Mechanism of action of endothelin in rat cardiac muscle: cross-bridge kinetics and myosin light chain phosphorylation

Gunther H. Rossmanith, Joseph F. Y. Hoh*, Lynne Turnbull and Russell I. Ludowyke†

*Biomedical Systems Research Group, School of Mathematics, Physics, Computing and Electronics, Macquarie University-Sydney, NSW 2109, *Department of Physiology and The Institute for Biomedical Research, University of Sydney, NSW 2006 and †Centre for Immunology, St Vincent's Hospital, Sydney, NSW 2010, Australia*

1. The molecular mechanism of inotropic action of endothelin was investigated in rat ventricular muscle by studying its effects on characteristics of isometric twitch, barium-induced steady contracture and the level of incorporation of $^{32}P_i$ into myosin light chain 2.

2. Exposure of rat papillary muscle to endothelin caused an increase in isometric twitch force but did not alter the twitch-time parameters.

3. Endothelin did not significantly change the maximum contracture tension but did cause an increase in contracture tension at submaximal levels of activation, without changes in the tension-to-stiffness ratio and kinetics of attached cross-bridges. Kinetics of attached cross-bridges were deduced during steady contracture from complex-stiffness values, and in particular from the frequency at which muscle stiffness assumes a minimum value, f_{min}. Endothelin did not alter f_{min}.

4. Endothelin caused an increase in the level of incorporation of $^{32}P_i$ into myosin light chain 2 without a concurrent change in the level of incorporation of $^{32}P_i$ into troponin I.

5. We conclude that the inotropic action of endothelin is not due to an increase in the kinetics of attached cross-bridges, nor due to a change in the force per unit cross-bridge, but may result from an increased divalent cation sensitivity caused by elevated myosin light chain 2 phosphorylation, resembling post-tetanic potentiation in fast skeletal muscle fibres.

Endothelin is a twenty-one amino acid peptide secreted by vascular endothelial cells that has powerful vasoconstrictive properties (Yanagisawa *et al.* 1988). Endothelin is also a potent positive inotropic agent in isolated cardiac tissues and myocytes from a number of mammalian species, including humans (Moravec, Reynolds, Stewart & Bond, 1989; Molenaar *et al.* 1993). Recent experiments show that the metabolism of endothelin in the coronary vasculature is influenced by blood flow and tissue oxygen tension, suggesting that this agent may have an important cardioregulatory function (McClellan, Weisberg, Rose & Winegrad, 1994). However, the mechanism of inotropic action of endothelin is not well understood. In rat cardiac muscle, endothelin has been shown to increase the amplitude of the twitch contraction without significantly increasing the cytosolic calcium transient (Kelly *et al.* 1990; Wang, Paik & Morgan, 1991; Kramer, Smith & Kelly, 1991; Qiu, Wang, Perreault, Meuse, Grossman & Morgan, 1992). These observations raise two questions regarding the mechanisms involved. First, at the mechanical level, what is responsible for the enhancement of force? Second, what are the molecular mechanisms underlying these changes, and how are they linked to the activation of the endothelin receptor?

Possible mechanisms to explain the effect of endothelin on the isometric twitch and the associated lack of effect on the calcium transient include an increase in tension per unit cross-bridge, enhanced calcium sensitivity, or a change in cross-bridge kinetics. Very little work has so far been done on the mechanics of endothelin-stimulated cardiac muscle which reflects events at the cross-bridge level, the bulk of the literature being concerned solely with isometric twitch contractions. Analysis of the effect of inotropic agents during steady isometric contracture has revealed that some agents influence only the number of attached cross-bridges, while others also enhance the kinetics of cross-bridge cycling (Hoh, Rossmanith, Kwan & Hamilton, 1988; Hoh, Rossmanith & Hamilton, 1991). As regards the molecular mechanism of action of endothelin, it has been proposed that endothelin stimulates the sarcolemmal Na^+–H^+ exchanger by a protein kinase C (PKC)-mediated pathway, resulting in a rise in pH_i (Kramer *et al.* 1991). Myocardial cell

alkalosis is associated with an increase in Ca^{2+} sensitivity (Mayoux, Coutry, Lechene, Marotte, Hoffmann & Ventura-Clapier, 1994), thereby providing a possible mechanism for the inotropic action of endothelin. However, the observed degree of alkalinization can only partially account for this inotropic action of endothelin (Kramer *et al.* 1991). It is therefore likely that an alternative mechanism for endothelin-induced inotropy exists.

In skeletal muscle, the phenomenon of post-tetanic potentiation (PTP) is associated with an enhanced Ca^{2+} sensitivity brought about by the phosphorylation of myosin light chain 2 (MLC2) (for reviews see Hoh, 1992; Sweeney, Bowman & Stull, 1993). In skinned cardiac tissue, phosphorylation of MLC2 by exogenous myosin light chain kinase (MLCK) has been shown to cause an increase in calcium sensitivity (Morano, Bachle-Stolz, Katus & Ruegg, 1988; Clement, Puceat, Walsh & Vassort, 1992). PKC, which is stimulated by the action of endothelin, has been shown to phosphorylate cardiac MLC2 *in vitro* in a manner indistinguishable from the action of the more specific, calcium-activated MLCK (Venema, Raynor, Noland & Kuo, 1993; Venema & Kuo, 1993). The physiological role of MLC2 phosphorylation remains in doubt.

In this paper, we investigate the mechanical and molecular mechanism of the inotropic action of endothelin in rat ventricular tissue using analysis of mechanical and biochemical parameters. We present evidence consistent with the hypothesis that endothelin exerts positive inotropic effects through an increased divalent cation sensitivity mediated by increased MLC2 phosphorylation.

METHODS

Mechanical apparatus and temperature control

A detailed description of the mechanical apparatus and temperature control is given in a previous paper (Rossmanith, Hoh, Kirman & Kwan, 1986). All experiments were carried out at 25 °C.

Muscle preparation

All experiments reported here were carried out on ventricular muscle of male Sprague–Dawley rats. The rats had a mean age of 48 ± 4 days ($n = 70$). The rats were killed by decapitation and each heart was immediately removed and placed in solution 1 kept at 25 °C (for composition of solutions, refer to Solutions section). The left ventricle was opened and a papillary muscle selected (length, 2·98 ± 0·17 mm; diameter, 0·87 ± 0·04 mm; $n = 57$, muscles used in phosphorylation studies not included). The tendon of the muscle was tied. The papillary muscle was dissected from the heart by cutting the tendon at one end and removing a portion of the ventricular wall at the other, and then attached to the force gauge and length driver as previously described (Rossmanith *et al.* 1986). The muscle was then immediately immersed in the muscle bath containing solution 1. These procedures were in accordance with The Animal Research Act and Regulation of Australia, 1990 and were monitored by the Animal Care and Ethics Committee of Macquarie University-Sydney.

Solutions

The standard bathing solution (solution 1) used was a modified Krebs–Henseleit solution (KHS) and had the following composition (mM): NaCl, 120; KCl, 4·69; $CaCl_2$, 1·5; $MgCl_2$, 0·54; KH_2PO_4, 1·02; $NaHCO_3$, 25; and D-glucose, 10. A calcium-free KHS (solution 2) was obtained by omitting $CaCl_2$ from the KHS. A phosphate-free solution (solution 3) was obtained by omitting KH_2PO_4 from the KHS. Ba^{2+} contracture solutions were obtained by adding the required amounts of $BaCl_2$ to solution 2 (see below). All solutions were kept at pH 7·4 by continuous bubbling with carbogen (95% O_2–5% CO_2). For determination of MLC2 phosphorylation, the homogenizing buffer was based on that of Silver & Stull (1982) and consisted of (mM): sodium pyrophosphate, 100 (pH 8·8); EGTA, 5; NaF, 50; β-mercaptoethanol, 15; phenylmethylsulphonyl fluoride, 1; together with 10 μg ml^{-1} aprotinin; 10 μg ml^{-1} leupeptin; 2 μg ml^{-1} pepstatin; and 10% glycerol at 0 °C.

The drugs used were endothelin-1 (Auspep, Parkville, VIC, Australia) and isobutylmethylxanthine (IBMX) (Sigma). Stock solution of endothelin was made up by dissolving the drug in deionized water containing 1 μg ml^{-1} bovine serum albumin. Stock solution of IBMX was made up by dissolving the drug in solution 2. All chemicals and drugs were of analytical grade.

Computer control and data analysis

A detailed description of the computer control and data analysis procedures has been reported in earlier publications (Rossmanith, 1986; Rossmanith *et al.* 1986). The signal used to perturb muscle length was pseudo-random binary noise (PRBN). As a time-domain signal, PRBN is akin to step changes in muscle length, giving rise to characteristic tension transients. Envisaged in the frequency domain, the effect of PRBN is to expose the muscle simultaneously to length changes embracing a whole set of frequencies, and is therefore more time efficient than the sinusoidal method which exposes the muscle to one frequency at a time. The amplitude of the signal was tailored to the length of the particular muscle preparation, and to not exceed 0·1% of the operating length (L_0), defined below.

Data are presented as means ± S.E.M. Student's t test and one-way analysis of variance (ANOVA) and Fisher's protected least-significant difference (PLSD) test were used to determine differences between means. Values of $P < 0.05$ were considered to be significant. All data presented were analysed using StatView® SE+Graphics (Abacus Concepts, Inc., Berkeley, CA, USA (1987)).

Protocols for muscle activation and mechanics

After mounting, the muscle was stretched by approximately 30% of the length corresponding to the onset of passive tension, to arrive at the operating length, L_0 (Rossmanith *et al.* 1986; Saeki, Kato, Totsuka & Yanagisawa, 1987). Electrical stimulation of the muscle preparation was achieved via platinum wire electrodes located 5 mm on either side of the muscle. The rectangular stimulating pulses had a strength of 40 V, a width of 1 or 2 ms and a pulse frequency of 0·33 Hz. The isometric twitch was displayed on a digitizing oscilloscope (model 5223, Tektronix, Beaverton, OR, USA) and on a chart recorder (model WX 4422, Graphtec Corp., Tokyo, Japan).

Isometric twitch

After a standard twitch-stabilization period of 30 min in solution 1, the isometric twitch response was recorded by means of a Polaroid oscilloscope camera (model C59A, Tektronix) and was taken as the control response. Endothelin at a concentration that was determined from dose–response curves to elicit a near-maximal inotropic response (100 nM) was added to the muscle bath and the time course of its effect on the twitch profile was followed over a 30 min period (Vigne, Lazdunski & Frelin, 1989; Moravec *et al.* 1989; Li, Stewart & Rouleau, 1991).

Protocols used in studying the effect of endothelin on barium contractures and tension-to-stiffness ratio

To induce barium contracture, the muscle was washed in several changes of solution 2 until the twitch force had become negligibly small, and steady Ba^{2+} contractures were then induced by the addition of $BaCl_2$. Because endothelin takes a long time to act, and in order to avoid causing fatigue to the isolated muscle preparation by prolonged contractures at high levels of activation, two protocols were used to study the effect of endothelin on barium contractures.

Protocol 1. Endothelin (100 nM) was added to a stabilized twitching muscle in solution 1. After the potentiating effect of endothelin on the isometric twitch had reached steady state, a series of brief Ba^{2+} contractures were induced by adding $BaCl_2$ in concentrations ranging between 0·5 and 70 mM, at which maximal tension was attained. Because endothelin is not washed out by solution changes (Li *et al.* 1991), the total time of exposure of the muscle to endothelin is the sum of the times during twitch and during Ba^{2+} contracture. With this protocol, the potentiated contracture for a given level of Ba^{2+} was rapidly achieved, and this protocol was used to measure the potentiating effect of endothelin at different levels of Ba^{2+} activation.

Protocol 2. Endothelin (100 nM) was added to the bathing solution after the barium contracture had reached a steady level. This protocol was used to see the time course of action of endothelin on contracture tension as well as the effect of endothelin on the tension-to-stiffness ratio.

The tension-to-stiffness ratio was obtained by dividing the steady contracture tension by the high-frequency stiffness. High-frequency stiffness values were obtained by measuring the tension response to small-amplitude (0·05% L_0) sinusoidal length changes at high frequency. What constitutes a high frequency is relative to the speed of the muscle. For fast skeletal muscle, 2 kHz is considered an appropriate frequency (Julian & Morgan, 1981). Rat cardiac muscle is about tenfold slower than rat fast skeletal muscle, and hence we used 200 Hz for our stiffness measurements.

For investigating the effect of endothelin on maximum contracture tension, the tensions were normalized with respect to cross-sectional areas. For each muscle the average cross-sectional area was calculated from the volume of the muscle, taken as a truncated cone, divided by the length of the muscle.

For investigating the effect of endothelin on tension *versus* $[Ba^{2+}]$ relations, we utilized protocol 1. We compared the tensions elicited by 0·5 and 10 mM Ba^{2+}, in the presence and absence of endothelin. Two groups of muscle were used, a control group and a group in which the isometric twitch had already been potentiated by endothelin. To allow pooling of data within each group, and comparison of data between groups, each tension was expressed as a percentage of the maximum tension generated by that muscle.

Kinetics

After the establishment of steady contracture with 0·5 mM $BaCl_2$, the computer was enabled and the dynamic length and tension signals recorded for analysis of complex stiffness. High-frequency (200 Hz) stiffness was measured from sinusoidal length and force signals displayed on the digitizing oscilloscope. Once these controls were obtained, endothelin (100 nM) was added to the muscle bath (protocol 2). PRBN length–tension records and high-frequency stiffness values were collected every 5 min up to approximately 20 min after the endothelin addition. It has been shown (Rossmanith *et al.* 1986) that at these levels of barium activation, complex-stiffness spectra do not change appreciably over a period of 30 min. The muscle was then relaxed with solution 1 and twitch re-established to verify that the mechanical functionality of the muscle had not been compromised by the experimental procedure. Protocol 1 (see above) was used to study the effect of endothelin on the frequency at which muscle stiffness assumes a minimum value (f_{min}) for periods exceeding 20 min without compromising the complex-stiffness spectra.

Measurement of myosin light chain 2 phosphorylation in papillary preparations

Papillary muscles from the left ventricle were dissected as described above with the following exceptions. Both ends of the papillary muscle were tied and then the muscle was tied between the two arms of a metal spring. Each muscle, together with the spring, was dissected from the heart by cutting the tendon at one end and a portion of the ventricular wall at the other. The muscle and spring were placed in solution 3 (phosphate free) continuously infused with carbogen. The papillary muscles were incubated in this resting state with 300 μCi ml^{-1} of [^{32}P]orthophosphoric acid (Amersham, UK) for 4 h. Samples were then washed in fresh solution 3 for 10 min. This washing was repeated three times to ensure removal of free $^{32}P_i$ from the buffer. Endothelin (100 nM) was then added to

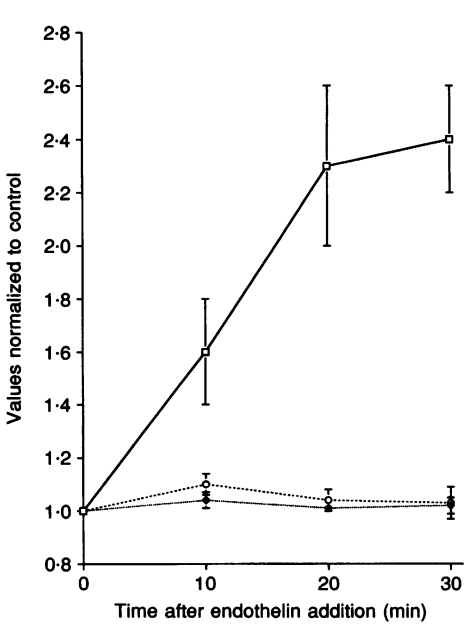

Figure 1. Time course of the effect of endothelin on isometric twitch

The time course of the effect of endothelin on isometric peak twitch tension (□), time-to-peak tension (TTPT, ◆) and time-to-half-relaxation (TT1/2R, ○). Values plotted ($n = 17$) are normalized relative to control. The control value was 146·5 ± 8·5 ms for TTPT and 152 ± 11·7 ms for TT1/2R.

the experimental vessels. Control muscles were incubated in solution 3 in the absence of endothelin. At 15, 30 and 45 min, experimental samples and controls were removed from the incubation solutions and freeze clamped with large forceps cooled with liquid nitrogen. Frozen samples were homogenized immediately in 100 μl of homogenizing buffer using disposable plastic pestles and tubes (Contes, USA). Samples were placed on ice for 15 min before centrifugation at 13 000 g for 10 min at 4 °C. The supernatant was then added to an equal volume of Laemmli sample buffer (Laemmli, 1970) and heated at 100 °C for 6 min. Aliquots of each sample were electrophoresed on 12·5% SDS–PAGE gels and subsequently stained with Coomassie Blue.

A number of protein bands in the region of interest were assayed by laser scanning densitometry. Equivalent amounts of total protein for each sample were then electrophoresed in a second 12·5% SDS–PAGE gel which was used for analysis of MLC2 phosphorylation. Equivalence of protein loading was confirmed in the second gel by laser densitometry. The MLC2 and troponin I (TnI) bands were excised, trypsinized, the supernatant lyophilized and phosphopeptides separated by flatbed isoelectric focusing (IEF) as described previously (Ludowyke, Peleg, Beaven & Adelstein, 1989; Miller, Alber, Varin Blank, Ludowyke & Metzger, 1990). The phosphopeptide bands were analysed from autoradiograms by laser scanning densitometry or by using a phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA).

The one-dimensional (1-D), IEF-based separation of MLC2 phosphopeptides reveals the same number of identifiable phosphopeptides as two-dimensional (2-D) separation (Ludowyke *et al.* 1989; Miller *et al.* 1990). A major advantage of the 1-D IEF method is that it allows the separation of phosphopeptides of MLC2 and TnI on the same gel. Previously, 2-D phosphopeptide maps of TnI revealed three major phosphopeptides, which were all increased when TnI was phosphorylated by PKC (Venema & Kuo, 1993). The 1-D separation system employed here revealed two major phosphopeptides in control or endothelin-activated TnI. It is likely that two of the three major TnI phosphopeptides run together in the 1-D

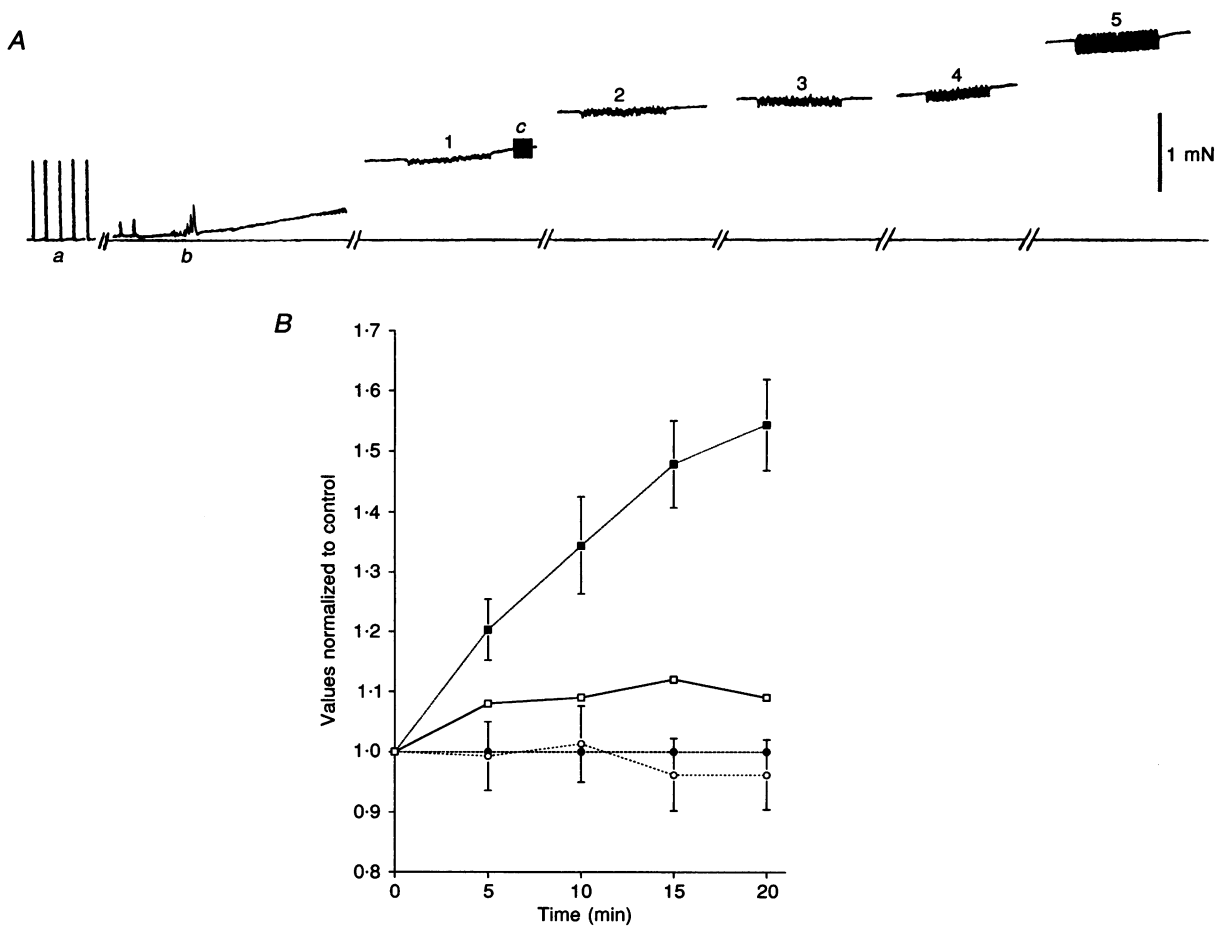

Figure 2. Effect of endothelin on barium-induced contracture

A, chart recording of barium-induced contracture in a papillary muscle: *a*, stabilized isometric twitch just prior to the addition of Ca^{2+}-free solution; *b*, addition of Ba^{2+} contracture solution (0·5 mM $BaCl_2$) and initial rise of contracture tension; *c*, addition of endothelin (100 nM), and its effect on contracture tension shown in the tension segments 2–5, taken 5, 10, 15 and 20 min after the addition of endothelin. The perturbations seen in the contracture tension records, which represent a duration of 32 s, are in response to the PRBN length sequences. Segment 1 is the control contracture tension. *B*, the time course of the effect of endothelin on contracture tension (■), ratio of tension-to-stiffness (○), and frequency of minimum stiffness, f_{min} (●). The variation of contracture tension with time, in the absence of endothelin, is indicated by □. Values plotted are normalized relative to control. The control value for f_{min} was 2·0 ± 0·1 Hz ($n = 4$).

IEF separation here. Our results showed that endothelin did not induce a change in the total TnI phosphorylation or that of the two separable phosphopeptides. Therefore there was no advantage in further separating the phosphopeptides in the 2-D system.

RESULTS

Effect of endothelin on isometric twitch

The time course of the effect of endothelin on the magnitude and speed of the isometric twitch is shown in Fig. 1. Endothelin increased isometric twitch force in ventricular muscle by $140 \pm 20\%$ ($n = 17$) within 30 min, the major portion of this increase occurring within the first 20 min. This potentiation in isometric twitch force was not accompanied by a significant alteration in the time course of the isometric twitch contraction, as indicated by the effect of endothelin on time-to-peak tension (TTPT) and time-to-half-relaxation (TT1/2R).

Effect of endothelin on contracture tension and tension-to-stiffness ratio

Endothelin did not significantly ($P = 0{\cdot}79$) alter the maximum tension per cross-sectional area, being $(1{\cdot}5 \pm 0{\cdot}3) \times 10^3$ N m^{-2} ($n = 4$) and $(1{\cdot}6 \pm 0{\cdot}2) \times 10^4$ N m^{-2} ($n = 6$), respectively, in the presence and absence of endothelin. However, endothelin did increase the contracture tension, expressed as a percentage of maximal tension, at submaximal levels of activation. This was investigated in particular for 0·5 and 10 mM Ba^{2+}. For the control group, the tensions elicited by 0·5 and 10 mM Ba^{2+} were $11{\cdot}9 \pm 2{\cdot}1\%$ ($n = 6$) and $44{\cdot}3 \pm 4{\cdot}1\%$ ($n = 6$), respectively, of maximum tension. The contracture tension elicited by 0·5 mM Ba^{2+} approximately corresponded to peak isometric twitch tension ($100 \pm 10\%$, $n = 16$). For the group where endothelin had first potentiated the isometric twitch, the contracture tensions elicited by 0·5 and 10 mM Ba^{2+} were $25{\cdot}2 \pm 4{\cdot}2\%$ ($n = 9$) and $71{\cdot}4 \pm 3{\cdot}5\%$ ($n = 9$), respectively, of maximum tension. Analysis of variance indicated that both 0·5 and 10 mM Ba^{2+} elicited higher tension in the endothelin-treated muscles compared with the control tensions, the levels of significance being $P = 0{\cdot}03$ and $P = 0{\cdot}0003$, respectively. No significant variation was observed in the tension-to-stiffness ratio, in the presence or absence of endothelin, as the level of barium activation was increased up to maximum activation. In the absence of endothelin, the tension-to-stiffness ratio was $2{\cdot}2 \pm 0{\cdot}1$ at 10 mM Ba^{2+} and $2{\cdot}0 \pm 0{\cdot}1$ ($n = 9$) at maximum activation.

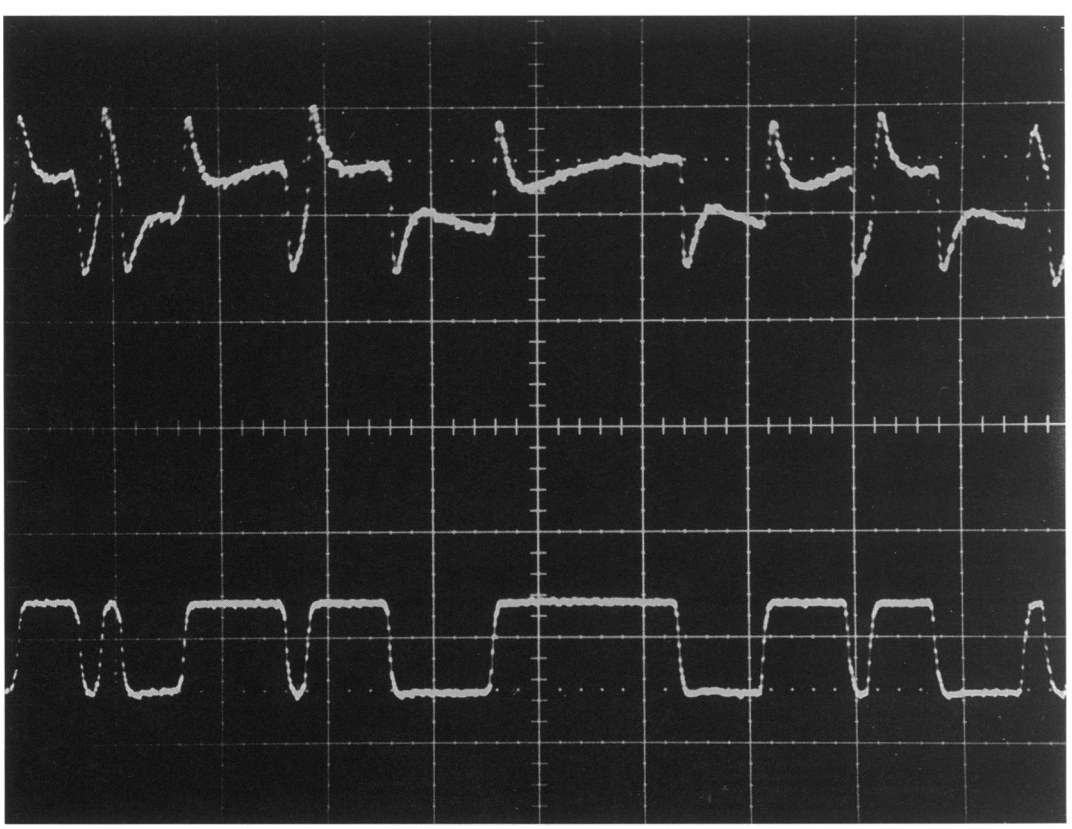

Figure 3. Record of a segment of the dynamic force response of rat papillary muscle to perturbation of muscle length

Digital oscilloscope record of a segment of the dynamic force (upper trace) of rat papillary muscle in steady Ba^{2+}-induced contracture in response to perturbation of muscle length (lower trace) in the form of a 511-length PRBN sequence. One division of force, length and time represent 280 μN, 1·5 μm and 0·2 s, respectively.

The corresponding values in the presence of endothelin were $2{\cdot}5 \pm 0{\cdot}3$ and $2{\cdot}1 \pm 0{\cdot}3$ ($n = 9$). These results were obtained by means of protocol 1 (see Methods). The observation that endothelin did not significantly alter the maximum tension per cross-sectional area permitted pooling of contracture tensions at submaximal levels of Ba^{2+} activation when these were expressed as a percentage of maximal tension.

Effect of endothelin on kinetics

These studies were carried out in contracture tensions elicited by 0·5 mM Ba^{2+}, using protocol 2 (see Methods) where endothelin was added after the muscle had attained steady barium contracture. Without the addition of endothelin, the variation in this contracture tension was less than 10% of control ($n = 5$) over a 20 min period (Fig. 2*B*). Analysis of variance of the tension values at 5 min intervals showed that there was no significant difference between any of the contracture tensions over this time period. In contrast, endothelin stimulation induced a progressive increase in contracture tension with time (Fig. 2*A*), reaching a $54 \pm 7{\cdot}5\%$ ($n = 17$) increase in contracture tension by 20 min. In addition, over the period of time when endothelin potentiated contracture tension by up to 54%, no significant variation was seen in the tension-to-stiffness ratio (Fig. 2*B*), as indicated by analysis of variance.

Analysis of the PRBN length changes and the resulting force changes as shown in Fig. 3 gives rise to the characteristic dynamic stiffness and phase data shown in Fig. 4. The data display a minimum in dynamic stiffness at a characteristic frequency, f_{min}, accompanied by the minimum/maximum feature in the phase response. It has

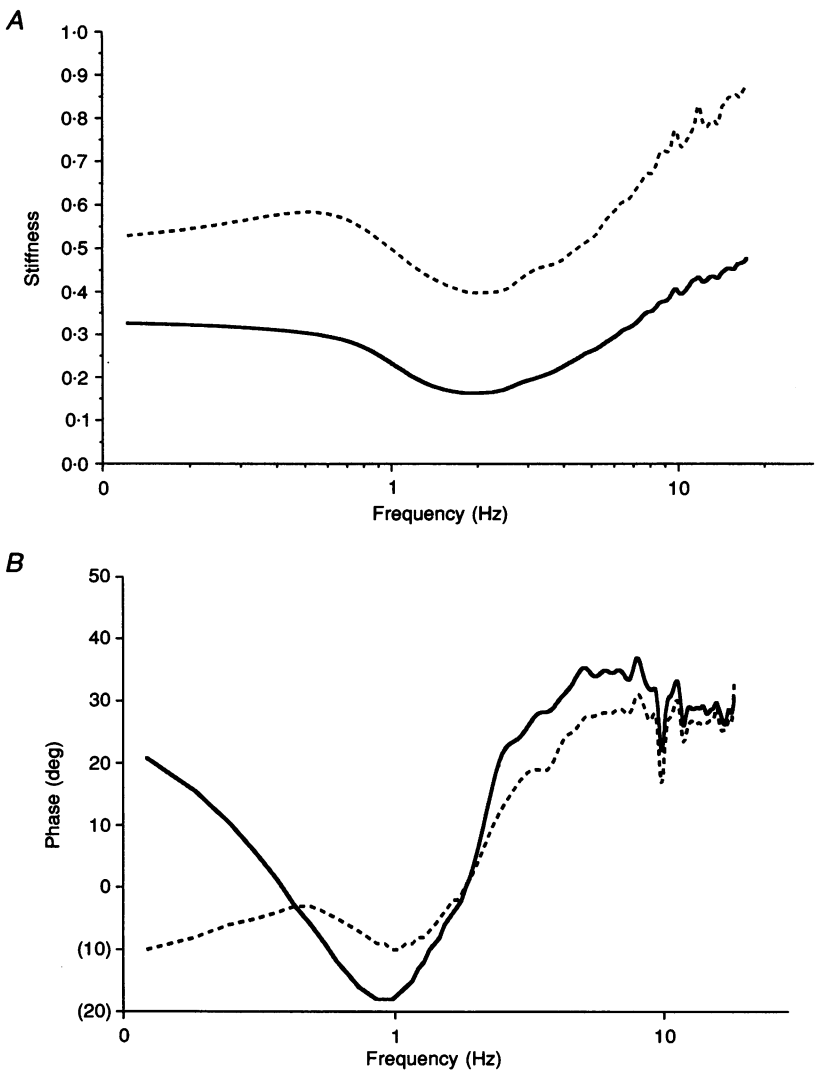

Figure 4. Dynamic stiffness and phase plots from rat ventricular muscle

Dynamic stiffness (*A*) and phase plots (*B*) from rat ventricular muscle under steady-state 0·5 mM Ba^{2+} contracture in the absence (continuous trace) and presence (dashed trace) of endothelin. PRBN length signal and corresponding dynamic force response, segments of which are shown in Fig. 3, gave rise to dynamic stiffness and phase data at 340 equally spaced frequencies in the range 0·1 to 19 Hz. A stiffness value of 1 is equivalent to 11·1 N m^{-1}.

been established in previous studies (Rossmanith *et al.* 1986; Hoh *et al.* 1988, 1991) that ventricular muscle from juvenile rats has an f_{min} value of 2 Hz at 25 °C. This was again confirmed in this study where control f_{min} values were 2·0 ± 0·1 Hz ($n = 4$). Endothelin caused no significant variation in the absolute values of f_{min} (2·0 ± 0·1 Hz, $n = 13$) from the control values, even when the total exposure time of the muscles to endothelin was extended to 60 min with the alternate protocol. IBMX has been previously shown (Hoh *et al.* 1991) to increase cross-bridge kinetics in cardiac muscle, as indicated by an increase in f_{min}. We verified, by using IBMX, that each papillary muscle reported here was able to have its cross-bridge kinetics changed by this inotropic intervention (data not shown).

Effect of endothelin on myosin light chain 2 phosphorylation

SDS–PAGE and autoradiographic analysis indicated that a basal level of $^{32}P_i$ radiolabelling occurred in a number of proteins in rat cardiac tissue, including tropomyosin, troponin T (TnT), TnI and MLC2, and that the addition of endothelin consistently stimulates a marked increase in radiolabelling of only one of these proteins, MLC2 (Fig. 5*A*). There were no consistent changes seen in the level of phospholabelling of TnI or TnT.

Phosphopeptide mapping of MLC2 from control tissue showed two major phosphopeptides, as seen previously in cardiac myofibrils (Venema *et al.* 1993). Endothelin causes

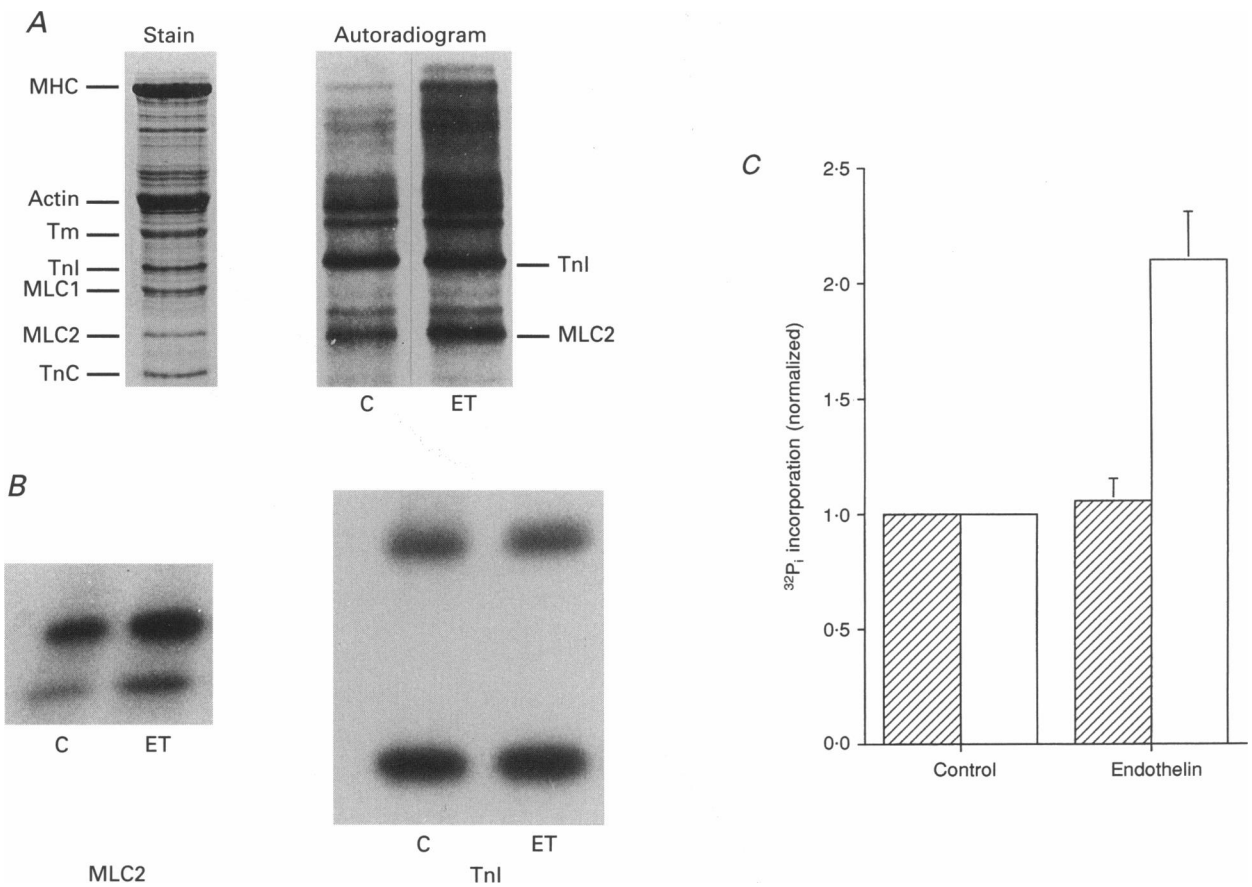

Figure 5. Phosphorylation of isolated rat cardiac papillary muscle and IEF phosphopeptide mapping of MLC2 and TnI

Papillary muscles were phosphorylated for 4 h with $^{32}P_i$ and, following washing, incubated with either no addition (control, C) or 100 nM endothelin (ET) for 15 min. Samples were homogenized, and proteins separated on 12·5% SDS–PAGE gels. MLC2 and TnI were excised and trypsin-digested, and their phosphopeptides were separated on the same one-dimensional isoelectric focusing gel (cathode at bottom of *B*). In *A*, proteins were identified by Coomassie Blue staining as shown on the left; the autoradiograms on the right show the endothelin-induced increase in phosphorylation of the MLC2. MHC, myosin heavy chain; Tm, tropomyosin; TnI, troponin I; MLC1, myosin light chain 1; MLC2, myosin light chain 2; TnC, troponin C. *B*, autoradiograms of phosphopeptide maps of MLC2 and TnI showing the endothelin-induced increased phosphorylation of both MLC2 phosphopeptides, with no change in the corresponding TnI phosphopeptides. Results are representative of three different experiments. *C*, autoradiograms of phosphopeptide maps of MLC2 (□) and TnI (▨) as shown in *B* were analysed by laser scanning densitometry. Relative densitometric units from 15 to 45 min ($n = 8$) are normalized relative to control.

an equal increase in phospholabelling of both these MLC2 phosphopeptides, but did not increase the level of phospholabelling of the two major TnI phosphopeptides above their basal levels (Fig. 5*B*). Analysis of these phosphopeptide maps showed that there was less than 10% variation ($n = 8$) in the level of phospholabelling of TnI in response to endothelin stimulation, whilst over the same time period, there was a 100% increase in the level of phosphorylation of MLC2 (Fig. 5*C*, $n = 8$). There was no significant difference in the level of MLC2 phosphorylation by endothelin between 15 and 45 min.

DISCUSSION

Effect of endothelin on isometric twitch

We confirm numerous earlier studies showing that endothelin potentiated isometric twitch tension of rat cardiac tissue with little or no change in the time course of the isometric twitch. However, studies involving rabbit and guinea-pig cardiac tissue did report a significant effect of endothelin on the time course of the isometric twitch, noting great increases in the duration of contraction and TT1/2R and reductions in the TTPT (Ishikawa, Yanagisawa, Kimura, Goto & Masaki, 1988; Li *et al.* 1991). These results point to a species difference in the effect of endothelin on cardiac tissue.

Potentiating the magnitude of the isometric twitch–force profile without altering its time course is also characteristic of another inotropic agent, ouabain (Hoh *et al.* 1991). In contrast with ouabain, whose inotropic action can be attributed to an increase in the magnitude of the calcium transient (Allen & Blinks, 1978; Wier & Hess, 1984), no appreciable change in the calcium transient is associated with the action of endothelin on rat ventricular myocytes (Kelly *et al.* 1990). This suggests that endothelin potentiates cardiac muscle twitch force by enhancing calcium sensitivity.

Effect of endothelin on contracture tension

Our results show that endothelin did not significantly alter the contracture tension at maximal Ba^{2+} activation, but did enhance the contracture tension at submaximal levels of Ba^{2+} activation. The levels of extracellular [Ba^{2+}] ranging from 0·5 to 70 mM presumably correspond to levels of intracellular concentration of the ion, spanning partial-to-full saturation of troponin C. Our results can thus be interpreted by saying that endothelin enhanced the force response at divalent ion concentrations corresponding to partial saturation of TnC. Earlier evidence supporting this notion (Kelly *et al.* 1990) was deduced from an increase in magnitude of the shortening of myocytes against internal load at a given submaximal level of activation rather than from an analysis of the effect of endothelin on the force–pCa relationship.

Since it has been reported that the action of endothelin is accompanied by alkalosis, the question arises whether alkalosis could explain the effect of endothelin on mechanics reported here. A recent investigation into the effect of alkalosis on hypertrophied rat heart fibre bundles (Mayoux *et al.* 1994) reported an increase in calcium sensitivity in skinned fibres together with an increase of approximately 20% in the tension at maximal Ca^{2+} in response to an increase in pH from 7·1 to 7·4. Our results showed that endothelin did not potentiate maximum contracture tension. So unless alkalosis can cause an increase in Ca^{2+} sensitivity without an increase in the tension at maximal TnC saturation, it is unlikely that alkalosis plays a significant role in the results described in this paper.

Effect of endothelin on the tension-to-stiffness ratio

The apparent increase in divalent cation sensitivity produced by endothelin in cardiac muscle cells could be due to an increase in the number of cross-bridges acting, or due to an increase in the force per unit cross-bridge. The observation that endothelin did not potentiate contracture tension elicited by maximal barium activation would suggest that the force per unit cross-bridge is not increased. We further investigated this by measuring the tension-to-stiffness ratio in the presence and absence of endothelin. High-frequency stiffness has been correlated with the number of cross-bridges participating in the generation of force (Julian & Morgan, 1981), and therefore the tension-to-stiffness ratio is a correlate of the force per unit cross-bridge. The result showed that endothelin did not change the tension-to-stiffness ratio even though the contracture tension was increased under its influence. This was observed as contracture tension increased as a function of time under the influence of endothelin at a constant level (0·5 mM) of barium activation (protocol 2, Fig. 2), as well as at different levels of barium activation induced after the muscle has reached a maximal endothelin-induced twitch response (protocol 1). We therefore conclude that endothelin does not alter the force per unit cross-bridge, but most likely acts by increasing the number of the cross-bridges acting for a particular submaximal level of saturation of TnC by calcium or barium ions.

Effect of endothelin on f_{min}

In this study, endothelin did not result in a shift in the stiffness minimum frequency, f_{min}, in rat ventricular muscle, indicating that those cross-bridge kinetics that are captured by f_{min} are unchanged by the action of endothelin (Fig. 4). It has been shown (Rossmanith & Tjokorda, 1993; Tjokorda, 1996) that in terms of a three-state model of cross-bridge cycling in which there are two attached states and one detached state, f_{min} is insensitive only to the rate constant of attachment. This insensitivity of f_{min} to endothelin is consistent with the lack of effect of endothelin on the twitch-time parameters, TTPT and T1/2R, in the light of unchanged Ca^{2+} transients. This contrasts with the mechanism of action of inotropic agents that work by increasing the level of intracellular cAMP, such as IBMX, and β-adrenergic receptor agonists, which result in increasing cross-bridge kinetics as indicated by an increase in f_{min} (Hoh *et al.* 1991) and abbreviation of the time

course of the isometric twitch. The lack of change in these stiffness spectra also makes it unlikely that endothelin alters the cross-bridge on-time, and further reinforces the interpretation of the tension-to-stiffness data that endothelin does not change the force per unit cross-bridge.

In summary, our mechanical analyses of endothelin-stimulated cardiac muscle are consistent with endothelin permitting more cross-bridges to attach to the thin filaments at a given level of TnC saturation by calcium or barium ions, without interfering with their kinetic characteristics as reflected by the complex-stiffness spectra. This conclusion differs substantially from that reported by McClellan, Weisberg & Winegrad (1996) using very different methods. They reported that endothelin at 1–10 nmol l^{-1} decreased the maximal speed of shortening and ATPase activity while increasing isometric force, and proposed that the cross-bridge duty cycle was increased. It is not clear at present whether endothelin at these low doses has an action different from that reported here.

Myosin light chain 2 phosphorylation, calcium sensitivity and skeletal muscle mechanics

The twitch potentiating action of endothelin on cardiac muscle is very interesting when compared with the phenomenon of post-tetanic potentiation (PTP) in fast skeletal muscle, whereby the isometric twitch is potentiated following a brief tetanus (Close & Hoh, 1968). In both phenomena, isometric twitch is potentiated without any change in twitch time course, and the enhancement of the twitch results from an increase in calcium sensitivity of the myofibrils. A substantial body of evidence supports the notion that the molecular basis of PTP is the phosphorylation of MLC2. During PTP in skeletal muscle, MLC2 is phosphorylated by MLCK, a calmodulin-regulated enzyme activated by the high cytosolic calcium present during tetanus (for reviews see Hoh, 1992; Sweeney *et al.* 1993). MLC2 phosphorylation causes the cross-bridge to move away from the thick filament toward the thin filament (refer to Fig. 17 in Metzger, Greaser & Moss, 1989), facilitating cross-bridge attachment to the actin binding sites, and thereby enhancing the number of cross-bridges activated at a given level of calcium.

Myosin light chain 2 phosphorylation as the mechanism of inotropic action of endothelin

We tested this hypothesis after labelling the intracellular pools of ATP using ^{32}Pi. We found that the administration of endothelin did not change the level of phosphorylation of TnI, in agreement with Gando, Nishihira, Hattori & Kanno (1995), but increased the phosphorylation of MLC2 relative to TnI by more than 50% within the time frame wherein near-maximal inotropic action of endothelin is manifest (15–45 min). Phosphopeptide analysis of MLC2 from control and endothelin-treated cardiac muscle showed that the same two peptides were labelled in both instances, suggesting that endothelin enhanced the phosphorylation of the same site or sites as in control muscle. Since phosphorylation of MLC2 in cardiac muscle is known also to enhance calcium sensitivity (Morano *et al.* 1988; Clement *et al.* 1992), these results strongly support the hypothesis that endothelin enhances calcium sensitivity by phosphorylation of MLC2 in cardiac muscle. Our findings agree with some of the results reported by Damron and co-workers (Damron, Darvish, Murphy, Sweet, Moravec & Bond, 1995) in that they also found that endothelin increased the relative level of phosphorylation of MLC2 in rat cardiac muscle. However in contrast to our findings, these authors also reported that endothelin, under conditions which substantially differ from ours, moderately increased the level of phosphorylation of TnI.

Pathways for myosin light chain 2 phosphorylation in the heart

It is unlikely that endothelin-induced cardiac MLC2 phosphorylation is mediated by the same enzyme as in skeletal muscle, since MLCK activity is low in the heart (Herring & England, 1986) and there is no enhancement of the calcium transient necessary to activate the Ca^{2+}-sensitive enzyme during endothelin intervention in the rat myocytes. Venema and co-workers (Venema *et al.* 1993) have recently shown that PKC is able to phosphorylate cardiac MLC2 in myofibrillar preparations to the same extent as does MLCK, and that these two kinases phosphorylated MLC2 in such a manner that the label occurred in the same two major phosphopeptides in each instance. These two kinases are therefore likely to have the same consequences of increasing myofibrillar calcium sensitivity. These workers also showed that any effect of PKC-catalysed phosphorylation of MLC2 on calcium sensitivity would be overcome by the inhibitory effects of the phosphorylation of TnI in myofibril preparations. As we do not see any effects of endothelin on TnI phosphorylation, but that endothelin increases phosphorylation of MLC2 at the two major sites, we propose that MLC2 is phosphorylated by PKC, which is known to be activated in response to the binding of endothelin to its receptors (Griendling, Tsuda & Alexander, 1989). It has been recently shown that PKC-ϵ is the major isotype of PKC in rat cardiac tissue, and that it is activated by endothelin and adrenaline as evidenced by its translocation from the cytosol to other subcellular sites (Bogoyevitch , Parker & Sugden, 1993). This isotype of PKC is independent of divalent cations (Rybin & Steinberg, 1994), and is therefore unlikely to be adversely affected by barium ions during muscle contracture. Consistent with this notion, Damron *et al.* (1995) have also shown that after prolonged incubation of myocytes in Ca^{2+}-free solution the level of MLC2 phosphorylation in response to endothelin stimulation is not affected.

Endothelin and α_1-adrenoceptor stimulation appear to share a common subcellular signalling pathway in cardiac myocytes which involves the hydrolysis of phosphatidylinositol and activation of PKC (Brown, Buxton & Brunton, 1985; Vigne *et al.* 1989; Iwakura *et al.* 1990). Indeed, α_1-adrenoceptor stimulation does increase calcium sensitivity of myofibrils

(Iwakura *et al.* 1990) as well as enhancing the level of phosphorylation of cardiac MLC2 to a small extent (Venema & Kuo, 1993).

In summary, we propose that in terms of its mechanism of action, endothelin is a novel class of inotropic agent which exerts its inotropic effect by stimulating the phosphorylation of MLC2 via PKC, thereby causing an enhanced calcium sensitivity and an increased rate of cross-bridge attachment without interfering with subsequent cross-bridge kinetic steps. In as much as endothelin shares the same intracellular signalling pathway with α-adrenergic agents, the proposed mechanism is also applicable to α-adrenergic stimulation.

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Author's email address

G. H. Rossmanith: gunther@mpce.mq.edu.au

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