Endothelin Increases Myofilament Ca$^{2+}$ Sensitivity in $\alpha$-Toxin–Permeabilized Rabbit Mesenteric Artery

Junji Nishimura, Suzanne Moreland, Hee Yul Ahn, Tomoyuki Kawase, Robert S. Moreland, and Cornels van Breemen

This study was designed to investigate the mechanism of endothelin-1 (ET-1) contractions in Staphylococcus $\alpha$-toxin–permeabilized vascular smooth muscle. Rabbit small mesenteric arteries permeabilized with $\alpha$-toxin were mounted for isometric or isotonic force recording or were processed for determination of myosin light chain (MLC) phosphorylation levels. Addition of 100 nM ET-1 plus 10 $\mu$M GTP significantly enhanced myofilament Ca$^{2+}$ sensitivity as compared with the addition of Ca$^{2+}$ alone (EC$_{50}$, 0.47 $\mu$M Ca$^{2+}$ for Ca$^{2+}$ alone and 0.13 $\mu$M Ca$^{2+}$ for ET-1 plus GTP). This enhanced sensitivity was reversed by GTPxS. ET-1-induced contractions were relaxed at a constant [Ca$^{2+}$] by the addition of 30 $\mu$M cAMP or cGMP, demonstrating a direct effect of the cyclic nucleotides on contractile regulation. Inhibition of protein kinase C activity by 100 nM staurosporine relaxed ET-1 plus GTP-induced contractions, and pretreatment with 40 $\mu$M chelerythrine inhibited the ET-1 plus GTP increase in force. At 0.32 $\mu$M Ca$^{2+}$, steady-state levels of shortening velocity were not increased by ET-1 plus GTP, although steady-state levels of MLC phosphorylation were significantly enhanced. The ET-1-induced increase in MLC phosphorylation was not altered by changes in [Ca$^{2+}$], whereas the shortening velocity was Ca$^{2+}$ dependent, suggesting that the increase MLC phosphorylation may be the result of protein kinase C, rather than MLC kinase, activation. These results are consistent with the hypothesis that ET-1 increases myofilament Ca$^{2+}$ sensitivity by a $G$ protein–dependent pathway and subsequent activation of protein kinase C. We propose that protein kinase C increases myofilament Ca$^{2+}$ sensitivity during ET-1 stimulation either by phosphorylation of a thin-filament regulatory protein or by downregulation of the MLC phosphatase. (Circulation Research 1992;71:951–959)

KEY WORDS • myosin light chain phosphorylation • crossbridge cycling • myofilament Ca$^{2+}$ sensitivity • protein kinase C • endothelin • vascular smooth muscle

Endothelin-1 (ET-1) is a potent naturally occurring vasoconstrictor, which was first isolated by Yanagisawa et al$^1$ from cultured porcine endothelial cells. Since this initial discovery, ET-1 has been shown to stimulate contraction of many smooth muscle tissues.$^2$ Moreover, ET-1 is now known to be only one of a family of peptides, including endothelin-2, endothelin-3, and the sarafotoxins.$^3$ Contraction of smooth muscle in response to ET-1 was originally proposed to be mediated by calcium influx through voltage-dependent calcium channels.$^4$ Subsequent studies have shown that the calcium source is tissue and species specific, because ET-1–induced contractions can be supported by calcium influx through dihydropyridine-sensitive or -insensitive calcium channels or by inositol 1,4,5-trisphosphate–triggered release of intracellular calcium, depending on the smooth muscle cell studied.$^2,^3$

Regardless of the source of calcium, a primary step in the initiation of a smooth muscle contraction is phosphorylation of the 20-kd myosin light chain (MLC) catalyzed by the Ca$^{2+}$- and calmodulin-dependent MLC kinase, which is widely believed to be the primary regulator of smooth muscle contraction.$^3$ Morgan and Morgan$^6$ were the first to demonstrate another type of regulation. These investigators showed that the Ca$^{2+}$ sensitivity of contraction is increased during agonist activation of vascular smooth muscle as compared with membrane depolarization by KCl. The phenomenon of Ca$^{2+}$ sensitization has since been shown to occur in numerous intact smooth muscle tissues in response to a variety of agonists.$^7$ Of particular importance to this study was the work of Karaki and coworkers$^8$ who demonstrated that ET-1 stimulation of smooth muscle significantly increased the Ca$^{2+}$ sensitivity of contraction.

Although the simultaneous measurement of force and intracellular [Ca$^{2+}$] in intact smooth muscle has provided important information concerning agonist-
induced changes in Ca\textsuperscript{2+} sensitivity, it was not until the development of the _Staphylococcus_ \(\alpha\)-toxin–permeabilized preparation that the intracellular mechanisms involved could be examined. Nishimura et al.\textsuperscript{10} first demonstrated that \(\alpha\)-toxin–permeabilized vascular smooth muscle retained receptor- and G protein–dependent activation systems. Agonist activation of the \(\alpha\)-toxin–permeabilized tissue, in the presence of GTP, was shown to significantly enhance myofilament Ca\textsuperscript{2+} sensitivity. This was corroborated by Kitazawa et al.\textsuperscript{10} in both vascular and nonvascular smooth muscle. Permeabilization of smooth muscle with \(\alpha\)-toxin offers distinct advantages over detergent or glycerol techniques. In the \(\alpha\)-toxin–permeabilized preparation, endogenous proteins are retained at physiological concentrations, although the ionic content of the intracellular space can be manipulated by changing the bathing medium. Another advantage of the \(\alpha\)-toxin–permeabilized preparations is the maintenance of receptor-linked signal transduction systems.

The goal of this study was to use the \(\alpha\)-toxin–permeabilized small mesenteric artery to examine the mechanism(s) involved in the ET-1–induced increase in myofilament Ca\textsuperscript{2+} sensitivity. The dependence of this enhanced Ca\textsuperscript{2+} sensitivity on G proteins and the possible role of protein kinase C as a mediator of the enhancement were investigated in this study. We also examined the effect of cyclic nucleotides as modulators of a contraction in response to ET-1. In addition, the crossbridge parameters of stiffness, maximal shortening velocity, and MLC phosphorylation were determined during ET-1–induced contractions and during contractions in response to Ca\textsuperscript{2+} alone.

**Materials and Methods**

Adult male New Zealand White rabbits (2–5 kg) were killed by CO\textsubscript{2} inhalation with subsequent exsanguination. The mesenteric arteries in the region of the jejunum were removed and placed in aerated (100% O\textsubscript{2}) physiological salt solution (PSS). Second- and third-order branches of the mesenteric artery (=250 \(\mu\)m o.d.) were isolated and cleaned of adhering fat and connective tissue. The composition of PSS for the experiments examining force was (mM) NaCl 140, KCl 5, CaCl\textsubscript{2} 1.5, MgCl\textsubscript{2} 1, dextrose 10, and HEPES 5, pH 7.2. In the experiments examining stiffness, shortening velocity, and MLC phosphorylation levels, the PSS contained (mM) NaCl 140, KCl 4.7, CaCl\textsubscript{2} 1.6, MgSO\textsubscript{4} 1.2, dextrose 5, Na\textsubscript{2}HPO\textsubscript{4} 1.2, MOPS 2, pH 7.4, and Na\textsubscript{2} EDTA 0.02. All experiments were performed at room temperature.

Force recordings were obtained from small rings (250–300 \(\mu\)m in width) mounted in an 0.8-ml volume chamber by means of two tungsten wires passed through the lumen of the ring; one wire was attached to the chamber, and the other was attached to a force transducer (U gage, Sinko Co., Ltd., Tokyo) mounted on a micromanipulator (model US1, Narashige, Tokyo). The tissue was stretched to a resting force that resulted in maximal active force development in response to 145 mM KCl-PSS (equimolar substitution for NaCl). The solution bathing the tissue was changed by the addition of 5 ml of a new solution to one end of the chamber while aspirating the overflow by a vacuum pump at the other end. The longitudinal axis of the tissue ring was in parallel with the direction of the flow of solution to ensure maximal luminal perfusion. Maximal contraction of the intact tissue ring was evoked by the addition of 10 \(\mu\)M norepinephrine in 145 mM KCl-PSS.

Mechanical measurements were obtained from small helical strips (2.5–4.5 mm in length, 20–80 \(\mu\)g wet weight) of the mesenteric arteries. Nearly circumferential strips were obtained to optimize cellular orientation. The tissues were mounted by means of small tweezer, one of which was connected to a force transducer and the other to a motor in an apparatus designed by Dr. Konrad Güth, Wissenschaftliche Geräte, Heidelberg. The estimation of maximal velocity of shortening \(\left(V_s\right)\) was performed by isotonic quick releases to afterloads ranging from 0.1 to 0.5 times the force at the instant of the release. The change in length between 1 and 2 seconds of isotonic release was used to estimate shortening velocity at a single afterload. Isotonic shortening velocity from at least five different releases to various afterloads was used to estimate \(V_s\).\textsuperscript{11} The estimation of tissue stiffness was performed by imposing a 500-Hz sine wave length change (0.05% tissue length for optimal active force development \([L_o]\) and measuring the resultant change in force with respect to length. The servo lever was interfaced to a microcomputer for the initiation of experimental protocols, collection of data, and data analysis (software was written by Dr. Konrad Güth and modified by Dr. Steven Petrou, University of Massachusetts Medical School, Worcester, Mass.).

Permeabilization of the mesenteric tissues was performed as previously described.\textsuperscript{9} Briefly, the tissues were exposed to 10 mM ATP for approximately 30 minutes, which resulted in a phasic contraction and desensitization of the purinergic receptors. All subsequent solutions contained a minimum of 1 mM ATP to maintain purinergic receptor desensitization. The tissues were exposed for 45 minutes to calcium-free PSS containing 10 \(\mu\)M norepinephrine, 10 mM ATP, and 2 mM EGTA to deplete intracellular calcium stores. Permeabilization of the smooth muscle cells was performed by the addition of 30 \(\mu\)g/ml _Staphylococcus_ \(\alpha\)-toxin (2,500 hemolytic units/ml) for either 15–30 minutes if obtained from the National Institutes of Health (kindly provided by Dr. Hohman) or for 60 minutes if obtained from Gibco-BRL Life Sciences. The \(\alpha\)-toxin was administered in an intracellular solution composed of (mM) EGTA 2, potassium methanesulfonate 100, MgCl\textsubscript{2} 5.38, Na\textsubscript{2}ATP 5.35, creatine phosphate 10, and Tris maleate 20, pH 7.0. Ca\textsuperscript{2+}–containing solutions were prepared by the addition of an appropriate concentration of CaCl\textsubscript{2} to the intracellular solution (all contracting solutions contained 10 mM EGTA) to produce the desired free \([Ca^{2+}]_o\). The apparent calcium EGTA association constant used for these calculations was \(10^5\text{M}^{-1}\). The calculated free \([Mg^{2+}]_o\) in all solutions was 1 mM, and the calculated total \([MgATP]_o\) was 4 mM. Calmodulin and creatine kinase were not included in any solution. The 1,000-d exclusion size of the pores formed by the \(\alpha\)-toxin ensures endogenous physiological concentrations of both proteins in the permeabilized preparations.

MLC phosphorylation levels were determined on long (=10 mm) strips of the mesenteric arteries. The strips were treated exactly as those used for force and mechanical determinations except they were not mounted. Previous studies using both intact and Triton
X-100 detergent–skinned fibers of swine carotid artery have shown that load has no effect on MLC phosphorylation levels. After permeabilization and equilibration, the tissues were exposed to an appropriate activation solution for 20 minutes, at which time they were rapidly frozen in a dry ice–acetone slurry containing 6% trichloroacetic acid. The tissues were slowly thawed to room temperature and then transferred to a vial containing 50 μl of a homogenization solution previously frozen in liquid nitrogen and composed of 1% sodium dodecyl sulfate, 10% glycerol, and 20 mM dithiothreitol. The vial, containing tissue, solution, and a small stainless-steel ball, was cooled in liquid nitrogen and then placed in a dental amalgamator (Wig-A-Bug, Crescent Dental Manufacturing, Lyons, Ill.) for homogenization of the tissue. After homogenization, the vial was allowed to reach 4°C, and the contents were transferred to a storage tube. The vial was rinsed with 50 μl homogenization solution, which was added to the storage tube and then stored at −80°C. Within 1 week of homogenization, the samples were thawed and subjected to two-dimensional electrophoresis as described previously. After electrophoresis, the separated proteins were subjected to high field intensity Western blotting to nitrocellulose membranes. Visualization of the blotted proteins was performed using a colloidal gold protein stain (Amersham Corp., Arlington Heights, Ill.) as described by Kitazawa et al. Quantitation of the stained blots was performed by scanning densitometry of the nitrocellulose paper made transparent by immersion in decalin, as described by Maruyama et al. Values are reported as moles inorganic phosphate (P_i) per mole MLC by integration of the spot corresponding to the phosphorylated MLC as a percent of the total of both the phosphorylated and unphosphorylated MLC.

The [Ca^{2+}]_i necessary to produce a half-maximal response (EC_{50}) was calculated by the four-parameter logistic equation described by DeLean et al. All values are expressed as mean±SEM. Group mean values were compared using unpaired two-tailed Student’s t test. A value of p<0.05 was taken as significant.

Results

The α-toxin–permeabilized smooth muscle preparation retains functional sarcolemmal receptors coupled through a G protein system to signal transduction pathways. Adrenergic and cholinergic receptor activation in the presence of GTP or GDPβS result in enhanced myofilament Ca^{2+} sensitivity in α-toxin–permeabilized tissues. Figure 1A shows a representative tracing of an experiment designed to examine the G protein–dependent Ca^{2+}-sensitizing action of ET-1. A small increase in force was observed on addition of a low [Ca^{2+}]_i (0.18 μM) buffered by 10 mM EGTA. The addition of 100 nM ET-1 caused a significant, but variable, increase in the level of force developed (31±10% of the maximal Ca^{2+}-induced force), which could be relaxed by 1 mM GDPβS. Figure 1B shows a representative tracing of a similar experiment. The addition of GTP to the fiber significantly increased the ET-1–induced enhancement of force (73±2% of maximal Ca^{2+}-induced force) and abolished the tissue variability. Presumably, the tissue variability resulted from the different endogenous [GTP] remaining in the fibers after α-toxin permeabilization. This tracing also demonstrates that the increase in force in response to ET-1 plus GTP was reversed by the addition of 1 mM GDPβS.

Experiments similar to that depicted in Figure 1B were performed at various levels of [Ca^{2+}]_i. The addition of 100 nM ET-1 plus 10 μM GTP increased the myofilament sensitivity over the entire range of [Ca^{2+}]_i examined (Figure 2). The EC_{50} value for contraction in response to Ca^{2+} alone was 0.47±0.06 μM. In the presence of 100 nM ET-1 plus 10 μM GTP, a significantly lower EC_{50} value of 0.13±0.01 μM Ca^{2+} was calculated (p<0.05).

The results shown in Figures 1 and 2 demonstrate that a combination of ET-1 plus GTP significantly enhances myofilament Ca^{2+} sensitivity. The mechanism for this receptor-coupled sensitization is unknown. To investigate the possible involvement of protein kinase C in the ET-1 plus GTP–induced increase in Ca^{2+} sensitivity, we used the relatively specific protein kinase C inhibitors staurosporine (Figure 3) and chelerythrine (Figure 4). Staurosporine (100 nM) had no significant effect on steady-state force maintenance in response to 0.5 μM Ca^{2+} alone. Under conditions of similar force development, staurosporine produced a significant relaxation of strips contracted with ET-1 plus GTP (lower [Ca^{2+}]_i than for Ca^{2+} alone). Because nonspecific effects of staurosporine could account for the relaxation of force in response to ET-1 plus GTP, another protein kinase C inhibitor, chelerythrine, was tested. Figure 4 shows that pretreatment of the α-toxin–permeabilized

![Figure 1](https://example.com/image1.png)

**Figure 1.** Effect of GTP and GDPβS on contractions induced by endothelin-1 (ET-1) in the α-toxin–permeabilized rabbit mesenteric artery. Panel A: Representative tracing showing the effect of 100 nM ET-1 on a Ca^{2+}-induced contraction. The addition of ET-1 significantly increased force development, which was reversed by the addition of 1 mM GDPβS. Panel B: Representative tracing showing the effect of 10 μM GTP on the augmented force development in response to 100 nM ET-1. GTP significantly increased the ET-1 enhancement of the Ca^{2+}-dependent contraction. Similar to the tracing in panel A, the addition of 1 mM GDPβS relaxed the ET-1 plus GTP increase in force development. Tracings are representative of three or more experiments.
ET-1 plus GTP produced approximately 90% of maximal force in the absence of chelerythrine at this [Ca\(^{2+}\)], as shown in Figure 2. The relaxation by staurosporine of force in response to ET-1 plus GTP but not in response to Ca\(^{2+}\) alone and the inhibition by chelerythrine of the increase in force by ET-1 plus GTP suggest the possible involvement of protein kinase C in the agonist-induced increase in myofilament Ca\(^{2+}\) sensitivity. However, another staurosporine- and chelerythrine-sensitive mechanism cannot be completely ruled out.

The MLC kinase/phosphatase system is known to be a primary component of contractile regulation as well as crossbridge kinetics in smooth muscle. Figure 5 shows the results of experiments designed to determine the level of MLC phosphorylation and the mechanical parameters of stiffness and \(V_0\) during contractions in response to 0.32 \(\mu\)M Ca\(^{2+}\) alone and with the inclusion of 100 nM ET-1 plus 10 \(\mu\)M GTP. The significant increase in stiffness in ET-1 plus GTP as compared with Ca\(^{2+}\) alone demonstrated that the enhanced force was the result of an increased number of attached cross-bridges. Steady-state levels of \(V_0\) were not affected by ET-1 plus GTP (0.016 ± 0.001 L\(_{\text{os}}\)/sec in Ca\(^{2+}\) alone, 0.017 ± 0.003 L\(_{\text{os}}\)/sec in ET-1 plus GTP; \(p > 0.05\)). Of particular interest was the significant increase in the steady-state levels of MLC phosphorylation as a result of ET-1 plus GTP stimulation in the presence of 0.32 \(\mu\)M Ca\(^{2+}\) (0.18 ± 0.02 mol P\(_{\text{i}}\)/mol MLC at rest, 0.36 ± 0.03 mol P\(_{\text{i}}\)/mol MLC in Ca\(^{2+}\) alone, and 0.50 ± 0.04 mol P\(_{\text{i}}\)/mol MLC in ET-1 plus GTP; \(p < 0.05\)).

Because the addition of ET-1 plus GTP increased steady-state MLC phosphorylation levels but not \(V_0\), we examined the Ca\(^{2+}\) dependence of both these parameters. At 0.5 \(\mu\)M Ca\(^{2+}\), the steady-state estimates of \(V_0\) were 0.028 ± 0.001 L\(_{\text{os}}\)/sec in the presence of Ca\(^{2+}\) alone and 0.024 ± 0.003 L\(_{\text{os}}\)/sec in the presence of Ca\(^{2+}\) plus ET-1 plus GTP (\(p > 0.05\)); these estimates were higher than the values collected at 0.32 \(\mu\)M Ca\(^{2+}\). Therefore, \(V_0\) was Ca\(^{2+}\) dependent but not affected by the addition of ET-1 plus GTP. Alterations in the [Ca\(^{2+}\)] significantly affected steady-state MLC phosphorylation levels in response to Ca\(^{2+}\) alone but not steady-state MLC phosphorylation levels in response to Ca\(^{2+}\) plus ET-1 plus GTP. Stimulation by Ca\(^{2+}\) alone increased steady-state MLC phosphorylation levels from a basal value of 0.18 ± 0.02 mol P\(_{\text{i}}\)/mol MLC to 0.24 ± 0.04 mol P\(_{\text{i}}\)/mol MLC with 0.09 \(\mu\)M Ca\(^{2+}\), 0.26 ± 0.03 mol P\(_{\text{i}}\)/mol MLC with 0.18 \(\mu\)M Ca\(^{2+}\), and 0.36 ± 0.03 mol P\(_{\text{i}}\)/mol MLC with 0.3 \(\mu\)M Ca\(^{2+}\). In contrast, stimulation by Ca\(^{2+}\) plus ET-1 plus GTP increased steady-state MLC phosphorylation levels from the same basal value to 0.46 ± 0.09 mol P\(_{\text{i}}\)/mol MLC with 0.09 \(\mu\)M Ca\(^{2+}\), 0.46 ± 0.09 mol P\(_{\text{i}}\)/mol MLC with 0.18 \(\mu\)M Ca\(^{2+}\), and 0.50 ± 0.04 mol P\(_{\text{i}}\)/mol MLC with 0.3 \(\mu\)M Ca\(^{2+}\).

Activation of \(\alpha\)-toxin–permeabilized vascular smooth muscle has been previously shown to be modulated by cyclic nucleotides. Figure 6 shows representative tracings from experiments designed to examine the potential modulatory role of cAMP and cGMP in contractions in response to ET-1 plus GTP. The addition of 30 \(\mu\)M cAMP or cGMP resulted in a significant relaxation of the contraction in response to 0.18 \(\mu\)M Ca\(^{2+}\) plus ET-1 plus GTP. The relatively high concentrations of cAMP and cGMP required to induce relaxation may be due in part to the activity of cyclic nucleotide phospho-

**Figure 2.** Graph showing the Ca\(^{2+}\)-force relation in the absence (○) and presence (●) of 100 nM endothelin-1 (ET-1) plus 10 \(\mu\)M GTP in the rabbit mesenteric artery. The addition of agonist plus GTP significantly increased the myofilament Ca\(^{2+}\) sensitivity for contraction. Values were normalized as a percent of the maximal contraction in response to 10 \(\mu\)M Ca\(^{2+}\) and are expressed as mean±SEM of three to five determinations.

**Figure 3.** Tracings showing the effect of staurosporine in \(\alpha\)-toxin–permeabilized rabbit mesenteric arteries contracted by 0.5 \(\mu\)M Ca\(^{2+}\) alone (panel A) or 0.18 \(\mu\)M Ca\(^{2+}\) plus 100 nM endothelin-1 (ET-1) plus 10 \(\mu\)M GTP (panel B). The addition of staurosporine to contractions of similar magnitude produced relaxation in the case of the ET-1 plus GTP–induced force but had no effect on the force induced by Ca\(^{2+}\) alone. These tracings are representative of at least three experiments.
diestases. This suggestion is supported by the tracing in Figure 7 showing that 10-fold lower concentrations of the nonhydrolyzable 8-bromo analogues of cAMP and cGMP produced similar relaxations. The contracting solution in this as in all experiments described in this study was buffered with 10 mM EGTA. In addition, in selected experiments the Ca<sup>2+</sup> ionophore A23187 or ionomycin was included in all solutions to ensure that the sarcoplasmic reticulum was not involved in any of the responses studied. Therefore, the cyclic nucleotide–induced relaxation of the ET-1 plus GTP contraction did not result from a change in the cytosolic [Ca<sup>2+</sup>]; rather, the cyclic nucleotides must directly modulate the mechanism(s) responsible for the Ca<sup>2+</sup> sensitivity.

**Discussion**

The α-toxin–permeabilized smooth muscle preparation was first described by Kerrick and colleagues. More recently, it was found that this preparation retains receptor- and G protein–coupled alterations in the myofilament Ca<sup>2+</sup> sensitivity. This may be related to the fact that α-toxin forms small pores of approximately 2–3 nm in diameter, which admit small ions and water-soluble molecules but do not allow diffusional removal of essential cytoplasmic proteins. Therefore, in sharp contrast to many other hyperpermeabilized preparations, integral regulatory components such as calmodulin- and cyclic nucleotide–dependent protein kinases are maintained at physiological concentrations. Using this preparation, we and others have demonstrated that GTP-dependent agonist stimulation enhances the Ca<sup>2+</sup> sensitivity of force development.

In this study, we have extended these earlier findings to demonstrate that ET-1, through a G protein–coupled system, significantly enhances the Ca<sup>2+</sup> sensitivity of myofilaments in rabbit small mesenteric arteries. Moreover, we have demonstrated that this enhancement of force generation is sensitive to staurosporine and chelerythrine, suggesting either a role for protein kinase C in the increased Ca<sup>2+</sup> sensitivity or, at minimum, a staurosporine- and chelerythrine-sensitive mechanism not involved in contractions elicted by Ca<sup>2+</sup> alone. Our results demonstrate that an increased number of attached crossbridges supports the increased force, that V<sub>o</sub> does not increase concomitantly with the Ca<sup>2+</sup> sensitivity, and that total MLC phosphorylation is significantly increased during steady-state force maintenance.

![Figure 4](http://circres.ahajournals.org/)

**Figure 4.** Tracing showing the effect of chelerythrine in α-toxin–permeabilized rabbit mesenteric arteries contracted by 0.3 μM Ca<sup>2+</sup> plus 100 nM endothelin-1 (ET-1) plus 10 μM GTP. After a 1-hour incubation in relaxing solution containing 40 μM chelerythrine, 0.3 μM Ca<sup>2+</sup> elicited force that developed to a level similar to that reached in response to the same [Ca<sup>2+</sup>] before chelerythrine exposure. The addition of 100 nM ET-1 plus 10 μM GTP did not increase force in the presence of chelerythrine. The [Ca<sup>2+</sup>] was buffered by 10 mM EGTA in these experiments.

![Figure 5](http://circres.ahajournals.org/)

**Figure 5.** Bar graph showing the effect of 100 nM endothelin-1 plus 10 μM GTP on force (F), stiffness (S), myosin light chain (MLC) phosphorylation (P), and maximal velocity of shortening (V<sub>e</sub>). P<sub>i</sub>, inorganic phosphate; L<sub>x</sub>, tissue length for optimal active force development. The [Ca<sup>2+</sup>] was 0.32 μM and was buffered by 10 mM EGTA. All measurements were performed in the steady state 20 minutes after activation with Ca<sup>2+</sup> alone (open bars) or with endothelin-1 plus GTP (+Endothelin bars). Determinations of force and stiffness were normalized as a percent of the maximal response to 10 μM Ca<sup>2+</sup>. All values are the mean±SEM for four to six determinations. *p<0.05 as compared with Ca<sup>2+</sup> alone.

![Figure 6](http://circres.ahajournals.org/)

**Figure 6.** Effects of cAMP and cGMP on contractions in response to 0.18 μM Ca<sup>2+</sup> plus 100 nM endothelin-1 (ET-1) plus 10 μM GTP in the α-toxin–permeabilized mesenteric artery. Representative tracings of at least three experiments show the relaxant action of 30 μM cAMP (panel A) or 30 μM cGMP (panel B) during steady-state force maintenance in response to ET-1 plus GTP. In both experiments, the [Ca<sup>2+</sup>] was buffered by 10 mM EGTA.
similarities between the ET-1 and norepinephrine contractions of α-toxin–permeabilized fibers suggest related transduction pathways, although our results do not specifically address this question. However, Takuwa et al.21 have reported that the ET-1 receptor is coupled to a pertussis toxin–insensitive G protein, whereas Boonen and De Mey22 have reported that the norepinephrine receptor is coupled to a pertussis toxin–sensitive G protein (but compare with Reference 23). At the present, it is not possible to rule out that ET-1 and norepinephrine activate different G proteins and different signal transduction systems and that this activation results in similar increases in myofilament Ca2+ sensitivity.

As has been previously reported for Ca2+- and phorbol ester–induced contractions,17 the ET-1–induced force was also relaxed by cyclic nucleotides, suggesting a cyclic nucleotide–dependent desensitization of the myofilaments. Ozaki et al.24 have recently corroborated this finding in canine gastric smooth muscle. These investigators demonstrated that cAMP (3–300 μM) relaxed α-toxin–permeabilized gastric smooth muscle at a constant intracellular [Ca2+]. Although it is generally accepted that in intact fibers cAMP acts primarily by lowering intracellular [Ca2+],25 the relaxations recorded in α-toxin–permeabilized fibers were not accompanied by changes in cytosolic [Ca2+] because of the presence of 10 mM EGTA and the Ca2+ ionophore ionomycin. The rather high concentrations of cAMP and cGMP required to induce relaxation in our study, although consistent with our previous results17 and those of Ozaki et al.,24 may have been due to the fact that exogenously applied cyclic nucleotides were rapidly broken down by endogenous phosphodiesterases. In support of this possibility is our finding that 10-fold lower concentrations of 8-bromo-cAMP and 8-bromo-cGMP were equally effective in relaxing the ET-1–contracted fibers. The physiological relevance of the observed downregulation of myofilament Ca2+ sensitivity by cAMP and cGMP requires further study.

The precise mechanism(s) by which the ET-1 activation of G protein increases myofilament Ca2+ sensitivity is unknown. The results of this study demonstrate that staurosporine relaxes and chelerythrine inhibits the enhanced force development, suggesting the involvement of protein kinase C. Using intact vascular smooth muscle, we have previously demonstrated that staurosporine at concentrations in the range of 100 nM does not affect MLC phosphorylation levels during ET-1 stimulation.26 In addition, we did not observe a relaxation when this concentration of staurosporine was added to an α-toxin–permeabilized tissue contracted in response to Ca2+ alone. Chelerythrine has been reported to be more selective for inhibition of protein kinase C than are the other more commonly used inhibitors.27 This increased selectivity occurs primarily because chelerythrine competes with substrate for the active site of protein kinase C and does not inhibit ATP binding, the mechanism by which staurosporine inhibits protein kinase C activity.28 The effects of chelerythrine on MLC kinase activity have not, to our knowledge, been examined; however, the finding that chelerythrine did not attenuate a contraction in response to Ca2+ alone would suggest that, at the concentrations and conditions used in this study, inhibition of MLC kinase activity was not involved. Therefore, the effects of

---

**Figure 7.** Effects of nonhydrolyzable cyclic nucleotides on contractions in response to 0.18 μM Ca2+ plus 100 nM endothelin-1 (ET-1) plus 10 μM GTP in the α-toxin–permeabilized mesenteric artery. Representative tracings of experiments show the relaxant action of 3 μM 8-bromo-cAMP (8Br-cAMP, panel A) or 3 μM 8-bromo-cGMP (8Br-cGMP, panel B) during steady-state force maintenance in response to ET-1 plus GTP. The control time course of contraction in response to 0.18 μM Ca2+ plus ET-1 plus GTP is also shown (panel C). In all experiments, the [Ca2+] was buffered by 10 mM EGTA.

The conclusion that ET-1 increases Ca2+ sensitivity through a G protein–dependent process is based, in part, on the finding that the response to ET-1 is significantly augmented by the addition of GTP. ET-1 alone induced some increase in Ca2+ sensitivity, most likely in conjunction with endogenous GTP remaining in the artery after permeabilization. This conclusion is supported by the demonstration that, on repeated norpinephrine stimulation of α-toxin–permeabilized fibers in the absence of exogenous GTP, the enhancement of force declines as a function of the number of contraction–relaxation cycles (authors' unpublished observations). The mandatory role of G proteins is also suggested by the finding that activation by either ET-1 alone or by ET-1 plus GTP was reversed by GDPβS. Similar GDPβS–induced relaxation of adrenergic- and cholinergic-stimulated force has been observed in several α-toxin–permeabilized smooth muscles.9,10,20 The magnitude of the ET-1–induced increase in the Ca2+ sensitivity of force is similar to that previously demonstrated after norpinephrine activation of α-toxin–permeabilized rabbit mesenteric arteries.19 Overall,
Stauroporine and chelerythrine may be specifically due to inhibition of protein kinase C, although we cannot totally rule out another mechanism that is sensitive to both compounds but not activated during contractions stimulated by Ca²⁺ alone.

Several other groups have also suggested an important role for protein kinase C in ET-1–induced contractions. Griending et al. demonstrated that ET-1 stimulation of cultured rat aortic smooth muscle cells was associated with a large increase in diacylglycerol and activation of protein kinase C. Ohlstein et al. showed that 100 nM stauroporine inhibited ET-1–induced contractions of intact rabbit thoracic aorta in response to ET-1, analogous to the results in our study using permeabilized rabbit arteries. ET-1 contractions of intact rat aortas are also stauroporine sensitive, and ET-1 induces a significant increase in diacylglycerol content in cultured smooth muscle cells. Taken together, these studies strongly suggest that activation of protein kinase C is intimately involved in the cascade of events following ET-1 receptor activation.

An alternative explanation for the enhanced myofilament Ca²⁺ sensitivity in response to ET-1 activation involves a central role for a regulated MLC phosphatase. Kuriyama and coworkers were the first to demonstrate that the GTPγS-dependent enhancement of Ca²⁺ sensitivity in permeabilized smooth muscle was accompanied by increased MLC phosphorylation levels. This increase in MLC phosphorylation, measured either in the presence of GTPγS or at the peak of force development in response to an agonist plus GTP, has been corroborated in other laboratories. We have also observed an increase in MLC phosphorylation levels in α-toxin–permeabilized fibers stimulated with GTPγS as compared with those stimulated with Ca²⁺ alone (authors’ unpublished observations). Somlyo and colleagues have suggested that agonist- and G protein–dependent activation “downregulates” MLC phosphatase activity, resulting in increased MLC phosphorylation levels at any given [Ca²⁺]. This hypothesis is based on the increase in MLC phosphorylation observed in the early phase of contractions of permeabilized smooth muscle. On the similarity of the increase in myofilament Ca²⁺ sensitivity between agonist-induced force and that developed in the presence of the phosphatase inhibitor okadaic acid, and on the decrease in the rate of dephosphorylation during relaxation of a GTPγS-induced contraction as compared with a contraction in response to Ca²⁺ alone. Our results demonstrating a significant increase in the steady-state levels of MLC phosphorylation are in apparent agreement with this hypothesis. However, we have recently shown that MLC phosphorylation levels during a contraction of the α-toxin–permeabilized mesenteric artery in response to norepinephrine plus GTP at constant [Ca²⁺] (0.3 μM) are only transiently elevated, falling with time to levels similar to those in response to the same [Ca²⁺] alone. Therefore, G protein–dependent downregulation of an MLC phosphatase may be important in the initial development of a contraction, but it apparently cannot account for the maintenance of the agonist-induced contraction.

If the ET-1 plus GTP–induced increase in MLC phosphorylation is at serine-19, as recently shown for the initial phase of a contraction of permeabilized tracheal tissue, then these results are not consistent with a primary role for MLC phosphorylation in the regulation of crossbridge cycling. Since we found that Vₐₕ was not enhanced by ET-1 plus GTP. However, it is important to note that the role of serine-19 MLC phosphorylation in the regulation of crossbridge cycling is controversial. Activation of protein kinase C by stimulation with phorbol esters is known to result in MLC phosphorylation at sites other than serine-19. In the present study, we did not determine the site of phosphate incorporation into the MLC; however, the increase in MLC phosphorylation was not Ca²⁺ sensitive, suggesting that MLC kinase may not be responsible for the phosphorylation. In contrast, Adam et al. have shown by phosphopeptide mapping that, in ET-1–contracted swine carotid artery strips, phosphorylation of the MLC was primarily at sites consistent with activation of MLC kinase; only 17% of the MLC phosphorylation was at sites consistent with activation of protein kinase C. It is possible that the well-described tissue and species diversity in the mechanisms of ET-1–induced contractions of vascular tissues (see Reference 27) accounts for the difference in the results of Adam et al. and of the present study.

If protein kinase C is involved in the ET-1 plus GTP–induced enhancement of myofilament Ca²⁺ sensitivity, as suggested by the effects of stauroporine and chelerythrine, then there are several possible mechanisms by which this may occur. Phosphorylation of MLC by protein kinase C inhibits MLC kinase–catalyzed MLC phosphatase and decreases actin-activated myosin ATPase activity. However, protein kinase C–catalyzed MLC phosphorylation is not believed to be important physiologically. Therefore, effects of protein kinase C on the thick filaments do not appear to be likely. Although not addressed in the present study, it is interesting to speculate that the putative thin-filament regulatory proteins, calponin and caldesmon, may be involved in the agonist-induced increase in myofilament Ca²⁺ sensitivity. Unphosphorylated calponin can bind to actin and significantly depress actin-activated myosin ATPase activity. The inhibition is reversed after calponin phosphorylation by either Ca²⁺–calmodulin–dependent protein kinase II or protein kinase C. Unphosphorylated caldesmon has also been shown to depress actin-activated myosin ATPase activity; an increase in cytosolic Ca²⁺ or phosphorylation by either Ca²⁺–calmodulin–dependent protein kinase II or protein kinase C reverses this inhibition. Increased protein kinase C activity may therefore relieve the inhibitory effects of caldesmon and account for the ET-1 plus GTP–induced increase in force shown in our study. Consistent with this hypothesis is the work of Adam et al. and Abe et al., who demonstrated a sustained increase in caldesmon phosphorylation after stimulation of intact vascular smooth muscle with ET-1.

Another possible explanation for the ET-1 plus GTP increase in Ca²⁺ sensitivity is that protein kinase C catalyzes phosphorylation of the MLC phosphatase (or activates a phosphatase inhibitor by phosphorylation), which results in downregulation of the enzymatic activity. This mechanism would integrate the role for protein kinase C described in this study with the idea of a regulated MLC phosphatase proposed by Somlyo and coworkers. This is an attractive hypothesis that is
consistent with the increase in MLC phosphorylation levels during steady-state force shown in the present study in response to ET-1 stimulation as well as the early increase in MLC phosphorylation levels in response to adrenergic stimulation.\textsuperscript{10,34,36} Moreover, this mechanism could explain the similarity between agonist-induced and phorbol ester–induced contractions and the staurosporine and chelerythrine sensitivity of the enhanced force development. Whether this mechanism is plausible will require further investigation.

In summary, the results of this study have demonstrated that ET-1 stimulation increases myofilament Ca\textsuperscript{2+} sensitivity by a G protein–dependent mechanism in the α-toxin–permeabilized artery. The ET-1–induced increase in force development can be relaxed by cyclic nucleotides without changing the cytosolic [Ca\textsuperscript{2+}]. The increased force can be reversed by staurosporine or blocked by chelerythrine, presumably by inhibition of protein kinase C activity. The enhanced force development in response to ET-1 exposure is supported by an increased number of attached crossbridges over those activated by the same [Ca\textsuperscript{2+}] alone. Although the steady-state levels of MLC phosphorylation are increased during ET-1 stimulation, this increase appears to be Ca\textsuperscript{2+} insensitive. Steady-state crossbridge cycling rates are not changed by addition of ET-1 plus GTP. These findings are consistent with the hypothesis that ET-1 receptor occupation induces activation of protein kinase C, which in turn phosphorylates an integral regulatory protein, possibly calponin, caldesmon, or MLC phosphatase. Phosphorylation of the regulatory protein results in an increased myofilament Ca\textsuperscript{2+} sensitivity.

Acknowledgment

The authors would like to express their sincere thanks to Dr. R.J. Hohman for generously providing α-toxin.

References


18. Cassidy P, Hoar PE, Kerrick WGL: Irreversible thio phosphorylation and activation of tension in functionally skinned rabbit ileum strips by \textsuperscript{35}S\textsuperscript{3}ATP\textsuperscript{3}Y. J Biol Chem 1979;254:11148–11153


22. Boonen HCM, De Mey JGR: G-proteins are involved in contractile responses of isolated mesenteric resistance arteries to agonists. Naunyn Schmiedebergs Arch Pharmacol 1990;342:462–468


Endothelin increases myofilament Ca\textsuperscript{2+} sensitivity in alpha-toxin-permeabilized rabbit mesenteric artery.

J Nishimura, S Moreland, H Y Ahn, T Kawase, R S Moreland and C van Breemen

doi: 10.1161/01.RES.71.4.951

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1992 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/71/4/951

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/