ELSEVIER

European Journal of Pharmacology 308 (1996) 351–355

ejp

Short communication

Dual effects of endothelin-1 on the L-type Ca^{2+} current in ventricular cardiomyocytes

Elizabeth Kelso [a,*], Paul Spiers [b], Barbara McDermott [a], Norman Scholfield [b], Bernard Silke [a]

[a] *Department of Therapeutics and Pharmacology, Queen's University of Belfast, Whitla Medical Building, 97 Lisburn Road, Belfast BT9 7BL, UK*
[b] *School of Biomedical Sciences, The Queen's University of Belfast, Whitla Medical Building, 97 Lisburn Road, Belfast BT9 7BL, UK*

Received 7 May 1996; accepted 10 May 1996

Abstract

The effects of endothelin-1 on the L-type Ca^{2+} current were studied in rabbit ventricular cardiomyocytes, using both the 'perforated' and the conventional 'ruptured' whole-cell patch-clamp techniques. Endothelin-1 exerts a dual effect on ventricular cardiomyocytes using experimental conditions which minimize intracellular dialysis; endothelin-1 produced both an increase (10^{-9} M) and a decrease (10^{-8} M) in the L-type Ca^{2+} current using the perforated patch-clamp technique. However, using the ruptured patch-clamp technique, endothelin-1 produced a similar decrease in L-type Ca^{2+} current at 10^{-8} M, but no effect was observed at a concentration of 10^{-9} M. These effects suggest multiplicity in the receptor-effector coupling mechanism.

Keywords: Endothelin-1; Cardiomyocyte, ventricular; Ca^{2+} current

1. Introduction

Endothelin-1, a 21 amino acid polypeptide produced by endothelial cells, is not only among the most potent known vasoconstrictor substances, but is also a potent positive inotropic agent in isolated myocardium (Moravec et al., 1989). Based on early reports, it was speculated that the positive inotropic effect of endothelin-1 was influenced by an increase in the voltage-dependent L-type Ca^{2+} current (I_{Ca}) (Yanagisawa et al., 1988). However, subsequent studies clearly indicate that endothelin-1 can produce a positive inotropic effect independent of changes in the I_{Ca} (Rubanyi and Polokoff, 1994).

Studies examining the direct effects of endothelin-1 on I_{Ca} are confusing; positive (Lauer et al., 1992; Bkaily et al., 1995), negative (Ono et al., 1994) and an absence of (Tohse et al., 1990) effects have been reported in cardiac tissues. In this study, the concentration-dependent effects of endothelin-1 were examined, in ventricular cardiomyocytes isolated from rabbit myocardium, using a perforated patch-clamp technique as well as the conventional ruptured patch-clamp technique. The ruptured patch-clamp technique allows diffusible cytoplasmic constituents to be dialysed by the contents of the electrode solution, leading to loss of intracellular constituents. The perforated patch configuration, on the other hand, has the advantage of preventing loss of cytosolic components; the antibiotic, nystatin, forms voltage-insensitive ion pores in the cell membrane, which allow monovalent ions to cross the membrane but is impermeable to multivalent ions, second messengers, proteins, and, in general, molecules with > 0.8 nm diameter (Takens-Kwak et al., 1992). Hence, stable recordings may be maintained for longer periods and the intracellular milieu is minimally disturbed. The aim of the present study was to clarify the role of endothelin-1 in the regulation of the I_{Ca}. Using the perforated patch-clamp recording method, in addition to the conventional whole-cell recording technique, it was possible to clarify the differences reported in the literature regarding the effects of endothelin-1 on I_{Ca}.

2. Materials and methods

2.1. Cell isolation

Ventricular cardiomyocytes were obtained from male New Zealand white rabbits (2.5–3 kg) following enzy-

* Corresponding author. Tel.: +44 1232 335770; fax: +44 1232 438346.

PII S0014-2999(96)00366-4

matic dissociation using collagenase, according to a method described previously (Kelso et al., 1995). Briefly, rabbits were anaesthetized using sodium pentabarbitone (50 mg/kg i.v.) following heparinization (400 IU/kg, i.v.) and the hearts were removed and perfused, via an aortic cannula, with a modified Krebs Ringer buffer (KRB) containing (mM): NaCl 110, KCl 2.6, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11 (pH 7.4, 37°C) and collagenase (0.12% w/v). Loosened cells were released by means of mechanical chopping, and Ca^{2+} tolerance was restored gradually following two washes in KRB containing 250 μM and 500 μM $CaCl_2$, respectively. Isolated cells were placed in a storage medium (M199 with Earle's salts) supplemented with (mM): creatine 5, taurine 5, carnitine 2, streptomycin 100 IU/ml and penicillin 100 μg/ml (pH 7.4) at 25°C. Suspensions of cardiomyocytes were > 70% viable as estimated by their elongated rod-shaped morphology.

2.2. Recording techniques

An aliquot of cell suspension was placed in a transparent recording chamber and allowed to settle for 10 min before perfusing with a modified Tyrode's solution containing (mM) NaCl 137, KCl 5.4, $CaCl_2$ 3, $MgCl_2$ 1.2, HEPES 5 and glucose 10 (pH 7.4). Action potentials were elicited by passing a current pulse, of 6–12 ms duration, through the patch electrode using an Axopatch 1D patch-clamp amplifier. The I_{Ca} was recorded in voltage-clamp mode, and electrodes were filled with (mM): K-gluconate 110, KCl 20, $MgCl_2$ 2, HEPES 10, EGTA 11, $CaCl_2$ 1, GTP 0.1 and creatine phosphate 2.5 (pH 7.2), when using the ruptured patch-clamp technique. This electrode solution was supplemented with 150 μg/ml nystatin (Sigma) when using the perforated patch-clamp technique. A stock nystatin solution (50 mg/ml) was prepared in dimethylsulphoxide and was stable at −20°C for 1 month. Daily aliquots of this solution were prepared following dilution and ultrasonication, resulting in a final concentration of 150 μg/ml in 0.2% dimethylsulphoxide which was stable for 2 h. This concentration of dimethylsulphoxide applied to the cells had no observable effect on the I_{Ca}. Patch electrodes were fabricated from thin-walled borosilicate capillaries with a filament (GC 150TF; Clarke Electrochemical), by means of a horizontal lazer puller (Sutter Instruments, Model P2000), and had tip resistances of 1–3 MΩ when filled with the electrode solution. Using the ruptured patch-clamp technique, access to the cell interior was achieved by applying a brief pulse of negative pressure to the electrode after a gigaseal was formed. In contrast, using the perforated patch-clamp technique, access was achieved by nystatin which gradually partitioned into the membrane forming small pores; as more pores formed, the series resistance decreased while the capacitive transients increased. Typically, suitable access was achieved within 20 min after gigaseal formation. Following stabilization, Ca^{2+} currents were elicited, using either the ruptured or perforated patch-clamp techniques, by stepping the membrane voltage for 200 ms from a holding potential of −40 mV to test potentials of −30 to +60 mV at 1 s intervals and 10 mV increments. All currents were stored on computer for subsequent analysis using customized software.

2.3. 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. Action potentials, at 90% of repolarization, and I_{Ca} values were expressed as mean ± S.E., and data were analysed using analysis of variance followed by a multiple comparison test (Dunnett's); $P < 0.05$ was taken as indicating statistical significance.

3. Results

3.1. Perforated patch-clamp method

The I_{Ca} was stable for up to 45 min, using the perforated patch-clamp technique; this was a longer time than was required to complete a cumulative dose-response experiment using endothelin-1. Stable recordings were obtained following 5 min exposure, either in the absence or presence of endothelin-1 at the various concentrations used; dose-response experiments were completed within 30 min. Endothelin-1 produced a 25 ± 6% increase ($P < 0.05$) in the peak I_{Ca} amplitude, at a concentration of 10^{-9} M, (−3.18 ± 0.25 nA) from a control peak amplitude of −2.57 ± 0.27 nA (Fig. 1). This increase was reversed at a concentration of 3×10^{-9} M (−2.33 ± 0.26 nA) and was not different from the control value in the absence of endothelin-1 (Fig. 1b). At 10^{-8} M, endothelin-1 produced a 31 ± 6% decrease ($P < 0.05$) in I_{Ca} amplitude to −1.76 ± 0.14 nA. A typical current trace showing the increase and decrease in a single cell is shown in Fig. 1c. The total inward current was completely abolished using 5 μM nifedipine (results not shown).

3.2. Ruptured patch-clamp method

To minimise current rundown, which is a problem using this method, endothelin-1 was applied to the cells in a non-cumulative manner. The I_{Ca} was stable for 10 min without rundown. Endothelin-1, at a concentration of 10^{-8} M, decreased (33 ± 4%) the peak I_{Ca} to −1.86 ± 0.18 nA from a control value of −2.9 ± 0.18 nA; this decrease was reversed upon washout of endothelin-1 (Fig. 2b). At the lower concentration of 10^{-9} M, however, endothelin-1 did

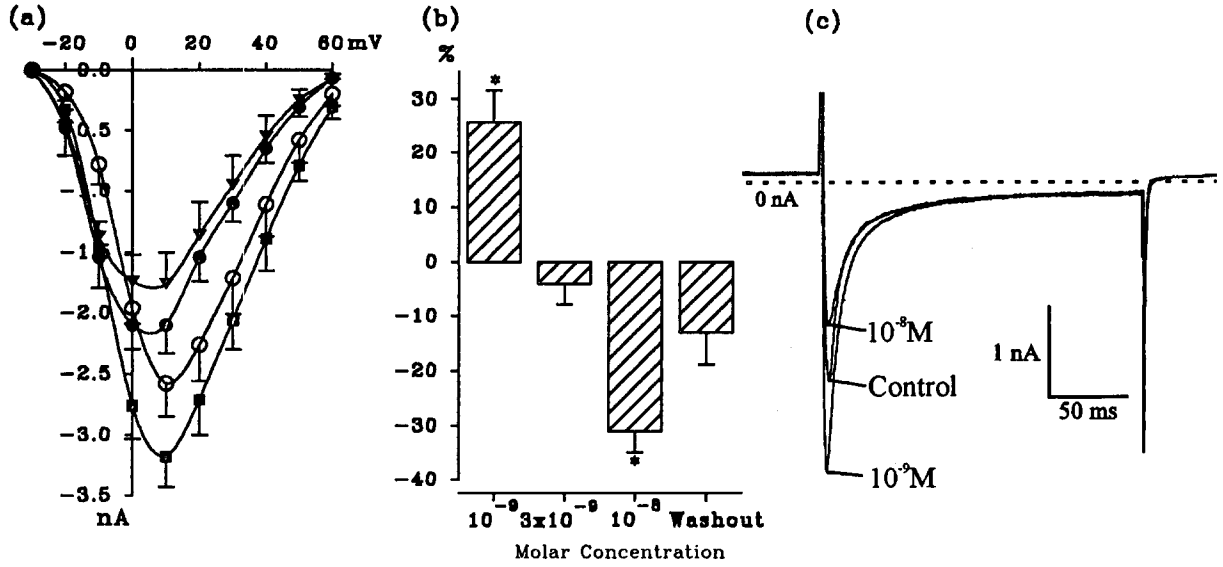

Fig. 1. Cumulative dose-response relationship of endothelin-1 on the I_{Ca} using the perforated patch-clamp technique. (a) Current-voltage relationships in the absence and presence of endothelin-1 (10^{-9} and 10^{-8} M); current amplitude was plotted as a function of membrane test potentials for depolarizations from a holding potential of −40 mV. Each point is the mean ± S.E. of $n = 6$. Open circles (○): control; filled squares (■): 10^{-9} M endothelin − 1; filled triangles (▼): 10^{-8} M endothelin-1; filled circles (●): washout of drug. (b) Concentration-dependent effects of endothelin-1 (10^{-9} to 10^{-8} M) on the peak I_{Ca} elicited from a holding potential of −40 mV to a test potential of +10 mV, represented as a percentage change from the control value in the absence of endothelin-1 (−2.57 ± 0.27 nA); $P < 0.05$ represents statistical significance from control values. (c) Current trace showing peak I_{Ca} at +10 mV in the absence (control) and presence of endothelin-1 at concentrations of 10^{-9} and 10^{-8} M.

not alter the peak current value from a control value of −2.3 ± 0.23 nA (Fig. 2a). Likewise, endothelin-1, at a concentration of 10^{-8} M, decreased the action potential duration ($P < 0.05$) to 241 ± 34 ms from a control value of 311 ± 29 ms. However, no apparent effect in action potential duration was observed in the presence of endothelin-1 at 10^{-9} M (322 ± 34 ms). The current-voltage relationships revealed that the effects of endothelin-1 were not associated with a shift along the voltage axis using either technique.

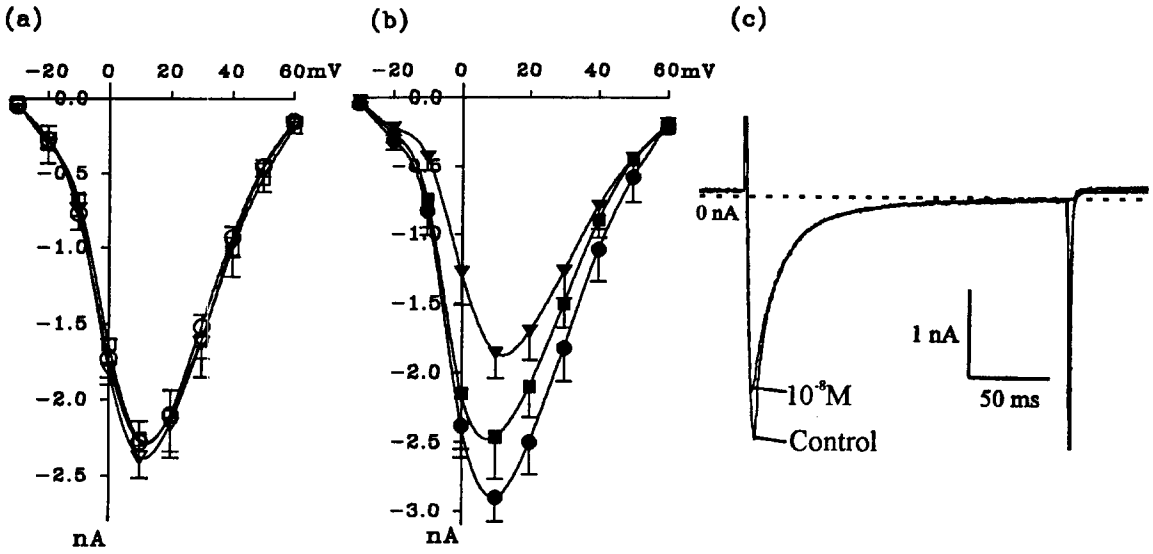

Fig. 2. Effects of endothelin-1 on the I_{Ca} using the ruptured patch-clamp technique. (a) Current-voltage relationships in the absence and presence of 10^{-9} M endothelin-1 ($n = 7$). Open circles (○): control; open triangles (▽): 10^{-9} M endothelin-1; open squares (□): washout of drug. (b) Current-voltage relationships in the absence and presence of 10^{-8} M endothelin-1 ($n = 8$). Filled circles (●): control; filled triangles (▼): 10^{-8}M endothelin-1; filled squares (■): washout of drug. (c) Current trace showing peak I_{Ca} at +10 mV in the absence (control) and presence of endothelin-1 at a concentration of 10^{-8} M.

4. Discussion

Endothelin-1 exerts a dual effect on the I_{Ca}, in rabbit ventricular cardiomyocytes, using the perforated patch-clamp technique. At 10^{-9} M, endothelin-1 increased the I_{Ca}, but this effect was reversed at higher concentrations, and at a concentration of 10^{-8} M, endothelin-1 decreased the I_{Ca}. However, using the ruptured patch-clamp technique, endothelin-1 only produced a negative effect at the elevated concentration (10^{-8} M); no effect was observed at lower concentrations. These results suggest that using experimental conditions which allow the intracellular constituents to remain largely undisturbed, endothelin-1 is capable of increasing the I_{Ca}. While most previous studies, examining the effects of endothelin-1 on the I_{Ca}, either report an increase or a decrease in peak amplitude, the present experiments show that endothelin-1 produces both effects dependent on concentration, indicating the complexity of the dynamics in the cell signalling system. Previous reports (Ono et al., 1994; Bkaily et al., 1995) which demonstrate a decrease in the I_{Ca} have shown that this response is mediated by the endothelin-A receptor subtype. However, the question remains to be answered whether the positive effect of endothelin-1 on I_{Ca} occurs due to activation of an alternative receptor subtype, for example, endothelin-B receptor subtype.

Lauer et al. (1992) also reported that endothelin-1 caused both an increase and decrease in the I_{Ca} in rabbit ventricular cardiomyocytes, but these effects were obtained in different populations of ventricular cardiomyocytes and using altered experimental conditions; the positive effect of endothelin-1 (2×10^{-8} M) on I_{Ca} was observed using an electrode solution supplemented with GTP, whereas the negative effect, obtained using an equimolar concentration of endothelin-1, was observed in the absence of GTP. In the present study, however, endothelin-1 did not increase the I_{Ca} using the conventional ruptured patch-clamp technique and electrodes with elevated concentrations of GTP (100 μM). Whether the variation in results is a reflection of the degree of intracellular dialysis or whether a small positive effect by endothelin-1 is unapparent due to other experimental limitations can only be speculated. Using the ruptured patch-clamp technique, endothelin-1 produced a small increase in the duration of the action potential, although this effect was not significant, but would tend to suggest that the I_{Ca} may be affected. However, using the perforated patch-clamp technique, both positive and negative effects of endothelin-1 on the I_{Ca} were clearly demonstrated suggesting that a loss or disturbance of some intracellular component is a likely cause of discrepancy.

Multiple signalling pathways are likely to be involved in the actions of endothelin-1 in ventricular myocardium. Endothelin-1 activates both endothelin-A and endothelin-B receptor subtypes present in these cells (James et al., 1994). One receptor subtype can couple, in the same cell, to different signal transduction pathways (Hilal-Dandan et al., 1992). Whether the dual effects of endothelin-1 on I_{Ca} result as a consequence of multiple signalling pathways following activation at a single receptor subtype, or whether more than one receptor subtype is involved is yet to be determined. Both receptor subtypes couple to phospholipase C (Vogelsang et al., 1994) resulting in the hydrolysis of phosphatidylinositol to produce inositol-1,4,5-triphosphate and 1,2-diacylglycerol. The latter may play a role in activating voltage-dependent Ca^{2+} channels via activation of protein kinase C. However, inositol-1,4,5-triphosphate stimulates release of Ca^{2+} from the intracellular stores resulting in an increase in intracellular Ca^{2+}. Elevated intracellular Ca^{2+} levels can inhibit endothelin-1-induced Ca^{2+} influx, and therefore may explain the heterogeneity at different concentrations. On the other hand, Takenaka et al. (1992) have suggested that mobilization of intracellular Ca^{2+} by inositol-1,4,5-triphosphate may activate Ca^{2+}-dependent Cl^- permeable channels resulting in an efflux of Cl^- and membrane depolarization which, in turn, may activate voltage-operated channels and causes Ca^{2+} influx.

In conclusion, we have established a dual effect of endothelin-1 on the I_{Ca} which is dependent on concentration, whereas previous studies have shown either a positive or negative effect on I_{Ca} when experimental conditions are altered between cells. Although multiple signalling mechanisms are likely to account for the heterogeneity regarding the effects of endothelin-1 on the I_{Ca}, it is clear that experimental methodologies have contributed to the disparite findings in the literature.

References

Bkaily, G., S. Wang, M. Bui and D. Menard, 1995, ET-1 stimulates Ca^{2+} currents in cardiac cells, J. Cardiovasc. Pharmacol. 26, S293.

Hilal-Dandan, R., K. Urasawa and L.L. Brunton, 1992, Endothelin inhibits adenylate cyclase and stimulates phosphoinositide hydrolysis in adult cardiac myocytes, J. Biol. Chem. 267, 10620.

James, A.F., L.H. Xie, Y. Fujitani, S. Hayashi and M. Horie, 1994, Inhibition of the cardiac protein kinase A-dependent chloride conductance by endothelin-1, Nature (London) 370, 297.

Kelso, E.J., B.J. McDermott and B. Silke, 1995, Actions of the novel vasodilator, flosequinan, in isolated ventricular cardiomyocytes, J. Cardiovasc. Pharmacol. 25, 376.

Lauer, M.R., M.D. Gunn and W.T. Clusin, 1992, Endothelin activates voltage-dependent Ca^{2+} current by a G protein-dependent mechanism in rabbit cardiac myocytes, J. Physiol. 448, 729.

Moravec, C.S., E.E. Reynolds, R.W. Stewart and M. Bond, 1989, Endothelin is a positive inotropic agent in human and rat heart in vitro, Biochem. Biophys. Res. Commun. 159, 14.

Ono, K., G. Tsujimoto, A. Sakamoto, K. Eto, T. Masaki, Y. Ozaki and M. Satake, 1994, Endothelin-A receptor mediates cardiac inhibition by regulating calcium and potassium currents, Nature (London) 370, 301.

Rubanyi, G.M. and M.A. Polokoff, 1994, Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology, Pharmacol. Rev. 46, 325.

Takenaka T., M. Epstein, H. Forster, D.W. Landry, K. Iijima and M.S. Goligorsky, 1992, Attenuation of endothelin effects by a chloride channel inhibitor, indanyloxyacetic acid, Am. J. Physiol. 262, F799.

Takens-Kwak, B.R., H.J. Jongsma, M.B. Rook and A.C.G. Van Gin-

neken, 1992, Mechanism of heptanol-induced uncoupling of cardiac gap junctions: a perforated patch-clamp study, Am. J. Physiol. 262, C1531

Tohse, N., Y. Hattori, H. Nakaya, M. Endou and M. Kanno, 1990, Inability of endothelin to increase Ca^{2+} current in guinea-pig heart cells, Br. J. Pharmacol. 99, 437.

Varro, A., P.P. Nanasi and D.A. Lathrop, 1991, Voltage-clamp characteristics of ventricular cardiomyocytes in the rabbit, Cardioscience 2, 233.

Vogelsang M., A. Broede-Sitz, E. Schafer, H. Reinhard and O.E. Brodde, 1994, Endothelin ET_A-receptors couple to inositol phosphate formation and inhibition of adenylate cyclase in right atrium, J. Cardiovasc. Pharmacol. 23, 344.

Yanagisawa M, H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, K. Goto and T. Masaka, 1988, A novel potent vasoconstrictor peptide produced by vascular endothelial cells, Nature (London) 332, 411.