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Stimulation of L-Type Ca^{2+} Current by the Endothelin Receptor A-Selective Antagonist, BQ-123, in Ventricular Cardiomyocytes Isolated from Rabbit Myocardium

Elizabeth J. Kelso,† J. Paul Spiers,‡ Barbara J. McDermott,*
C. Norman Scholfield‡ and Bernard Silke**

*Department of Therapeutics and Pharmacology, and ‡School of Biomedical Sciences, The Queen's University of Belfast, Whitla Medical Building, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland

ABSTRACT. BQ-123 is extensively used as an antagonist at endothelin (ET) receptors, having selectivity at the ET_A receptor subtype. In this study, the effects of BQ-123 *per se* on action potentials, L-type calcium currents, and potassium currents, were examined in ventricular cardiomyocytes isolated from adult, male, New Zealand White rabbits, using the patch-clamp technique. BQ-123 (1μM) increased ($P < 0.02$) the duration of the action potential to 267 ± 36 ms from a control duration of 228 ± 30 ms. BQ-123 did not have any effect on the inward rectifier or transient outward potassium currents, but increased ($P < 0.02$) the L-type Ca^{2+} current to −2.76 ± 0.3 nA from a control value of −2.45 ± 0.28 nA. The increases in both duration of the action potential and L-type Ca^{2+} current were reversed upon washout (233 ± 28 ms and −2.32 ± 0.31 nA, respectively) and were not different from the control values in the absence of BQ-123. In contrast, the endothelin receptor antagonists, BQ-788, PD155080 and PD145065 (1–10 μM) did not affect the L-type Ca^{2+} current. These results indicate that, unlike PD155080, BQ-788 and PD145065, the conventional ET_A receptor-selective antagonist, BQ-123, exerts a unique positive effect on the L-type Ca^{2+} current in ventricular cardiomyocytes isolated from rabbit myocardium. The mechanism of action of BQ-123, therefore, is not confined to ET receptor antagonism. BIOCHEM PHARMACOL **55**;6:897–902, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. BQ-123; endothelin-1; ventricular cardiomyocytes; ET_A receptor antagonist; BQ-788; PD145065; PD155080; Ca^{2+} current

Endothelin (ET)§ receptor-selective antagonists are valuable tools, not only for enhancing the understanding of (patho) physiological responses associated with the peptide but also for characterizing the function of ET receptors associated with different tissues. Two distinct subtypes of ET receptor, ET_A and ET_B, have been pharmacologically distinguished by the different potencies of the ET isopeptides towards the receptors [1–5]; ET-1 has a higher affinity than ET-3 for the ET_A receptor subtype [6, 7], whereas ET-1 and ET-3 have a similar affinity for the ET_B receptor subtype [8, 9]. Recently, the increase in development and subsequent use of receptor-selective compounds indicates a greater complexity in receptor classification [3]. ET_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 [10]. In addition, differential antagonist selectivities are apparent between the ET_B receptor subtypes located on vascular endothelium (ET_{B1}) and smooth muscle (ET_{B2}) [3, 10].

BQ-123 (cyclo[-D-Trp-D-Asp-Pro-D-Val-Leu-]) is among the first ET-1 receptor-selective antagonists to have been developed, and is still the most widely used. It is a cyclic pentapeptide synthesised by amino-acid substitution of the natural precursory substance, BE-18257A, itself a weak antagonist of ET_A receptors, isolated from the cultured broth of *Streptomyces misakiensis* [11]. BQ-123 is an extremely potent and highly selective antagonist at ET_A receptors with IC_{50} values, in binding experiments, of 7.3 nM (porcine aortic smooth muscle cells) and 18 μM (porcine cerebellum) for ET_A and ET_B receptor subtypes, respectively [11]. This compound has been used to identify at least three ET-receptor subtypes in ventricular myocar-

† Correspondence to: Dr. E. J. Kelso, Department of Therapeutics and Pharmacology, The Queen's University of Belfast, Whitla Medical Building, 97 Lisburn Road, Belfast. BT9 7BL. TEL: (01232) 335770; FAX: (01232) 438346; E-mail: e.kelso@qub.ac.uk

§*Abbreviations:* APD, action potential duration; BE-18257A, cyclo(D-Glu-L-Ala-D-Val-L-Leu-D-Trp-); BQ-123, cyclo[-D-Trp-D-Asp-Pro-D-Val-Leu-]; BQ-788, *N*-[*cis*-(2,6-dimethylpiperizinyl)carbonyl](4Me)LLeu-(1-methoxycarbonyl)DTrp-DNle; BSA, bovine serum albumin; DMSO, dimethylsulphoxide; ET, endothelin; ET_A, endothelin receptor A; ET_B, endothelin receptor B; IC_{50}, concentration giving half maximal inhibition; KRB, Kreb's Ringer buffer; PD145065, Ac(D-2-(10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5-yl))Gly-LLeu-LAsp-LIle-LIle-LTrp.Na; PD155080, sodium 2-benzo[1,3]dioxol-5-yl-3-benzyl-4(4-methoxy-phenyl)-4-oxobut-2-enoate.

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dium of rabbit [12], and extensively as a tool to classify functional ET receptor responses in a number of vascular smooth muscle tissues. However, preliminary use of this compound to investigate the effects of ET-1 on the L-type Ca^{2+} current showed that ET-1 in combination with BQ-123 produced a small but consistent increase in peak current amplitude. In the present study, we examined the electrophysiological effects of this peptide antagonist and compared its effect on the L-type Ca^{2+} current with that of alternative compounds, including the non-peptide antagonist, PD155080 (ET_A receptor-selective) and also BQ-788 (ET_B receptor-selective) and PD145065 (ET_A/ET_B receptor-nonselective).

MATERIALS AND METHODS

Materials

BQ-123 was obtained from Bachem Inc., and BQ-788 (N-[*cis*-(2,6-dimethylpiperizinyl)carbonyl](4Me)LLeu-(1-methoxycarbonyl) DTrp-DNle), PD155080 (sodium 2-benzo[1,3] dioxol-5-yl-3-benzyl-4(4-methoxy-phenyl)-4-oxobut-2- enoate) and PD145065 (Ac(D-2-(10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5-yl))Gly-LLeu-LAsp-LIle-LIle-LTrp.Na) were gifts from Parke–Davis Pharmaceutical Co. All antagonists were dissolved in dimethyl sulphoxide (DMSO) and stored in aliquots of 10^{-4} M at −20°; the final concentration of DMSO was <0.01%. Collagenase (Type I) was purchased from Serva Feinbiochemica. Medium 199 was obtained from GIBCO Ltd. All other chemicals were of analytical grade; twice distilled water which had been de-ionized through a Millipore-Q system was used in all experiments.

Isolation of Ventricular Cardiomyocytes

Ventricular cardiomyocytes were obtained following enzymatic dissociation [14]. Briefly, New Zealand White male rabbits (16 weeks; 2.5–3 kg) were anaesthetised using sodium pentabarbitone (50 mg/kg i.v.) following heparinization (400 IU/kg, i.v.). The heart was excised and cannulated through the ascending aorta, before being perfused and equilibrated at 37° with a modified Kreb's Ringer buffer (KRB), containing (in mM) NaCl 110, KCl 2.6, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2 and glucose 11 (pH 7.4; 95% O_2:5% CO_2). Enzymatic digestion then followed, using KRB supplemented with 0.12% (w/v) collagenase (Serva Feinbiochemica). The hearts were then cut at the atrioventricular junction, sliced vertically towards the apex and loosened cells were released by means of mechanical chopping (McIlwain Chopper, Mickle Laboratory Engineering Co. Ltd.). The minced tissue was placed in the collagenase-containing perfusate which had been supplemented with 0.1% (w/v) BSA, and the mixture triturated using a 10 ml serological pipette for approximately 5 min. The dispersed 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 left to settle by gravity, at 37°. After approximately 5 min the supernatant was aspirated and the resulting cell material resuspended at a density of 1–2 mg protein/mL in a storage medium (M199 with Earle's salts, containing [in mM] creatine 5, taurine 5, carnitine 2, streptomycin 100 IU/mL, penicillin 100 μg/mL, pH 7.4) at 25°. Suspensions of cardiomyocytes were >70% viable as estimated by their elongated rod-shaped morphology.

Electrophysiology

An aliquot of cell suspension was placed in a transparent recording chamber and allowed to settle for 10 min before perfusing with a Tyrode's solution containing (in mM) NaCl 137, KCl 5.4, $CaCl_2$ 3, $MgCl_2$ 1.2, HEPES 5, glucose 10, pH 7.4. Action potentials (APs) were elicited by passing a current pulse, of 6–12 ms duration, through the recording patch electrode using an Axopatch 1D patch-clamp amplifier. L-type Ca^{2+} currents were recorded in voltage-clamp mode, and low resistance (1–3 MΩ) electrodes were filled with (in mM): K-gluconate 120, Na_2GTP 0.1, KCl 20, $MgCl_2$ 1.2, HEPES 10, and EGTA 11, $CaCl_2$ 1, creatine phosphate 2.5 (pH 7.2). Access to the cell interior was achieved by applying a brief pulse of negative pressure to the electrode after a gigaseal was formed. Following stabilization, the Ca^{2+} current was activated by clamping the membrane voltage for 200 ms from a holding potential of −40 mV to test potentials of −40 to +60 mV; the clamp step was induced every 2s. The amplitude of peak Ca^{2+} current was measured as the difference between the peak of the inward current and the steady state level at the end of the voltage pulse [15]. Current "rundown" was not significant over the time course of the experiments; that is, over a 10 min period. "Steady state" potassium currents were measured at the end of a 5 sec pulse from a holding potential of −40 mV to test potentials −100 and +60 mV. The transient outward potassium current was obtained in the presence of $CdCl_2$ (0.25 mM), and the amplitude of the peak current was measured as the difference between the peak outward and steady state currents [15]. All currents were stored on computer for subsequent analysis using customised software. The various ET antagonists were applied locally to the cell using a gravity fed microperfusion system (150 μl/min), which allowed the solution bathing the cell to be changed in approximately 2 sec. Current-voltage relationships were determined at 90 sec intervals following drug administration.

Data Analysis

Durations of AP at 90% repolarization (APD) and peak current values were expressed as mean ± SEM. Current-voltages were constructed, and peak L-type Ca^{2+} current values were compared at +10 mV. Comparisons were made

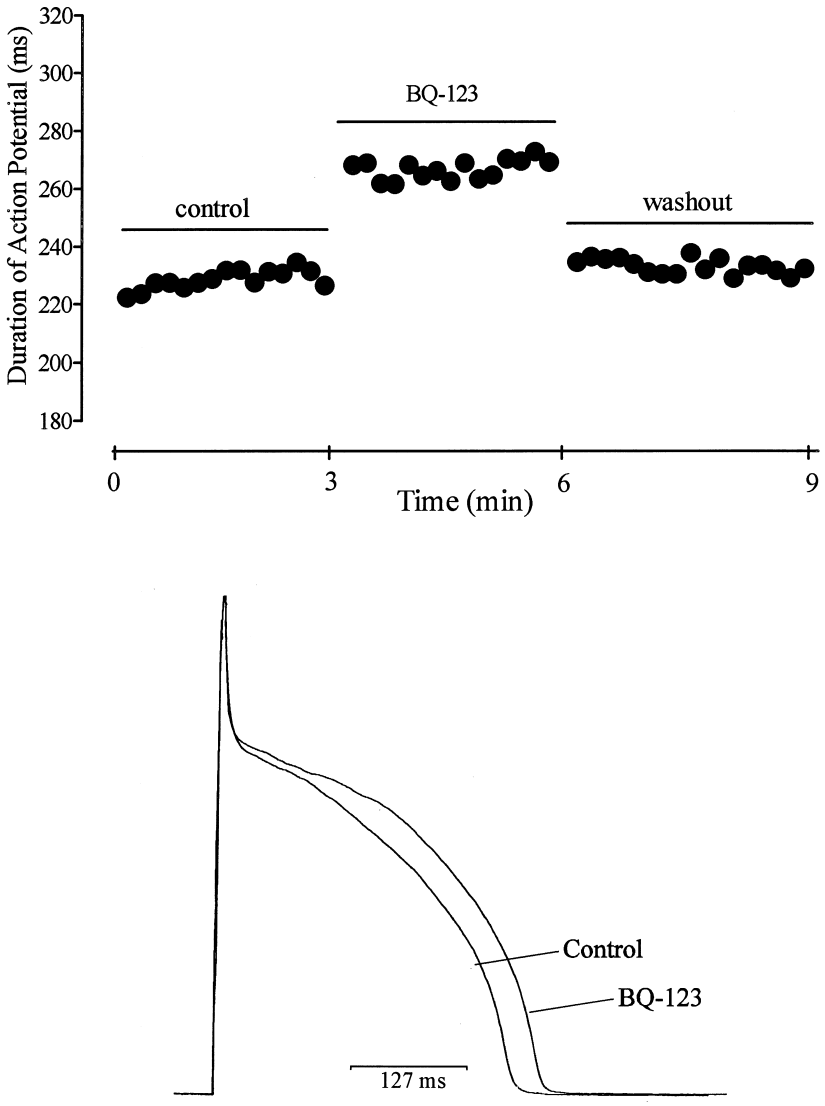

FIG. 1. Effects of BQ-123 (1 μM) on the duration of the action potential at 90% of repolarization in ventricular cardiomyocytes isolated from rabbit myocardium. (a) Recordings were taken before and after 30 sec exposure to BQ-123 for a period of 3 min, and also 30 sec after washout in the absence of BQ-123 for a further 3 min period ($n = 9$). (b) Typical action potential trace recorded before and after exposure to BQ-123.

using analysis of variance and Student's paired *t*-test. Statistical significance was established at $P < 0.02$.

RESULTS

BQ-123, at a concentration of 1 μM, produced an increase ($P < 0.02$) in APD to 267 ± 36 ms from a control duration of 228 ± 30 ms ($n = 9$) (Fig. 1). BQ-123 also increased ($P < 0.02$) the peak L-type Ca^{2+} current to −2.76 ± 0.3 nA from a control peak amplitude of −2.45 ± 0.28 nA ($n = 9$). The increases in both APD and the L-type Ca^{2+} current were reversed upon washout (233 ± 28 ms and −2.32 ± 0.31 nA, respectively) and were not different from the control values in the absence of BQ-123. The current-voltage relationship indicated that BQ-123 was not associated with a shift along the voltage axis (Fig. 2). Unlike BQ-123, the other ET antagonists, BQ-788, PD155080 and PD146065 (1–10 μM) had no effect on the L-type Ca^{2+} current, and peak current values are given in Table 1. At the end of each experiment, the total inward current in each of these experiments was completely abolished using 5 μM nifedipine (data not illustrated), confirming the presence of the L-type Ca^{2+} current. Contamination of the I_{Ca} was minimized by inducing voltage pulses from −40 mV, which inactivates the Na^+ current; the influence of other K^+ currents were minimised by subtracting the current at the end of the test pulse from the peak inward current [15].

The steady state potassium current in rabbit ventricular cells has been attributed primarily to current flowing through the inward rectifier channel [15], and was blocked using 200 μM $BaCl_2$ (data not illustrated). BQ-123 had no

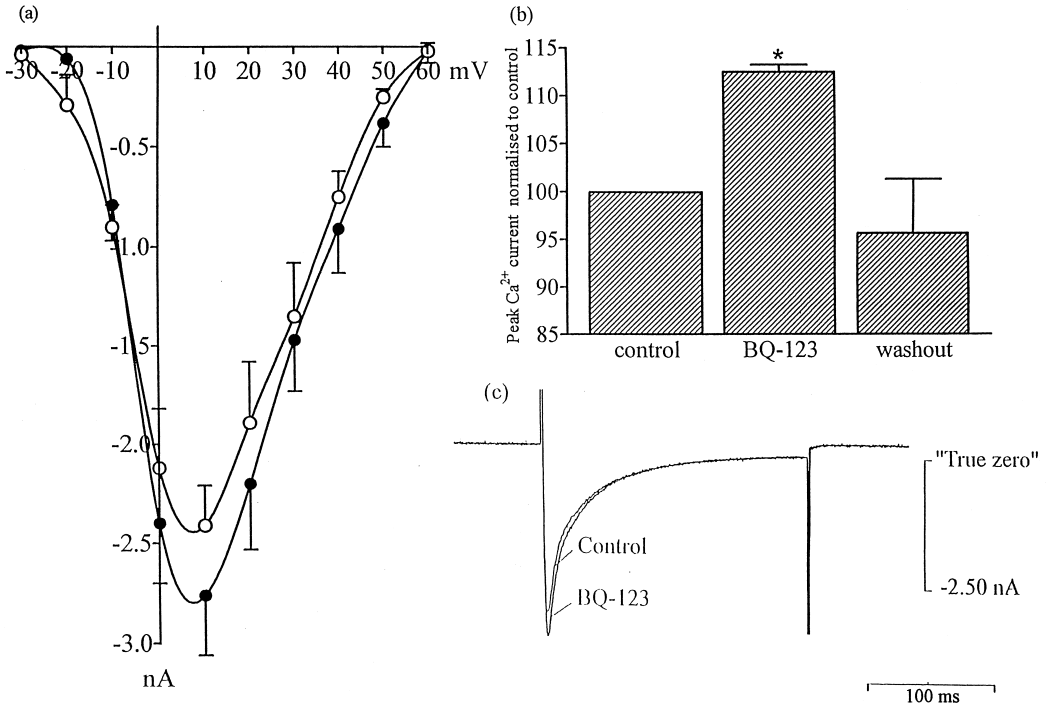

FIG. 2. Effects of BQ-123 (1 μM) on L-type Ca^{2+} current. (a) Current-voltage relationship of current before exposure (○) and after a 3 min exposure to BQ-123 (●) The L-type Ca^{2+} current was activated by clamping the membrane voltage for 200 ms from a holding potential of −40 mV to test potentials of −30 mV to +60 mV. (b) Bar-chart comparing the peak current amplitude recorded at +10 mV before and after exposure to BQ-123 (3 min), and also following a 3 min washout period in the absence of BQ-123. Values were normalised to control which was represented as 100%. (c) Typical current trace, at +10 mV, recorded before and after exposure to BQ-123. Data shown represent mean ± SEM (n = 9). $P < 0.02$ represents statistical significance from control values.

effect on this current (n = 8) (Fig. 3a) or the transient outward potassium current (n = 6) observed at potentials positive to −30 mV (Fig. 3b).

DISCUSSION

BQ-123 is widely used to characterise the receptor subtypes mediating the different effects of ET-1. In preliminary experiments we observed that BQ-123 not only reversed the effect of ET-1, but appeared to produce a small increase in the L-type Ca^{2+} current. This finding prompted an investigation of the electrophysiological effects of BQ-123 *per se*. BQ-123 increased the APD and this effect was accounted for by an increase in the L-type Ca^{2+} current. PD155080, an ET_A receptor-selective antagonist, which has similar affinity and selectivity to BQ-123 [16], however, did not produce any inherent effects on the L-type Ca^{2+} current. Furthermore, neither BQ-788, an ET_B receptor-selective antagonist, nor PD145065, an ET_A/ET_B receptor-non-selective antagonist, produced any effect on the L-type Ca^{2+} current.

The effects of ET-1 on the L-type Ca^{2+} current, using the patch-clamp technique, have been intensely investigated in recent years but the findings are still very contradictory. Both positive [17–19] and negative [20, 21] effects of ET-1 on the L-type Ca^{2+} current have been reported in cardiomyocytes. On the other hand, we have found that while ET-1 increased the L-type Ca^{2+} current at a concentration of 1 nM, the peptide decreased the L-type Ca^{2+} current at greater concentrations [13]. Since both effects of ET-1 could be obtained in the same preparation, it was important to ascertain whether BQ-123, having blocked the negative response, could be exposing a positive effect which may be associated with a different receptor subtype. However,

TABLE 1. Effects of endothelin receptor-selective and non-selective antagonists on peak L-type Ca^{2+} current

	BQ-123 (n = 9)	BQ-788 (n = 6)	PD155080 (n = 7)	PD145065 (n = 7)
Control	−2.48 ± 0.28	−2.35 ± 0.24	−2.86 ± 0.23	−2.75 ± 0.48
1μM	−2.79 ± 0.33*	−2.19 ± 0.25	−2.68 ± 0.26	−2.63 ± 0.48
Washout	−2.32 ± 0.31	−2.20 ± 0.24	−2.72 ± 0.31	−2.73 ± 0.46

Values are mean ± SE of peak L-type Ca^{2+} current values (nA).
* $P < 0.02$ difference from control.

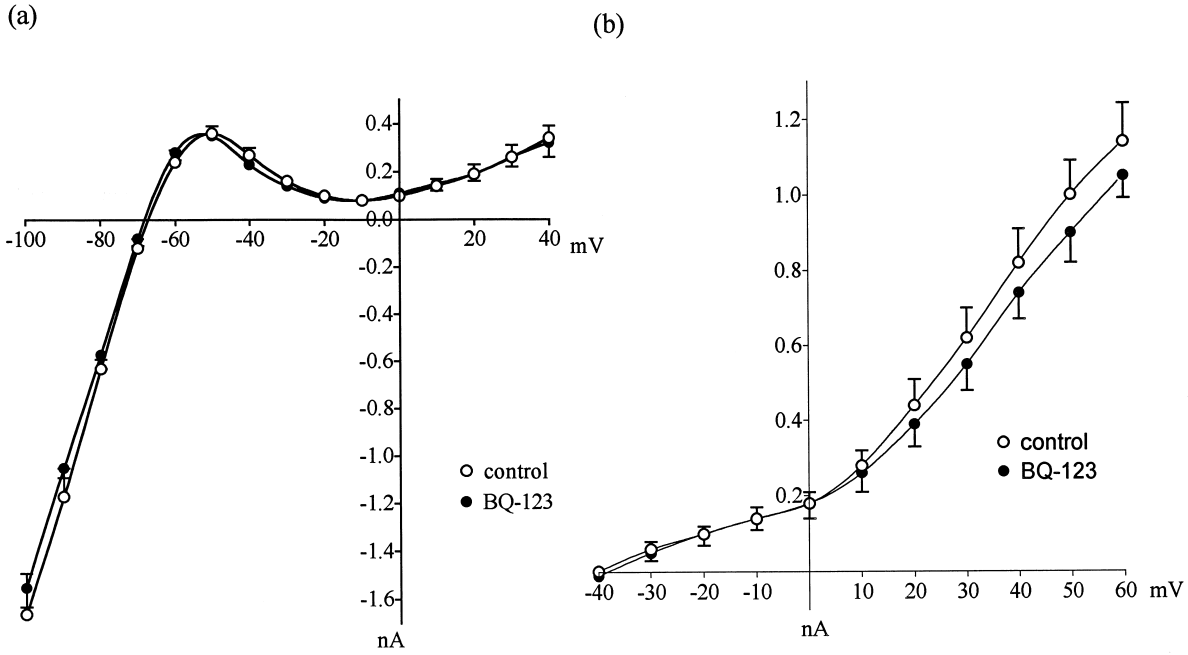

FIG. 3. Absence of effect of BQ-123 on potassium currents. (a) "Steady state" potassium current-voltage relationship in the absence and presence of BQ-123. Recordings were obtained at the end of a 5 sec pulse from a holding potential of −40 mV at a frequency of 0.2 Hz; voltage was stepped from −100 mV to +60 mV in 10 mV increments. (b) Current-voltage relationship of the peak transient outward potassium current from a holding potential of −40 mV to test potentials of −40 mV to +60 mV. All recordings were obtained in the presence of 0.25 μM $CdCl_2$. Data shown represent mean ± SEM (n = 6–8).

BQ-123, *per se*, produced a positive effect on the L-type Ca^{2+} current in ventricular cardiomyocytes, and is therefore not ideal as a tool for investigating the dual effect of ET-1 on L-type Ca^{2+} current in rabbit ventricular cardiomyocytes.

While the ET_A receptor-selective antagonist, PD155080, blocked the negative effect of ET-1 on the L-type Ca^{2+} current, an increase in current amplitude was observed following washout of both compounds [22]. Hence, it is conceivable that antagonism of the ET_A receptor-mediated negative effect on the L-type Ca^{2+} current may expose a residual positive effect acting at non-ET_A receptor subtypes. Whether BQ-123 could be unmasking a response at a subtype of non-ET_A receptor can only be speculated at this point. In a recent study, Ono *et al.* [20] found that the decrease in the L-type Ca^{2+} current produced by ET-1 (10 nM) was blocked by BQ-123 in guinea-pig and rabbit atrial cardiomyocytes. They recorded a progressive increase in the L-type Ca^{2+} current following exposure to BQ-123 alone, of a similar proportion to that observed in our study (~13%). Although the authors failed to comment on this observation, it would appear to be "real" as the L-type Ca^{2+} current is more likely to decrease in amplitude with time. Although Cheng *et al.* [21] reported that BQ-123 (1 μM) prevented the decrease in the L-type Ca^{2+} current in human atrial and ventricular cardiomyocytes, recordings were only documented for BQ-123 in the absence and presence of ET-1 without any prior control values.

There is little previous evidence of BQ-123 having an inherent effect in the absence of ET. Bigaud and Pelton [23] reported a decrease in femoral arterial blood pressure accompanied by a systemic vasodilation, following intravenous administration of BQ-123 (1.6 μg/kg), in the anaesthetized rat. However, these effects were contrary to haemodynamic data obtained from the conscious rat [11]. The mechanism for the response observed by Bigaud and Pelton [23] is uncertain, but may result from an inhibition of ET-1-mediated basal tone via the ET_A receptor. It was also interesting to note that BQ-123 (1 μM) increased the positive contractile effect of ET-1 at elevated concentrations (1–100 nM) in rabbit myocardium; however, these data were not discussed by the authors [12]. Sokolovsky [24] hypothesised the existence of a secondary binding site which allows BQ-123 to interact and/or interfere with endothelin binding. Whether interaction of BQ-123 with such a site produces a weak agonist response resulting in an increase in the L-type Ca^{2+} current or whether this effect is direct and consequently may not be ET receptor-mediated can only be speculated.

In summary, the present study clearly demonstrated a positive effect of BQ-123 on the L-type Ca^{2+} current, which was not apparent using other ET antagonists, in ventricular cardiomyocytes isolated from rabbit myocardium. Although the negative effect of ET-1 on the L-type Ca^{2+} current does appear to be mediated at least in part by the ET_A receptor-subtype as reported by others [20, 21] and using an alternative ET_A receptor-selective antagonist,

PD155080 [22], the mechanism of action of BQ-123 is not confined to ET_A receptor antagonism.

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