depolarization measured while intact for inclusion in the analysis. One-half of the pigs met this standard.

PA assay. Arteries were labeled with 32PO4 (50 µCi/ml in PO4-free PSS) for 2 h at 35°C. The segments were placed in nonlabeled solutions 10 min before exposure to ET-1. Arteries were then freeze clamped and placed immediately into 3 ml chloroform-methanol (1:2 vol/vol) for PA extraction and processing (22). The lipid phase was dried, resuspended in 30 µl chloroform, and spotted onto a Whatman LKGD plate (Whatman Lab). The plate was developed using a TLC solvent system of chloroform-pyridine-88% formic acid (10:6:1 vol/vol/vol). PA was separated with a retardation factor of 0.5 that was identified by standards (Sigma) (22). 32P was measured by means of a storage phosphor screen exposed to the TLC plate for 2 h. The screen was scanned on a Molecular Dynamics PhosphorImager and analyzed using Molecular Dynamics software. The PA values were expressed as a percentage of total 32P counts per lane.

Data analyses. The contractile responses were normalized in terms of maximal response to ET-1. The excitatory concentration causing a 50% response (EC50) was determined for each ring by linear interpolation between the logarithms of concentrations that produced responses just below and above 50%. Because the EC50 values were normally distributed on a logarithmic rather than arithmetic scale, logarithmic values were used to make statistical comparisons. These were transformed to arithmetic means for presentation in the text. Student’s t-test was used to test for differences between group means, with P < 0.05 taken to be significant.

RESULTS

Contractile responses of intact and permeabilized rings. Rings treated with α-toxin exhibited stable responses to ET-1 and to Ca as shown in Fig. 1. During our preliminary experiments, we found it necessary to add GTP to R2 to sensitize the contractile responses to ET-1, consistent with a previous report (42). It is significant that cumulative addition of ET-1 caused progressive force development in the presence of 0.1 µM Ca (Fig. 1A). This concentration simulated basal [Ca], in intact porcine coronary arteries (measured by fura 2) and was not associated with significant force development (29). Maximal force was not achieved by high ET-1 (30 nM) at 0.1 µM Ca, and subsequent addition of Ca to 3 µM resulted in a maximal response (Fig. 1A). This response and that to 10 µM Ca alone (Fig. 1B) exceeded the response to high K in intact rings. Cumulative addition of Ca in the absence of ET-1 also caused progressive increases in contraction (Fig. 1B). The averaged responses, as shown in Fig. 2, exhibited a sharp increase in slope between 0.2 and 1 µM Ca (log[Ca] = −6.70 to −6.00). The EC50 of 0.43 ± 0.08 µM (log[Ca] = −6.37) was similar to that reported for rabbit mesenteric and canine basilar arteries treated with α-toxin (42, 57) and to that observed in intact porcine coronary arteries (29). Measures of contraction and Ca activity (fura 2 method) during K depolariza-
tion showed half-maximal contraction at a \([Ca]\) equivalent to 0.39 µM (log\([Ca]\) = 6.41; see Fig. 2C in Ref. 29).

As noted above, we chose an EGTA concentration (2 mM) that would maintain a relatively normal sensitivity of the contractile system to Ca. It was important not to increase inadvertently the Ca sensitivity of the coronary arteries by the conditions employed in the \(\alpha\)-toxin protocol.

The contractile response to \([Ca]\) was shifted to the left (Ca sensitization) in the presence of ET-1 at a high concentration (10 nM), which yielded a 61% response at 0.1 µM Ca (log\([Ca]\) = -7.0; Fig. 2). On the other hand, 0.6 µM Ca (log\([Ca]\) = -6.22) was required to produce this effect in the absence of ET-1 (Fig. 2). This observation is consistent with previous reports that high concentrations of ET-1 caused an increased sensitivity of the contractile system to Ca (42, 57, 63). The ET-1 concentration-response relation for the same group of pigs appears in Fig. 3. The curves for intact and \(\alpha\)-toxin-treated rings are almost superimposable. The EC_{50} for treated rings (1.5 ± 0.4 nM, log[ET-1] = -8.82) was not significantly different from intact rings (1.9 ± 0.4 nM, log[ET-1] = -8.72). Hence, short-circuiting Ca channels with \(\alpha\)-toxin did not alter the contractile sensitivity to ET-1. The contractile sensitivity of our preparations to ET-1 was similar to that observed for porcine coronary arteries, (23, 24) although considerable range has been reported (15, 52).

![Fig. 1](image1.png)

**Fig. 1.** Force tracings of intact porcine coronary rings exposed to 80 mM K depolarization (80K) and to endothelin-1 (ET-1; A) or varying intracellular Ca concentration ([Ca]i) after treatment with \(\alpha\)-toxin (B). Staphylococcal \(\alpha\)-toxin was applied during break in recording, as indicated by bold arrows. Note that force development of treated rings in response to [Ca] of 10 µM exceeded that in response to K depolarization. Also, force development in response to [Ca] of 0.1 µM in presence of 30 nM ET-1 was 62% of maximal force, whereas no detectable response occurred in absence of ET-1. R₂, relaxing solution.

![Fig. 2](image2.png)

**Fig. 2.** Ca dependence of force development by porcine coronary arteries treated with \(\alpha\)-toxin. Responses (as percent of maximal response, means ± SE) were measured in absence of ET-1 (○: n = 5 pigs) and in presence of maximally effective concentrations (10 nM) of ET-1 (△: n = 3 pigs). ○, EC_{50} in absence of ET-1 (0.43 ± 0.08 µM). Maximal response to [Ca], averaged 140 ± 20% of response to K depolarization of intact arteries.

![Fig. 3](image3.png)

**Fig. 3.** ET-1 concentration response relation of intact porcine coronary arteries (○) and paired arteries permeabilized by treatment with \(\alpha\)-toxin (●) and exposed to 0.1 µM Ca (percent responses, means ± SE, n = 5 pigs). EC_{50} for intact rings (○; 1.9 ± 0.4 nM) was not significantly different from that for permeabilized rings (●; 1.5 ± 1.0 nM).
necessary to protect ET-1 from adsorption by siliconizing the containers and by adding acetic acid to the stock solution. The ET-1 responses of the preparations treated with staphylococcal α-toxin were stable and developed force comparable to that of intact rings.

PA acid production. Measures of ET-1 effects on \([^{32}P]\)PA levels were used to evaluate phospholipase activity. PA responses to ET-1 and other agonists have been shown to be sensitive indicators of PLC by the conversion of its product, diacylglycerol (DAG), to PA and also of PLD, which produces PA directly (21, 22, 31). The phosphor image measurements in these studies allowed \([^{32}P]\)PA to be identified (Fig. 4) and quantitated in the same step. This procedure eliminated the need to scrape the TLC plates for \(^{32}\)P counting as was done previously (21, 22, 31).

The time courses for contraction and \([^{32}P]\)PA responses were measured at maximal (100 nM) and half-maximal (1 nM) ET-1, as shown in Fig. 5. After equilibration at 35°C, the arteries were transferred to vials at 1°C for 90 s with ET-1 applied during the final 60 s before transfer back to 35°C. This protocol allowed the peptide to diffuse into the rings without effect before initiation of the \([^{32}P]\)PA and contractile response at 35°C. It reduced the half time for contraction to 40 ± 8 s (P < 0.05, n = 6 pigs) compared with 67 ± 8 s when ET-1 (100 nM) was added directly to paired rings at 35°C. However, at ET-1 equal to 1 nM, the temperature-step protocol did not significantly alter the relatively slow response (half time = 7.1 ± 1.0 min; Fig. 5A). The time courses for \([^{32}P]\)PA responses (Fig. 5B) were similar to those for contraction (Fig. 5A). At high ET-1 (100 nM), a significant increase in \([^{32}P]\)PA was observed at 60 s (close to the half time for contraction). At low ET-1 (1 nM), the increase in \([^{32}P]\)PA was delayed (5 min), as was the contractile response.

The steady-state contractile responses of the group of pigs shown in Fig. 6 (EC\(_{50}\) 1.3 ± 0.2 nM, log[ET-1] = −8.89) were similar to those shown in Fig. 3. Significant increases in contraction and in \([^{32}P]\)PA occurred at ET-1 concentrations as low as 0.3 nM (log[ET-1] = −9.52, P < 0.02). The \([^{32}P]\)PA dependence on increasing ET-1 did not exhibit a definite maximum over the concentration range tested, and therefore no attempt was made to calculate an EC\(_{50}\).
over, PA levels increase significantly in intact sensitivity to ET-1 than a channel mechanism. Moreover, phospholipase coupling mechanism having a lower response relation to the right, as would be predicted for half-maximal occupation of receptors by a ligand. It has response noted above is higher than that required for contractile responses in porcine coronary arteries exposed to ET-1 at concentrations less than the concentration required for the EC50 contractile response accounting for 95% of the total (50). Surprisingly little binding was observed in endothelial cells (50). None of the above-cited references reported significant binding of ET-1 to medial or adventitial fibroblasts. Therefore, our assumption that >90% of the [32P]PA responses to ET-1 were from smooth muscle appears to be reasonable on the basis of the autoradiographic evidence.

It is well documented that ET-1 causes an increase in phospholipase products and metabolites in vascular cells, including the PLC products inositol 1,4,5-trisphosphate and DAG, the phospholipase A2 (PLA2) product arachidonic acid, and the PLD product phosphatidylethanol (7, 34, 48, 49, 56, 60). ET-1 also induced increases in [32P]PA, which could either result from the action of PLD on phospholipids (e.g., phosphatidylcholine) or be produced secondarily from the phosphorylation of DAG (22, 31). De novo synthesis of PA from glucose represents a third pathway that can produce [32P]PA, which is important to our assumptions, since this pathway does not involve phospholipase activity (19).

The de novo synthesis of PA and DAG (via phosphohydrolase) has been measured in relevant vascular cell types (16, 33, 62). Increased extracellular glucose or glycerol dehydrostimulated DAG formation in cultured vascular endothelial and smooth muscle cells, with a delay of 1 day for the response to glucose and 4 h for that to glycerol (16, 62). A 3-day exposure to high glucose was required to reach a steady state, which is too slow to explain acute effects of ET-1 on [32P]PA via de novo synthesis. Unfortunately, direct measures of agonist effects on de novo synthesis (e.g., [14C]glucose incorporation into PA) have not been made. It has been suggested that internalization of occupied receptors significantly reduces the number of receptors available for G protein linkages (13, 32). Thus, unlike α-adrenergic receptors in the rat aorta, few spare receptors are available to ET-1 under conditions of prolonged exposure to high concentrations (e.g., 1 h at 1 nM or greater) (13, 55). For this reason, we limited our measures of [32P]PA to 10-min exposures to ET-1.

The coronary arteries are composed of three major cell types, endothelium, smooth muscle, and fibroblasts (2). The endothelial monolayer typically contributes <10% of the cell mass in distributing arteries, whereas the fibroblasts are found for the most part in the adventitia. The first autoradiographic study of human and porcine coronary arteries showed the specific high-affinity binding of 125I-labeled ET to be localized in the media with no detectable endothelial contribution (47). The perivascular binding was localized to nerves (which would be removed by our dissection). Quantitative autoradiography showed the binding sites to be of the ETA receptor type in human aorta and coronary arteries as well as in rat aorta and carotid and cerebral arteries (2, 6). These observations are consistent with other techniques (15, 38, 52). The application of electron microscope autoradiography allowed the binding of 125I-labeled ET-1 to be localized to smooth muscle membranes in human coronary arteries, with ETA receptor binding accounting for 95% of the total (50). Surprisingly little binding was observed in endothelial cells (50). None of the above-cited references reported significant binding of ET-1 to medial or adventitial fibroblasts. Therefore, our assumption that >90% of the [32P]PA responses to ET-1 were from smooth muscle appears to be reasonable on the basis of the autoradiographic evidence.

Several studies have established that the predominant ET-1 receptor in porcine coronary arteries is of the ETA type (15, 38, 52). The application of an ETA-selective antagonist (BQ-123) and ETB-selective agonist (IRL-1620) indicated that 10–20% of the contractile response may be mediated by ETB receptors (15, 52). The molecular evidence indicates that both receptor types are members of the G protein-coupled superfamilies of receptors and that activation of either receptor activates PLC (35). It appears that agonist recognition is the major functional difference between the receptors. We used ET-1 in this study because it binds to both receptor types with high affinity (dissociation constant 0.1–0.3 nM) (13, 30). It is of interest that the concentration required for the EC50 contractile response noted above is higher than that required for half-maximal occupancy of receptors by a ligand. It has been suggested that internalization of occupied receptors significantly reduces the number of receptors available for G protein linkages (13, 32). Thus, unlike α-adrenergic receptors in the rat aorta, few spare receptors are available to ET-1 under conditions of prolonged exposure to high concentrations (e.g., 1 h at 1 nM or greater) (13, 55). For this reason, we limited our measures of [32P]PA to 10-min exposures to ET-1.

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suggested that ANG II-induced PA formation by cardiac fibroblasts resulted in part from de novo synthesis (3). The evidence was indirectly based on the incomplete effect of protein kinase C (PKC) downregulation and DAG kinase blockade on ANG II-stimulated PA formation. On the other hand, direct measures of de novo synthesis of PA in fibroblasts showed no significant change in ras-transformed cells that exhibited a five-fold increase in PA concentration (33).

Stimulation of de novo synthesis with high glucose selectively incorporated palmitate, rather than arachidonate and phosphatidylcholine, into DAG of cultured rat aortic smooth muscle cells (62). On the other hand, stimulation of rat arteries with norepinephrine increased PA enriched with arachidonate (43). We also observed a significant increase in [3H]imyristate PA in response to ET-1 stimulation of rat aorta in which phosphatidylcholine was selectively labeled (31). These responses argue against (but do not disprove) a major role for de novo synthesis of PA during acute responses in intact arteries. We feel that the use of [32P]PA as a measure of phospholipase activity is justified. A need exists, however, for a systematic study to resolve the quantitative contribution and time course of the multiple pathways that lead to CA production.

Correlations between the sensitivity of phospholipase activity and functional end points to ET-1 have been inconsistent. Two laboratories have emphasized that direct activation of Ca and nonselective cation channels by ET receptors occurred at lower ET-1 concentrations than did the production of phospholipase products and that comparison of ET-1 concentration-response curves commonly have shown that the production of phospholipase products is to the right of that for contraction (8, 9, 24, 31, 40, 48). However, significant increases in phospholipase products have been observed in cultured smooth muscle cells exposed to low concentrations of ET-1 (<1 nM) (12, 40). Our measures of [32P]PA in native arteries (Fig. 6) showed that receptor-coupled activation of phospholipase activity occurred at an ET-1 concentration significantly below the EC50 for contraction. This stimulation of PA production has the potential to interact with the ET-1 effects on Ca entry via ion channels by increasing DAG and PKC activity, which has been linked to Ca sensitization (42, 46, 57, 63).

The specific role of PA in Ca sensitization is not established. First, it is well recognized that PA can result from the phosphorylation of DAG and thus would be an intermediary in the resynthesis of membrane phospholipids (22). Second, PA can be produced directly by the action of PLD on phospholipids (mostly phosphatidylcholine) and could be a source of DAG via the action of phosphatidate phosphohydrolase (22, 34). Third, a growing literature supports the concept that PA directly affects responses of non-smooth muscle cells. For instance, PA activated NADPH oxidase and PLD (a self-amplification process) (1, 4). PA also has been proposed to interact with selective receptors coupled to PLC and to PLP2 (10, 20, 45, 51). The extent to which PA functions as a metabolic intermediate, a second messenger, and/or a ligand for receptor activation of phospholipases in coronary arteries is unknown at this time.

The time course for PA production (Fig. 5) does not rule out a direct role for PA in coupling ET-1 receptor interactions with contractile responses. At high ET-1 (100 nM), significant increases in [32P]PA occurred during the rapid phase of tension development, as would be expected if phospholipase activation were part of the early signaling process (12, 31, 35, 46, 57, 63). The time course at low ET-1 (1 nM) exhibited a delay (~2 min; Fig. 5) in both the [32P]PA and the tension responses. As in the case of high ET-1, significant increases of [32P]PA occurred during tension development at low ET-1 (1 nM), consistent with the hypothesis that phospholipase activation contributed to the contractile response over a wide range of ET-1.

A systematic study of [Ca2+] tension relations in intact porcine coronary arteries derived the time course as a function of ET-1 concentration (59). Initially, the tension responses to ET-1 were much slower than changes in [Ca2+]. After some point in time (depending on ET-1 concentration), the tension responses exceeded that predicted from the [Ca2+] relation for K depolarization. This increased tension response during constant or decreasing [Ca2+] indicated that an increase in Ca sensitivity had occurred (59). The Ca sensitization began at between 1 and 2 min of exposure to high ET-1 (100 nM) and between 12 and 15 min of exposure to low ET-1 (1 nM) (Fig. 5 in Ref. 59). The timing of this response is consistent with our measures of increased [32P]PA at 1 min in ET-1 = 100 nM but not until 5 min in ET-1 = 1 nM (Fig. 5B). It therefore appears that low concentrations of ET-1 can sensitize the contractile system to [Ca2+] in intact coronary arteries, as well as in α-toxin-permeabilized preparations (Fig. 3).

It is well established that ET-1 also stimulates Ca influx into vascular cells (35, 46, 61). Several studies have shown that 45Ca uptake is sensitive to ET-1, with significant stimulation occurring below 1 nM (23, 40). Electrophysiological studies have shown that ET-1 causes a concentration-dependent depolarization in smooth muscle cells with an EC50 between 0.6 and 1 nM (28, 41). The activation of Ca channels was suggested to cause this depolarization because whole cell Ba currents were increased over threefold by ET-1 (11). These currents were blocked by a high concentration of nicardipine, which also shifted the ET-1-tension response curve 100-fold to the right (11). In another study, it was noted that ET-1 increased the Ba currents with an EC50 of 0.6 nM (18). These findings are consistent with the proposal that ET-1 at low concentrations activates Ca channels of the L-type (24, 35). Measures of single-channel activity in the cell-attached mode, however, showed that the application of ET-1 to the bath increased Ca channel activity in the patch, which was isolated from the bath solution (18, 53). This finding indicates that the ET-1 effect on Ca currents may occur via a second messenger system. As an alternate explanation, internalized ET-1-receptor complexes could diffuse into the membrane isolated by the cell-attached
patch electrode (13, 32). This possibility has not been tested. We tried to circumvent such potential problems by short-circuiting the membrane channels with α-toxin.

Additional channel mechanisms have been proposed to underlie ET-1-induced depolarization. ET-1 inhibited the activity of ATP-sensitive K channels (KATP) in porcine coronary smooth muscle (36). Such inhibition was associated with depolarization, which in turn could activate Ca entry via voltage-dependent Ca channels. The ICs50 (1.3 nM) for the ET1 effects on KATP was similar to that observed for other functional end points (36). ET-1 also has been shown to increase membrane conductance and to stimulate inward currents via nonselective cation channels (5, 8). The inward current was sensitive to reduction in extracellular Ca, as well as to other ions, and was blocked by Ni but not by nifedipine (5). This channel type has been identified in various smooth muscles and appears to be activated by receptor-linked G proteins that can be modulated by increases in [Ca2+]i (17). Hence, ET-1 receptor channel events that initiate depolarization can lead to Ca entry and increased [Ca2+]i. In turn, this may add to the process by further stimulating Ca-dependent cation channels. Likewise, ET-1-induced Ca release from SR could promote Ca entry via Ca-dependent regulation of nonselective cation channels (61). Thus ET-receptor channel mechanisms and ET-receptor PLC coupling would contribute to Ca entry and dependence of [Ca2+]i on extracellular Ca concentration during both phasic and tonic responses to ET-1.

In our experiments, the contribution of changes in [Ca2+]i to the contractile process was controlled by buffering [Ca2+]i in α-toxin permeabilized arteries. With protocols such as that shown in Fig. 1, we were able to determine the ET1 concentration dependence of Ca sensitization at constant [Ca2+]i. As shown in Fig. 3, the EC50 for contraction in permeabilized vessels was similar to that under intact conditions in which both Ca entry and Ca sensitization occur. The analysis was conducted after the ET1 responses stabilized (tonic conditions in which both Ca entry and Ca sensitization were operative under physiologically relevant conditions. Rather than operating only at high ET1, Ca sensitization mechanisms are essential for normal biological responses over an extended range of ET1 concentrations.

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