Endothelin 1 Enhances Myofilament Ca\(^{2+}\) Responsiveness in Aequorin-Loaded Ferret Myocardium

Jianxun Wang, George Paik, and James P. Morgan

The influence of endothelin 1 on intracellular Ca\(^{2+}\) transients and isometric contractions was investigated in ferret papillary muscles loaded with the Ca\(^{2+}\)-regulated bioluminescent indicator aequorin. In concentrations of \(3\times10^{-9}\) to \(1\times10^{-7}\) M, endothelin produced dose-dependent increases in the amplitudes of both aequorin light signals (maximum, \(31\pm12\%\)) and developed tension (maximum, \(64\pm13\%\)). The peak aequorin light (\([Ca^{2+}]_i\))–peak tension curve generated by increasing endothelin concentrations was steeper and shifted to the left of the curve generated by varying \([Ca^{2+}]_o\); however, the maximum developed tension produced by endothelin did not exceed that produced by 6 mM [Ca\(^{2+}\)]. The effect of endothelin on the amplitude of the aequorin light signal was less than the effect of [Ca\(^{2+}\)], for similar levels of tension development. Moreover, \(1\times10^{-7}\) M endothelin caused an upward shift in the peak aequorin light–peak tension curve generated by varying [Ca\(^{2+}\)], and increased the maximum twitch force by about 12%. The contractions were prolonged, whereas the time course of the Ca\(^{2+}\) transient was not changed in the presence of endothelin. When the function of the sarcoplasmic reticulum was inhibited by 6 \(\mu\)M ryanodine, \(10^{-7}\) M endothelin still increased the force generation without increasing the intracellular peak Ca\(^{2+}\), either during isometric twitches or during tetani. Additional results were as follows: 1) \(\beta\)-receptor blockade with (±)-bupranolol (3 \(\times10^{-7}\) M) or \(\alpha\)-receptor blockade with prazosin (5 \(\times10^{-8}\) M) did not influence the effect of endothelin on Ca\(^{2+}\) transients or force generation, and 2) endothelin did not exert its myocardial effects in saponin-skinned ferret papillary muscles. These results suggest that, although Ca\(^{2+}\) release from the sarcoplasmic reticulum contributes to a small degree, the positive inotropic effects of endothelin 1 on intact ferret myocardium are predominantly due to an increase in the Ca\(^{2+}\) responsivity of the myofilaments. We hypothesize that an endothelin receptor–second messenger pathway may be the mechanism by which endothelin 1 exerts its myocardial effects.

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Endothelin is a newly discovered intrinsic inotropic factor that is known to elicit marked constrictor activity in isolated mammalian blood vessels\(^1\)–\(^3\) and has also recently been shown to potentiate both animal and human myocardial contractions.\(^4\)–\(^7\) It has been reported that the density of myocardial endothelin receptors is high\(^8\)–\(^10\) and that the removal of endocardial endothelium modifies the amplitude and time course of contractions.\(^11\) The release of endothelin from endothelial cells is increased under certain conditions, such as hypoxia and stretch, and in the presence of angiotensin II and thrombin.\(^2\) In patients with myocardial infarction\(^12\) and cardiogenic shock,\(^13\) the blood levels of endothelin have been reported to be elevated. Therefore, it is reasonable to propose that endothelin may have an important regulatory role on myocardial contraction and relaxation in vivo.

Endothelin exerts its effects on smooth muscle through increased Ca\(^{2+}\) entry via sarcolemmal voltage-dependent Ca\(^{2+}\) channels\(^14\)–\(^16\); however, the mechanism by which endothelin enhances myocardial contractility remains unclear. In general, changes in the degree of myocardial activation may result from 1) changes in the intracellular Ca\(^{2+}\) concentration (i.e., [Ca\(^{2+}\)]) or 2) changes in the
responsiveness of myofilaments to activator Ca^{2+}. The present study was designed to determine whether one or both of these mechanisms are involved in endothelin's cardiotonic actions.

Materials and Methods

Experiments \((n=20)\) were performed on papillary muscles isolated from the right ventricles of young adult male ferrets (age, 14–16 weeks; body weight, 1.0–1.4 kg). The Ca^{2+}-regulated bioluminescent indicator aequorin was microinjected into the muscles, which were mounted vertically between a miniature clamp and an isometric force transducer (MBL/5514-02) with a 5-0 Tevdek thread. A narrow extension of the base of the glass muscle bath extended a short distance axially into an ellipsoidal reflector, allowing the muscle to be positioned near one focal point of the reflector; a photomultiplier (Thorn-EMI 9635 QA, Gencom Inc., Fairfield, N.J.) was mounted so that its cathode was at the other focal point. This symmetrical optical reflecting system has high optical efficiency and minimizes motion artifacts. Light and force signals were recorded on a VHS videocassette recorder (A.R. Vetter Co., Rebersburg, Pa.), and a multichannel analyzer (model 4094A, Nicolet Instrument Corp., Madison, Wis.) was used for averaging aequorin light signals (usually 20–40) to obtain a satisfactory signal-to-noise ratio.

One hour after injection, the muscle (at optimal length) was stimulated to contract at 4-second intervals with 5-msec square-wave pulses applied through a platinum electrode located at the lower end of the muscle, just above the muscle clamp. The experiments were conducted in the presence of \(3 \times 10^{-7} \text{ M} \) \((\pm)-\text{bupranolol} \) (except as indicated) to prevent the effects of any endogenous norepinephrine that might be liberated by the driving stimulus. The normal physiological salt solution contained \((\text{mM})\) NaCl 120, KCl 5.9, glucose 11.5, NaHCO3 25, NaH2PO4 \(\cdot\) H2O 1.2, MgCl2 \(\cdot\) 6H2O 1.2, and CaCl2 2.0. The solution was bubbled continuously with 95% O2–5% CO2 at 30°C and had a pH of 7.4. In those experiments in which \([\text{Ca}^{2+}]_o\) was varied, phosphate was omitted from the solution to avoid precipitation at high \([\text{Ca}^{2+}]_o\). To inhibit the function of the sarcoplasmic reticulum (SR) and to allow tetanization of ferret ventricular muscle, 6 \(\mu\text{M}\) ryanodine was added to the muscle bath. Tetani were elicited by rapid stimulation (10–15 Hz) through field electrodes with rectangular stimulus pulses of long duration (50 msec). Superimposable records were obtained when tetani were separated by a “rest” period lasting 6 minutes (during which time the muscle was stimulated to twitch at 0.25 Hz). This stability allowed us to average luminescence signals from several consecutive tetani to improve the signal-to-noise ratio.

Because endothelin 1 could not be washed out in our experiments, the various protocols were performed in separate preparations. Data were expressed as mean ± SEM and analyzed by paired t test; \(n\) represents the number of experiments.

In Vitro Aequorin Experiments

It is conceivable that endothelin 1 or its metabolites might diffuse into cells and interact directly with aequorin to alter the luminescence reaction or the sensitivity of aequorin to Ca^{2+}. Therefore, endothelin was tested in vitro using the basic method and calibration device described by Blinks et al.\(^{21}\) The following tests were performed: 1) To test for Ca^{2+}-dependent light emission, aequorin was added to a PIPES buffer solution (1 ml) containing \((\text{mM})\) EGTA 20, Ca^{2+}EGTA 20, KCl 158, and PIPES 48.6; after the luminescent reaction was initiated, 10 \(\mu\)l test solution (similar composition but containing endothelin) was rapidly injected into the reaction cuvette with a syringe (model CR-700-20, Hamilton Co., Reno, Nev.). 2) To test for Ca^{2+}-independent light emission, the steps were identical except that 20 mM Ca^{2+}EGTA was omitted. With regard to Ca^{2+}-dependent light emission, the difference in peak light before and after endothelin (100 nM) was 0.15±0.16 (relative units, \(n=13, p>0.3\)); for Ca^{2+}-independent light emission, the difference in peak light before and after endothelin (100 nM) was 0.25±0.71 (relative units, \(n=8, p>0.3\)). Therefore, endothelin 1 did not affect the intensity of the luminescence, indicating that it does not directly interact with aequorin in a way that would alter the experimental results.

Chemicals

Pure synthetic bioactive endothelin 1 (Sigma Chemical Co., St. Louis, Mo.) was dissolved in deionized water in a concentration of 8 \(\mu\text{M}\) and stored below −20°C until used. Ryanodine (Calbiochem, San Diego, Calif.) was dissolved in deionized water in a concentration of 0.5 mM. Prazosin (Sigma Chemical) and \((\pm)-\text{bupranolol} \) (Sanol, Monheim, FRG) were dissolved in deionized water in concentrations of \(1 \times 10^{-2}\) and \(5 \times 10^{-4}\) M, respectively.

Results

Influence of Endothelin 1 on \([\text{Ca}^{2+}]_o\) and Tension

In our experiments, most muscles responded to endothelin at a concentration of \(3 \times 10^{-9}\) M, and the maximum response was reached at a concentration of \(1 \times 10^{-7}\) M (cumulative administration) (Figure 1). The onset of the response was slow (usually taking 2–6 minutes), and 20–30 minutes was required for the response to reach a plateau after each dose. Once the response reached a maximum, it remained stable for at least 2 hours. In concentrations of \(3 \times 10^{-9}\) M and higher, endothelin produced concentration-dependent increases in the amplitudes of both the Ca^{2+} transient and isometric contraction (Figures 1 and 2). Figure 2 shows typical tracings (from one of seven similar experiments) for both Ca^{2+} transients and isometric contractions in the presence of various concentrations of endothelin. Two significant points are illustrated by Figures 1 and 2. First, the increase in tension development is greater than the increase in the amplitude of the Ca^{2+} transient. At maximum
FIGURE 1. Cumulative concentration–response curves for the effects of endothelin 1 on peak aequorin light (peak $[\text{Ca}^{2+}]_i$) and peak tension in ferret papillary muscles. Values plotted on scale of ordinates were percent change from control (in the absence of endothelin, 20.1±2.1 mN/mm$^2$ and 9.0±3.8 nA; n=6).

(Figure 1), the increase in the amplitude of peak tension was 64±13% (from 20.1±2.1 to 32.5±2.6 mN/mm$^2$, p<0.01), whereas the increase in the amplitude of peak aequorin light was only 31±12% (from 9.0±3.8 to 11.8±4.2 nA, p<0.05, n=6). Second, the contraction was prolonged and the relaxation was delayed in the presence of endothelin (Figure 2). From the tracings of Figure 2, it is evident that there was an increase in amplitude of the Ca$^{2+}$ transient that could be responsible for the increase in tension development; however, we concluded that other mechanisms were probably involved, because 1) the increase in $[\text{Ca}^{2+}]_i$ appeared relatively small at any given level of force development and 2) by itself, an increase in peak $[\text{Ca}^{2+}]_i$ would not be expected to change the time course of isometric contraction in ferret papillary muscles (data not shown), as we have previously reported. These results led us to investigate whether endothelin 1 also produces a change in myofilament Ca$^{2+}$ responsiveness.

Effect of Endothelin 1 on the Peak Aequorin Light–Peak Tension Relation

To determine whether endothelin increased the Ca$^{2+}$ responsiveness of the myofilaments, we compared 1) the peak aequorin light ($[\text{Ca}^{2+}]_i$)–peak tension curve generated by varying endothelin concentrations with that generated by varying $[\text{Ca}^{2+}]_o$ (Figure 3) and 2) the peak aequorin light–peak tension curve generated by varying $[\text{Ca}^{2+}]_o$ in the presence of endothelin (maximally effective dose, 1×10$^{-7}$ M) with that in the absence of endothelin (Figure 4). In Figure 3 (n=4), the peak aequorin light–peak tension curve generated by varying endothelin concentrations was shifted to the left, and the slope of the curve was also increased, although the maximum developed tension produced by endothelin equaled the tension produced by 6 mM $[\text{Ca}^{2+}]_o$. For a given increase in force of contraction, the increase in the peak aequorin light was much lower when force was increased by endothelin than when it was increased by changing $[\text{Ca}^{2+}]_o$. In Figure 4 (n=4), 1×10$^{-7}$ M endothelin shifted the peak light–peak tension curve upward, and the maximum twitch force...
Effects of endothelin on [Ca\(^{2+}\)], and Force in the Presence of Ryanodine

To determine whether the increases in the amplitude of the Ca\(^{2+}\) transient in the presence of endothelin were caused by an increase of Ca\(^{2+}\) release from the SR or an increase of Ca\(^{2+}\) entry via the sarcolemma and also to obtain more evidence about the myofibrillar Ca\(^{2+}\)-sensitizing effect of endothelin, the peak intracellular Ca\(^{2+}\) and force generation were compared before and after the administration of 1×10\(^{-7}\) M endothelin, in the presence of a maximally effective concentration of ryanodine (i.e., 6 \(\mu\)M). In the presence of 6 \(\mu\)M ryanodine, we noted that 1) the amplitudes of the aequorin light signals were decreased to 5–10% of the control and the time to peak Ca\(^{2+}\) was increased and 2) the amplitudes of isometric contractions were decreased to 15–25% of the control and the time to peak tension was increased (Figure 6A). We assume that Ca\(^{2+}\) release from the SR was inhibited by 6 \(\mu\)M ryanodine and that the remaining twitch force was mainly triggered by Ca\(^{2+}\) entry. Note that in the presence of 6 \(\mu\)M ryanodine, 1×10\(^{-7}\) M endothelin 1) increased the developed tension (from 6.3±1.3 to 12.1±2.7 mN/mm\(^2\), \(p<0.05\), \(n=4\)) and prolonged the contraction (Figure 6B) and 2) increased the steady-state force (from 9.5±2.5 to 13.0±3.6 mN/mm\(^2\), \(p<0.05\), \(n=4\)) (Figure 7), without visibly increasing the peak Ca\(^{2+}\) either during isometric twitches or during tetani. These results suggest that 1) endothelin 1 increases Ca\(^{2+}\) release from the SR, which at least partially accounts for the increase in amplitude of the Ca\(^{2+}\) transients in Figure 2, and 2) endothelin 1 increases the Ca\(^{2+}\) responsiveness of the myofilaments, which is consistent with the results above (Figures 3–5).

Influence of Adrenergic Blockade on the Actions of Endothelin 1

Although it has been suggested that endothelin exerts its effects through sarcolemmal endothelin receptors, \(^1,2^3\) it is possible that the activation of \(\alpha\) and \(\beta\)-receptors might also be involved. To exclude this possibility, comparisons were made before and after \(\alpha\)- or \(\beta\)-receptor blockade. Figure 8 shows that 3×10\(^{-7}\) M (±)-bupranolol did not significantly change the endothelin concentration–response curve. The selective \(\alpha\)-adrenergic blocker prazosin (1×10\(^{-6}\) to 5×10\(^{-6}\) M) was added to the organ bath when the contractile effect of endothelin reached steady state. Prazosin did not attenuate the effects of endothelin on either the Ca\(^{2+}\) transient or isometric contraction, except for slightly
abbreviating the contraction (Figure 9). Similar results were obtained in four experiments. The results shown in Figures 8 and 9 suggest that endothelin 1 did not exert its myocardial effects through either β- or α-adrenergic receptors.

In the presence of endothelin 1, there was no visible increase in resting tension of the muscles, and we did not detect development of multiple components or "after-glimmers" in the aequorin light signal. This information suggests that the concentrations of endothelin used did not produce Ca\textsuperscript{2+} overload in the experimental preparations.

**Discussion**

**Influence of Ca\textsuperscript{2+} Responsiveness of the Myofilaments**

The most important discovery of this study is that the positive inotropic effect of endothelin 1 is predominantly due to an increase in myofilament Ca\textsuperscript{2+} responsiveness. This conclusion is based on the results obtained by four different approaches that have previously been used to evaluate myofibrillar Ca\textsuperscript{2+} responsiveness in intact, aequorin-loaded cardiac muscle preparations. In the first approach, peak aequorin light (i.e., peak [Ca\textsuperscript{2+}]) was compared with peak tension during contractions as contractility was varied by changing [Ca\textsuperscript{2+}]$_{0}$, or by endothelin administration (Figure 3). In the second approach, dT/dt$_{max}$ was compared with peak aequorin light during contractions as contractility was varied as in the first approach (data not shown). In the third approach, peak aequorin light and peak tension were compared as in the first and second approaches, except that contractility was progressively varied by changing [Ca\textsuperscript{2+}]$_{0}$ first in the absence and then in the presence of endothelin (Figure 4). In the fourth approach, steady-state aequorin light and force were compared during tetani induced by high-frequency stimulation in the presence of 6 μM ryanodine, in the absence and presence of endothelin (Figure 7). All of these different approaches gave consistent results, suggesting that endothelin has a relatively greater effect on the isometric twitch than on the corresponding [Ca\textsuperscript{2+}] transient. Consistent with our results, Eid et al.[26] reported that endothelin increases shortening of isolated rat myocytes without significantly increasing [Ca\textsuperscript{2+}] recorded with fura 2.

**Mechanism of Action**

Our results indicate that endothelin 1 increased the intracellular Ca\textsuperscript{2+} transient and the Ca\textsuperscript{2+} responsiveness of the myofilaments of intact ferret myocardium; the issue addressed here is what the cellular mechanisms might be. In our experiments, endothelin did not produce any direct β-agonist or cAMP-dependent effects. The effects of β-agonists on mammalian papillary muscles are to abbreviate both the Ca\textsuperscript{2+} transient and isometric contraction and to decrease the Ca\textsuperscript{2+} responsiveness of the myofilaments.[25] Therefore, the endothelin-induced prolongation of the time course of the isometric contraction with no change in the time course of the corresponding [Ca\textsuperscript{2+}] transient (Figures 5 and 6B) argues against a β-adrenergic-mediated drug effect. One of the reasons for us to study the effects of the α\textsubscript{1}-receptor blocker prazosin was that the myocardial effect exerted by endothelin is qualitatively similar to the effect of the α\textsubscript{1}-agonist phenylephrine, which also
increases the Ca$^{2+}$ responsiveness of the myofilaments and prolongs the contraction.\textsuperscript{25} The results from Figure 9 show that endothelin 1 did not exert its myocardial effect through $\alpha$-receptors. Experimental studies with smooth muscle have also shown that endothelin does not produce $\beta$- or $\alpha$-agonist effects.\textsuperscript{1} Endothelin may exert its myocardial effects through endothelin receptor-intracellular second messenger pathways. The bases for this hypothesis are that 1) the density of endothelin receptors in myocardium is high,\textsuperscript{8,10,2} endothelin is a relatively large peptide so that its diffusion into cells might be limited, and 3) endothelin 1 did not exert its myocardial effect after saponin-skinning of ferret papillary muscles (as shown previously in our laboratory\textsuperscript{27}). In saponin-skinned fibers, the sarcolemma is destroyed, and the endothelin receptor-intracellular second messenger pathways do not normally operate. It has recently been suggested that an increase in phosphoinositide turnover appears to be involved in the responses of certain tissues to endothelin.\textsuperscript{26-32} Endothelin increased phosphoinositide hydrolysis in rat atrium\textsuperscript{32} and smooth muscle.\textsuperscript{29,30} Phosphoinositide hydrolysis liberates two products that have been shown to have important biological actions: inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol. Diacylglycerol can activate protein kinase C, thereby inducing the phosphorylation of certain proteins.\textsuperscript{33} Such an action might influence the Ca$^{2+}$ channels in either the sarcolemmal or SR membrane, and it is conceivable that such an action might be responsible for the modest increase in the amplitude of the Ca$^{2+}$ transients that we have observed. IP$_3$ can be released into the cytoplasm and has been found to trigger Ca$^{2+}$ release from the SR in mechanically skinned rat ventricular cells.\textsuperscript{34,35} Therefore, it seems reasonable to propose that an action of IP$_3$ on the SR might also be involved in the increase of the amplitude of Ca$^{2+}$ transients that we observed in our experiments.

The results shown in Figures 5 and 6B suggest that an increase in the Ca$^{2+}$ affinity of troponin C might be involved in the endothelin-induced increase in the Ca$^{2+}$ responsiveness of the myofilaments. Troponin C is the most important Ca$^{2+}$ receptor in myocardial cytoplasm and binds Ca$^{2+}$ almost as rapidly as it is made available.\textsuperscript{36} Therefore, it would be expected that an increase in the affinity of troponin C for Ca$^{2+}$ would enhance binding and slow dissociation of Ca$^{2+}$ from the regulatory sites. The combination of these two actions would enhance and prolong contraction, as observed in Figure 6B. Since endothelin can increase the phosphoinositide hydrolysis in heart muscle and IP$_3$ can be released into the cytoplasm, it is not unreasonable to propose that IP$_3$ might mediate the increase in the Ca$^{2+}$ responsiveness of the myofilaments. Although it has been reported that IP$_3$ does not change the Ca$^{2+}$ sensitivity of saponin-skinned bundles of guinea pig ventricular muscle,\textsuperscript{37} IP$_3$ has been reported to enhance the Ca$^{2+}$ sensitivity of the myofilaments of rabbit skeletal muscle.\textsuperscript{38} Whether IP$_3$ could be responsible for the endothelin-induced increase in myofilament Ca$^{2+}$ responsiveness or whether other effects on the myofilaments may be involved as well remains to be determined.

It is not unreasonable to speculate that, in certain circumstances, myocardium might also modulate myocardial contractility by releasing endothelial-derived compounds, such as endothelin. This hypothesis is supported by several observations: 1) endocar-
dial endothelium and vascular endothelium are derived from the same tissue,87 9) endothelin is synthesized in and released from endothelial cells,1 3) endothelin gene expression was also found in fetal atrium and ventricle,40,41 and 4) endothelin prolonged the contractions (Figure 5), whereas the removal of endocardium abbreviated the contractions.11 Further investigations need to be performed to test this hypothesis.

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