Receptor-Mediated Effects of Endothelin on the L-Type Ca\textsuperscript{++} Current in Ventricular Cardiomyocytes

ELIZABETH J. KELSO, J. PAUL SPIERS, BARBARA J. McDERMOTT, C. NORMAN SCHOLFIELD and BERNARD SILKE

ABSTRACT

The purpose of this study was to establish whether specific receptor subtypes are responsible for mediating the effects of endothelin-1 (ET-1) and endothelin-3 (ET-3) on the L-type calcium current (I_{Ca}) using a number of receptor-selective antagonists, including PD155080 (ET\textsubscript{A}), BQ-788, RES-701 and IRL-1038 (ET\textsubscript{B}) and the ET\textsubscript{A}/ET\textsubscript{B} receptor-non-selective antagonist PD145065. Ventricular cardiomyocytes were isolated from adult New Zealand White rabbits using Langendorff perfusion with collagenase. I_{Ca} was recorded using a whole-cell patch-clamp technique. ET-1 decreased, whereas ET-3 increased, I_{Ca} at equimolar concentrations of 10 nM. The decrease in I_{Ca} produced by ET-1 was completely blocked by PD155080 and PD145065 (1 and 10 \mu M); however, I_{Ca} was increased upon washout of PD155080. Although the decrease in I_{Ca} produced by ET-1 was partially blocked by BQ-788 (1 and 10 \mu M), ET-1 in combination with either RES-701 (1 and 10 \mu M) or IRL-1038 (1 \mu M) produced a decrease in I_{Ca} similar to that produced by ET-1 alone. The increase in I_{Ca} produced by ET-3 was completely abolished by either BQ-788 or IRL-1038 (1 \mu M). These data indicate that the decrease in I_{Ca} produced by ET-1 in rabbit ventricular cardiomyocytes is mediated by the ET\textsubscript{A} receptor subtype, because PD155080 completely inhibited this response. The ET\textsubscript{B} receptor-selective antagonists RES-701 and IRL-1038 did not alter the decrease in current produced by ET-1, although the response was partially sensitive to BQ-788, which may lack receptor-subtype selectivity in these cells. In contrast, the increase in I_{Ca} produced by ET-3 was mediated by the ET\textsubscript{B} receptor subtype, because BQ-788 and IRL-1038 abolished this response.

Endothelin is a 21-amino-acid polypeptide that was originally isolated from the culture medium of porcine aortic endothelial cells (Yanagisawa et al., 1988). Three structurally and pharmacologically distinct isoforms of endothelin have been identified: ET-1, ET-2, ET-3 (Haynes and Webb, 1993). Although initially identified as a potent vasoconstrictor peptide, ET-1 mediates a wide variety of pharmacological activities in various tissues (Rubanyi and Polokoff, 1994). Many reports have described the positive inotropic, positive chronotropic and hypertrophic effects of ET-1 on the myocardium (Ishikawa et al., 1989b; Moravec et al., 1989; Takanashi and Endoh, 1991). The cellular basis for the actions of ET-1 is highly complex; however, alterations in Ca\textsuperscript{++} homeostasis appear to be central to the cardiac actions of this isopeptide. Although it is not clear whether ET-1 plays a physiological role, the isopeptide is implicated in many cardiovascular pathophysiological disorders, including hypertension, atherosclerosis, cardiogenic shock and myocardial infarction (Cavero et al., 1990; Kohno et al., 1990; McMurray et al., 1992; Huggins et al., 1993). Elevated plasma levels of ET-1 after myocardial infarction are likely to accompany much higher local concentrations of the peptide in the myocardium.

Two distinct subtypes of endothelin receptor, ET\textsubscript{A} and ET\textsubscript{B}, have been pharmacologically distinguished by the different potencies of the endothelin isopeptides toward the receptors. ET-1 has a higher affinity for the ET\textsubscript{A} receptor subtype than do ET-2 and ET-3, whereas the isopeptides have a similar affinity for the ET\textsubscript{B} receptor subtype (Rubanyi and Polokoff, 1994). Recently, the development and subsequent use of receptor-selective compounds have made receptor classification more complex (Sudjawaro et al., 1994; Warner, 1994). An increasing number of atypical ET responses have been identified that point to populations of receptors characterized as partially atypical or subtypes of ET\textsubscript{A} and ET\textsubscript{B} (Bax and Saxena, 1994). ET\textsubscript{A} receptors have...
been subdivided, on the basis of their differential selectivities to the ET$_A$ receptor-selective antagonist BQ-123, into ET$_{A1}$ (BQ-123-sensitive) and ET$_{A2}$ (BQ-123-insensitive) receptors (SuJawara et al., 1994). In addition, the ET$_B$ receptor subtypes located on vascular endothelium (ET$_{B1}$) and those located on smooth muscle (ET$_{B2}$) differ in antagonist selectivity (Douglas et al., 1994).

Both ET$_A$ and ET$_B$ receptor subtypes have been identified, using radioligand binding assays, in cardiac tissues isolated from human (Molenaar et al., 1993), rabbit (Takanashi and Endoh, 1991), guinea pig (Ono et al., 1994) and rat (Koseki et al., 1989). Both receptor subtypes coexist in cardiomyocytes, as has been demonstrated using in situ hybridization techniques (Hori et al., 1992). Many of the intracellular actions of ET-1 in cardiac tissues are mediated through the ET$_A$ receptor subtype. For example, ET-1 inhibits a protein kinase-A-dependent chloride current via the ET$_A$ receptor subtype in guinea pig ventricular cardiomyocytes (James et al., 1994). Moreover, BQ-123 abolished the decrease, produced by ET-1, in the accumulation of cyclic AMP and $I_{Ca}$ in atrial cells (Ono et al., 1994). It is speculated that stimulation of the ET$_B$ receptor subtype is likely to produce electrophysiological effects very different from those described for the ET$_A$ receptor subtype. ET-1 has been reported both to increase (Lauer et al., 1990; Ono et al., 1993), rabbit (Takanashi and Endoh, 1991), guinea pig (Ono et al., 1994) and rat (Koseki et al., 1989) the $I_{Ca}$ in cardiomyocytes, depending on the concentration and experimental conditions (Lauer et al., 1992; Kelso et al., 1996). The purpose of the current study was to establish, using a number of recently developed receptor-selective and receptor-non-selective antagonists, whether specific receptor subtypes are responsible for mediating the effects of ET-1 on $I_{Ca}$. An integral part of this investigation was to examine also the effects of ET-3, which has an equal affinity for the ET$_A$ and ET$_B$ receptor subtypes.

Materials and Methods

Isolation of cardiomyocytes. Ventricular cardiomyocytes were obtained from male New Zealand White rabbits (2.5–3 kg) after enzymatic dissociation using collagenase (Kelso et al., 1995a). All procedures were undertaken in accordance with Guidance on the Operations of the Animals (Scientific Procedures) Act 1986. Rabbits were anesthetized using sodium pentobarbitone (50 mg/kg i.v.) after heparinization (400 I.U./kg i.v.), and the chest was opened and the heart quickly removed and cannulated, through the ascending aorta, on a modified Langendorff perfusion apparatus. Blood was flushed from the coronary vasculature with a Ca$^{2+}$-free modified Krebs Ringer buffer (KRB) containing 110 mM NaCl, 2.6 mM KCl, 25 mM NaHCO$_3$, 1.2 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$ and 11 mM glucose (pH 7.4, 37°C), which had just previously been aerated with 95% O$_2$/5% CO$_2$. The perfusate was subsequently supplemented with 0.12% (w/v) collagenase and recirculated for approximately 15 min while maintained at 37°C and continuously aerated with 95% O$_2$/5% CO$_2$. After enzymatic digestion, the hearts were cut at the atrioventricular junction, sliced vertically toward the apex and chopped into cubes of 0.7 mm using a mechanical tissue chopper (McIlwain Chopper, Mickle Laboratory Engineering Co. Ltd., Surrey, U.K.). The minced tissue was placed in the collagenase-containing perfusate that had been supplemented with 0.2% (w/v) BSA, and the mixture was triturated using a 10-ml serological pipette for approximately 5 min. The loosened cells were filtered through a nylon mesh gauze of pore size 200 μm and washed twice. Ca$^{2+}$ was restored by means of centrifugation at 25 × g twice, and the cells were resuspended in modified KRB solutions containing 250 μM and 500 μM CaCl$_2$, respectively. Finally, the cells were layered onto a solution of 4% (w/v) BSA containing 1 mM CaCl$_2$ and were left to settle by gravity at 37°C. After approximately 5 min the supernatant was aspirated and the resulting cell material resuspended at a density of 1 to 2 mg protein/ml in a storage medium (M199 with Earle’s salts, containing 5 mM creatine, 5 mM taurine, 2 mM carnitine, 100 I.U./ml streptomycin, 100 μg/ml penicillin, pH 7.4) at 37°C. Suspensions of cardiomyocytes were more than 70% viable as estimated by their elongated rod-shaped morphology.

Recording techniques. An aliquot of cell suspension was placed in a transparent recording chamber and allowed to settle for 10 min before bathing with a modified Tyrode’s solution containing 137 mM NaCl, 5.4 mM KCl, 3 mM CaCl$_2$, 1.2 mM MgCl$_2$, 5 mM HEPES and 10 mM glucose (pH 7.4). The $I_{Ca}$ was recorded in voltage-clamp mode using an Axopatch 1D patch-clamp amplifier, and electrodes were filled with 110 mM K-gluconate, 20 mM KCl, 2 mM MgCl$_2$, 10 mM HEPES, 11 mM EGTA, 1 mM CaCl$_2$, 0.1 mM Na$_2$GTP and 2.5 mM creatine phosphate (pH 7.2). Patch electrodes were fabricated from thin-walled borosilicate capillaries with a filament (1.5 mm in outside diameter, Clarke Electrochemical) by means of a horizontal lazer puller (Sutter Instruments, Model P2000) and had tip resistances of 1 to 3 MΩ when filled with the electrode solution. Access to the cell interior was achieved by applying a brief pulse of negative pressure to the electrode after a gigaseal was formed. After stabilization, Ca$^{2+}$ currents were elicited by stepping the membrane voltage for 200 ms from a holding potential of −40 mV to test potentials of −30 to +60 mV at 5-s intervals and 10-mV increments; recordings were made at 90-s and 180-s intervals before and after the application of each drug combination. Current “ rundown” was not significant over the time course of the experiments—that is, over a 10-min period. All currents were stored on computer for subsequent analysis using customized software. Drugs were applied locally to the cell using a gravity-fed micropipetion system at approximately 150 μl/min, which allowed the solution bathing the cell to be changed in approximately 2 s.

Data analysis. The $I_{Ca}$ was measured, using standard methodology, as the difference between the peak of the inward current and the steady-state current level at the end of the voltage pulse (Varro et al., 1991). Current-voltage relationships were constructed, and peak $I_{Ca}$ values were compared at +10 mV. $I_{Ca}$ values were expressed as mean ± S.E. and data were analyzed, by comparing before and after drug applications, using analysis of variance followed by a Dunnett’s multiple comparison test (n > 2) or Student’s t test (n = 2); P values less than .05 were taken as indicating statistical significance.

Materials. ET-1 was purchased from Bachem Inc. (Torrance, CA). RES-701 and IRL-1038 were obtained from the American Peptide Co. (Sunnyvale, CA), and BQ-788 PD155080 and PD145065 were gifts from Parke-Davis Pharmaceutical Co., Ann Arbor, MI. All antagonists were dissolved in DMSO and stored in aliquots of 10$^{-4}$ M at −20°C; the final concentration of DMSO was <0.01%. ET-1 was dissolved in dilute acetic acid (0.01%) and stored in aliquots of 10$^{-5}$ M at −20°C. Collagenase (type I) was purchased from Serva Feinbiochemica (Heidelberg, Germany). Medium 199 was obtained from Gibco Ltd. (Paisley, U.K.). All other chemicals were of analytical grade (U.K.); twice-distilled water that had been deionized through a Millipore-Q system (Millipore, Haverhill) was used in all experiments.

Results

Effects of ET-1 and ET-3 on the $I_{Ca}$. Figure 1A shows original current traces at test potentials between −30 mV and +50 mV, in the absence and presence of ET-1 (1 and 10 nM). At the end of each experiment, the current was completely abolished using nifedipine (5 μM), which substantiates that the current measured was indeed that of the $I_{Ca}$ (results not shown). Contamination of the $I_{Ca}$ was minimized.

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by inducing voltage pulses from −40 mV, which inactivates the Na$^+$ current. The influence of K$^+$ currents was minimized by subtracting the current at the end of the test pulse from the peak inward current (Varro et al., 1991). ET-1 (10 nM) decreased (P < .05) the I$_{ca}$ to −1.86 ± 0.18 nA from a control value of −2.90 ± 0.16 nA (n = 6); this inhibitory effect was maximum after 90 s, remained stable at 180 s and was partially reversed to −2.46 ± 0.30 nA (fig. 1, B and C) after a 180-s washout of the peptide. ET-1, at a concentration of 1 nM, did not significantly alter the I$_{ca}$ from control. The corresponding current-voltage relationships, shown in figure 1B, revealed that the effects of ET-1 were not associated with a shift along the voltage axis but decreased the I$_{ca}$ at all potentials. ET-1 had no effect on the background holding current.

Fig. 1. Effects of ET-1 on I$_{ca}$ in ventricular cardiomyocytes isolated from rabbit myocardium (N = 5). a) Representative tracings recorded from a single cell showing the I$_{ca}$ before and 180 s after the application of ET-1 (10 nM), from a holding potential of −40 mV to test potentials between −30 mV and +50 mV. b) Current-voltage relationships constructed as the difference between the peak of the inward current and the steady-state current level at the end of the voltage pulse (n = 6). Recordings were made after a 180-s exposure to each concentration of the drug, and washout was assessed after perfusion in control solution, in the absence of ET-1, for a further 180 s. c) The concentration-dependent effects of ET-1 on peak I$_{ca}$, at +10 mV, were illustrated as percent change relative to control. Vertical lines are S.E.M. *P < .005 represents a difference from control peak I$_{ca}$. †P < .01 represents a difference compared with peak I$_{ca}$ in the presence of ET-1 (10 nM). N represents number of animals; n represents number of cells.

ET-3, at concentrations of 1 and 10 nM, increased (P < .05) the amplitude of peak I$_{ca}$ to −3.05 ± 0.27 nA (fig. 2A, and B) and −2.77 ± 0.21 nA (fig. 2C), respectively, from control values of −2.49 ± 0.16 nA and −2.10 ± 0.13 nA, respectively. This increase was time-dependent over a period of 270 s (fig. 2B). The peak I$_{ca}$ continued to increase to −3.10 ± 0.26 nA (fig. 2B) and −2.84 ± 0.22 nA (graph not shown) for a further 90 s in the absence of ET-3. The current then declined in amplitude to −2.87 ± 0.25 nA and −2.32 ± 0.19 nA, respectively, after a 270-s washout of ET-3. Because the effect of this isopeptide on I$_{ca}$ was slower, requiring a longer experiment time than ET-1, we recorded the effects of ET-3 at concentrations of 1 and 10 nM separately in different experiments (fig. 2, A and C). The current-voltage relationships in the presence of ET-3 were not associated with a shift along the voltage axis, and ET-3 had no effect on the background holding current. The effect of ET-3 was partially reversed (P < .01) upon washout of the drug (fig. 2D).

**Influence of the ET$_A$ receptor subtype on the ET-1-induced decrease in I$_{ca}$** PD155080, at concentrations of 1 and 10 μM, had no effect on the I$_{ca}$. (n = 6) (peak I$_{ca}$ values of −2.72 ± 0.26 nA and −2.76 ± 0.28 nA, respectively, compared with a control value of −2.90 ± 0.33 nA) (graph not shown). In order to minimize current rundown, the effects of ET-1 in combination with PD155080 were examined in a separate group of cells from those in which the effects of the antagonist alone were examined, or from those where the effects of ET-1 alone were examined. ET-1 (10 nM) in the presence of PD155080 (1 and 10 μM) did not alter the peak I$_{ca}$ amplitude, as observed from the original current trace in figure 3A, or over the voltage range of −30 to +60 mV (fig. 3B). The peak I$_{ca}$ amplitudes recorded in the presence of ET-1 in combination with PD155080 (1 and 10 μM) were −2.53 ± 0.13 nA and −2.58 ± 0.14 nA, respectively, which was not different from the control value of −2.65 ± 0.15 nA (n = 6). Washout of ET-1 in combination with PD155080 resulted in a small but significant increase in the peak I$_{ca}$ amplitude (fig. 3C) to −2.88 ± 0.13 nA. However ET-1 in
combination with PD155080 did not affect the voltage dependence of activation on \(I_{Ca}\).

**Influence of the ET<sub>B</sub> receptor subtype on the ET-1-induced decrease in \(I_{Ca}\).** At concentrations of 1 and 10 \(\mu\)M, neither RES-701 (−2.50 ± 0.23 nA and −2.53 ± 0.29 nA, respectively, compared with a control value of −2.64 ± 0.24 nA) nor BQ-788 (−2.20 ± 0.23 nA and −2.28 ± 0.27 nA, respectively, compared with a control value of −2.35 ± 0.22 nA) alone had any effect on the \(I_{Ca}\) (\(n = 6–8\)) (graphs not shown). ET-1 (10 nM) in combination with RES-701 (1 and 10 \(\mu\)M) decreased (\(P < .05\)) the peak \(I_{Ca}\) amplitude (\(n = 10\)) to −1.63 ± 0.13 nA and −1.59 ± 0.23 nA, respectively, from a control value of −2.65 ± 0.24 nA. This effect was partially reversed upon washout of the peptides to a peak \(I_{Ca}\) amplitude of −2.26 ± 0.24 nA (fig. 4A). However, the decrease in current amplitude seen at all potentials was not associated with a shift along the voltage axis (fig. 4B). The magnitude of the decrease in peak \(I_{Ca}\) amplitude produced by ET-1 in combination with RES-701 (34 ± 4% to 36 ± 4%) was similar to the decrease produced by ET-1 alone (34 ± 4%; fig. 4C). Moreover, ET-1 (10 nM) in combination with IRL-1038 (1 \(\mu\)M) decreased (\(P < .05\)) the peak \(I_{Ca}\) amplitude (\(n = 4\)) to −1.77 ± 0.46 nA from a control value of −2.37 ± 0.50 nA (graphs not shown).

ET-1 (10 nM) in combination with BQ-788 (1 and 10 \(\mu\)M) decreased (\(P < .05\)) the peak \(I_{Ca}\) amplitude (\(n = 6\)) to −2.13 ± 0.20 nA and −2.28 ± 0.2 nA, respectively, from a control value of −2.57 ± 0.27 nA (fig. 5, A and B). This decrease in current amplitude was completely reversed upon washout (−2.56 ± 0.23 nA). In comparison with the decrease in peak \(I_{Ca}\) amplitude produced by ET-1 alone, BQ-788 produced a concentration-dependent antagonism of the ET-1-induced decrease in \(I_{Ca}\) (fig. 5C). BQ-788 at concentrations of 1 and 10 \(\mu\)M, in combination with ET-1, decreased the peak \(I_{Ca}\) amplitude by 17 ± 3% and 11 ± 3%, respectively, compared with the 34 ± 4% decrease produced by ET-1 alone.

**ET<sub>AB</sub> receptor-non-selective antagonist in the presence of ET-1.** ET-1 (10 nM), in combination with the ET<sub>AB</sub> receptor-non-selective antagonist PD145065 (1 and 10 \(\mu\)M), had no effect on the \(I_{Ca}\) (\(n = 8\)). Values of peak current amplitude in the presence of ET-1, in combination with PD145065, were −1.98 ± 0.26 nA and −2.05 ± 0.30 nA at
concentrations of 1 and 10 μM, respectively (fig. 6A) and were not changed from a control value of $-2.11 \pm 0.29$ nA. Hence, PD145065 completely inhibited the effect of ET-1 alone on the ICa (fig. 6B).

**Influence of the ET$_B$ receptor subtype on the ET-3-induced increase in ICa.** ET-3 (1 nM) in the presence of either BQ-788 or IRL-1038 (1 μM) did not alter the ICa from control values of $-2.07 \pm 0.11$ nA and $-2.11 \pm 0.15$ nA, respectively (fig. 7). Both ET$_B$ receptor-selective antagonists abolished the increase (26 ± 7%) produced by ET-3 (1 nM) (fig. 2D). The combination of ET-1 and ET-3, at equimolar concentrations of 10 nM, did not alter the ICa from a control value of $-2.20 \pm 0.18$ nA; that is, both the decrease produced by ET-1 (fig. 1) and the increase produced by ET-3 (fig. 2) were completely abolished.

**Discussion**

Both ET-1 and ET-3 have potent positive inotropic effects on cardiac tissues (Rubanyi and Polokoff, 1994). Although Ishikawa et al. (1988b) originally speculated that the positive inotropic effect of ET-1 could be attributed to an increase in the ICa, subsequent studies have yielded conflicting findings. ET-1 has been reported to increase (Lauer et al., 1992; Tong et al., 1995; Bkaily et al., 1995), to decrease (Tohse et al., 1990; Ono et al., 1994; Cheng et al., 1995) and even to have no effect on the ICa (Furukawa et al., 1992; Habuchi et al., 1992) in cardiac tissues. The decrease in ICa produced by ET-1 could be reversed by altering the experimental conditions to maintain elevated intracellular GTP (Lauer et al., 1992). Other investigators, however, found that ET-1 decreased the ICa even when GTP was added to the dialyzing pipette solution (Cheng et al., 1995). Recently, using the perforated whole-cell patch-clamp technique, we demonstrated that ET-1 increases the ICa at a concentration of 1 nM (Kelso et al., 1996). This effect was reversed, in the same cells, at greater than nanomolar concentrations, producing a decrease in current amplitude at concentrations of 10 to 100 nM. This finding prompted us to investigate whether multiple endothelin receptor subtypes were responsible for the effects of ET isopeptides on the ICa.

Using the ruptured whole-cell patch-clamp technique, we found that ET-1, at a concentration of 10 nM, decreased the ICa by 34 ± 4% from control values (fig. 1), which is in contrast to an increase of similar magnitude (32 ± 5%) produced by ET-3 (fig. 2). Because ET-3 has been referred to as an ET$_B$ receptor-selective agonist (Ono et al., 1994) it appeared that the ET$_B$ receptor subtype might be involved in the positive effect on the ICa (an increase in current amplitude), whereas the ET$_A$ receptor subtype was likely to be involved in the negative effect of ET-1 on the ICa (the decrease in current amplitude).

PD155080 is a non-peptide receptor-selective antagonist.
that has high potency and selectivity similar to those of BQ-123 for the ET_A receptor subtype (Doherty et al., 1995).

Unlike BQ-123, the novel antagonist PD155080 has no effect on the ICₐ per se (Kelso et al., 1995b). PD155080 completely inhibited the decrease in ICₐ produced by ET-1 (10 nM) in combination with BQ-788 at a concentration of 1 µM and after washout of the drugs. The ETB receptor-selective antagonist RES-701 (Tanaka et al., 1994) did not prevent the decrease in ICₐ produced by ET-1 (10 nM) in combination with BQ-788 (fig. 4). However, the ETB receptor-selective antagonist BQ-788 (Ishikawa et al., 1994) partially inhibited the ET-1-induced decrease in ICₐ in a concentration-dependent manner (fig. 5), which suggests that the agonist may be mediated, in part, through a BQ-788-sensitive ETB receptor subtype insensitive to RES-701. Such a receptor subtype has been described in vascular tissues (Sudjawaro et al., 1994). The ETB receptor subtype that mediates vasorelaxation in vascular endothelium was observed to be pharmacologically different from the subtype that mediates vasoconstriction in smooth muscle, and these subtypes have been tentatively termed ET_B₁ and ET_B₂, respectively (Douglas et al., 1994). Whereas BQ-788 appeared to inhibit both ET_B₁ and ET_B₂ with similar affinity, RES-701 inhibited only the ET_B₁ receptor-mediated response (Warner, 1994; Sudjawaro et al., 1994). It is possible that the ICₐ response in ventricular cardiomyocytes is mediated by a ET_B₂ receptor subtype similar to that found in smooth muscle. However, it is more likely that BQ-788 partially inhibited the decrease in ICₐ produced by ET-1 as a result of a lack of selectivity of the

**Fig. 4.** Influence of the ETB receptor-selective antagonist RES-701 on the ET-1-induced decrease in ICₐ (N = 5). a) Representative tracings of peak ICₐ, at +10 mV, obtained in a single cell before and after the application of ET-1 (10 nM) in combination with RES-701 (1 µM) and after washout of the drugs. b) Current-voltage relationships in the absence and presence of ET-1 (10 nM) in combination with RES-701 (1 µM). Recordings were obtained at 180-s intervals. c) Comparison of percent changes, relative to the control, in peak ICₐ, for ET-1 in the absence and presence of RES-701 (1 and 10 µM). The blank bar is included for comparative purposes (fig. 1), whereas the shaded bars represent the change in peak ICₐ, values from another group of cells (n = 10). Vertical lines are S.E.M. *P < .005 compared with peak ICₐ, in the absence of drugs. RES represents RES-701.

**Fig. 5.** Influence of the ETB receptor-selective antagonist BQ-788 on the ET-1-induced decrease in ICₐ (N = 6) a) Representative tracings of peak ICₐ, at +10 mV, obtained in a single cell before and after the application of ET-1 (10 nM) in combination with BQ-788 (1 and 10 µM). Recordings were obtained at 180-s intervals. c) Comparison of percent changes, relative to the control, in peak ICₐ, for ET-1 (10 nM) in the absence and presence of BQ-788 (1 and 10 µM). The blank bar is included for comparative purposes (fig. 1), whereas the shaded bars represent the change in peak ICₐ, values from another group of cells (n = 8). Vertical lines are S.E.M. *P < .05 compared with peak ICₐ, in the absence of drugs. †P < .005 compared with peak ICₐ, in the presence of ET-1.

The ET_A receptor-selective antagonist RES-701 (Tanaka et al., 1994) did not prevent the decrease in ICₐ produced by ET-1 (fig. 4). However, the ET_B receptor-selective antagonist BQ-788 (Ishikawa et al., 1994) partially inhibited the ET-1-induced decrease in ICₐ in a concentration-dependent manner (fig. 5), which suggests that the agonist may be mediated, in part, through a BQ-788-sensitive ETB receptor subtype insensitive to RES-701. Such a receptor subtype has been described in vascular tissues (Sudjawaro et al., 1994). The ET_B receptor subtype that mediates vasorelaxation in vascular endothelium was observed to be pharmacologically different from the subtype that mediates vasoconstriction in smooth muscle, and these subtypes have been tentatively termed ET_B₁ and ET_B₂, respectively (Douglas et al., 1994). Whereas BQ-788 appeared to inhibit both ET_B₁ and ET_B₂ with similar affinity, RES-701 inhibited only the ET_B₁ receptor-mediated response (Warner, 1994; Sudjawaro et al., 1994). It is possible that the ICₐ response in ventricular cardiomyocytes is mediated by a ET_B₂ receptor subtype similar to that found in smooth muscle. However, it is more likely that BQ-788 partially inhibited the decrease in ICₐ produced by ET-1 as a result of a lack of selectivity of the
antagonist for the receptor subtypes. Although BQ-788 has been identified as a potent and selective ETB receptor antagonist (Ishikawa et al., 1994; Fukuroda et al., 1994), a recent report (Peter and Davenport, 1996) indicates that BQ-788 has low affinity and little selectivity for the ETB receptor subtype in human ventricular tissue.

In contrast to ET-1, however, the positive effect of ET-3 appears to be mediated through the ETB receptor subtype. BQ-788 completely abolished the increase in ICa produced by ET-3, but this response was also completely inhibited by IRL-1038 (fig. 7). Although these opposing responses, produced by ET-1 and ET-3, are coupled to different receptor subtypes (ETA and ETB, respectively), it is clear that multiple mechanisms are involved in the actions of the isopeptides on the ICa in ventricular cardiomyocytes. It is interesting that the magnitude of the decrease and increase in ICa produced by ET-1 and ET-3, respectively, are similar. Cardiac tissues have a dense population of endothelin receptors (Takai et al., 1992); however, ventricular cardiomyocytes have a much greater proportion of ETA receptor subtypes than ETB receptor subtypes.

The mechanisms responsible for these effects are unclear. Both ETA and ETB receptor subtypes can couple to phospholipase C (Vogelsang et al., 1994), resulting in the hydrolysis of phosphatidylinositol to produce inositol-1,4,5-triphosphate and 1,2-diacyl glycerol. The latter may play a role in activating voltage-dependent Ca11+ channels via activation of protein kinase C. However, inositol-1,4,5-triphosphate stimulates release of Ca11+ from the intracellular stores, resulting in an increase in intracellular Ca11+. Elevated intracellular Ca11+ levels can inhibit ET-1-induced Ca11+ influx and therefore may explain the heterogeneity at different concentrations. ET-1 is also reported to inhibit the accumulation of cyclic AMP in adult cardiomyocytes (Jones, 1996), which would decrease the ICa as a consequence of reduced protein kinase A-dependent phosphorylation of sarcolemmal proteins. However, in other cell systems ET-1 can stimulate cyclic AMP generation (Sokolovsky et al., 1994). ET-1 stimulates the formation of cyclic AMP in Chinese hamster ovary cells that express the ETA receptor subtype alone but decreases cyclic AMP levels in cells that express the ETb receptor subtype alone (Aramori and Nakanishi, 1992). It is possible that in cardiac cells, the negative effect on accumulation of cyclic AMP is coupled to an ETB receptor subtype, resulting in a decrease in the ICa. However, the positive effect on the ICa is unlikely to be mediated by such a mechanism,
because ET-1 has been found to increase cyclic AMP levels only at picomolar concentrations (Sokolovsky et al., 1994).

In summary, the ET_A receptor subtype is coupled to a decrease in $I_{Ca}$, whereas the ET_B receptor subtype is responsible for an increase in $I_{Ca}$. A role for endothelin has been proposed in a variety of cardiovascular disorders, including myocardial infarction and cardiac ischemia (Ruhany and Polokoff, 1994; Warner, 1994). The ability to alter calcium homeostasis will influence such pathogenic states. Indeed, suppression of the $I_{Ca}$ because of elevated levels of ET-1 is likely to be important in protecting against ventricular arrhythmias and myocardial Ca$^{2+}$ overload. However, evidence for multiple receptor subtypes that have opposing actions may have important implications for future therapeutic intervention.

Acknowledgments

The authors thank the medicinal Chemistry Department at Parke-Davis Pharmaceutical Division, Ann Arbor, Michigan, for the generous supply of compounds: BQ-788, PD155080 and PD145065.

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