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Endothelin-1 mediated inhibition of the acetylcholine-activated potassium current from rabbit isolated atrial cardiomyocytes

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1 Endothelin-1 is a 21 amino acid peptide with potent inotropic and chronotropic actions in the heart. Relatively little is known about the underlying electrophysiological effects of the peptide. In this study, the effects of endothelin-1 (ET-1) on the acetylcholine-activated potassium current ($I_{K(ACh)}$) were investigated in the absence and presence of the receptor-selective antagonists, PD155080 (ET_A receptor-selective) and RES-701 (ET_B receptor-selective) in rabbit atrial cardiomyocytes.

2 Cells were obtained from New Zealand White rabbits (2.5-3 kg) by enzymatic dissociation with collagenase. Potassium currents were recorded, in the presence of nifedipine (5 μM), by use of the whole cell ruptured patch-clamp technique. Following stabilization, control recordings were made with standard pulse protocols, and drugs were applied by a gravity fed microperfusion system.

3 Endothelin-1 (10 nM) alone did not affect the 'steady state' potassium current. Acetylcholine (1 μM) increased ($P<0.05$) the potassium current to -1321 ± 290 pA, from a control value of -955 ± 191 pA, at a step potential of -100 mV. Acetylcholine also increased the holding current at -40 mV from $+80\pm9$ pA to $+242\pm38$ pA, and this effect was abolished ($P<0.05$) in the presence of endothelin-1 ($+44\pm13$ pA). The responses to acetylcholine were attributed to activation of the atrial muscarinic-activated potassium current ($I_{K(ACh)}$) as they were blocked by atropine (10 μM). Endothelin-1 (10 nM) in the presence of acetylcholine did not affect the 'steady state' potassium current (-882 ± 88 pA compared to a control value of -870 ± 98 pA, at -100 mV).

4 The ET_A receptor-selective antagonist, PD155080 (1 μM), prevented ($P<0.05$) the ET-1 induced inhibition of $I_{K(ACh)}$ at all potentials. PD155080, in the presence of endothelin-1 and acetylcholine, increased the inward component of the 'steady state' potassium current to -1030 ± 210 pA from a control value of -804 ± 224 pA at a step potential of -100 mV. Also the outward component was increased at a potential of -20 mV from $+90\pm17$ pA to $+241\pm47$ pA.

5 Unlike PD155080, the ET_B receptor-selective antagonist, RES-701 (1 μM), only prevented ($P<0.05$) the inhibitory effect of endothelin-1 on the inward component of the $I_{K(ACh)}$; at -100 mV, RES-701, in the presence of endothelin-1 and acetylcholine, increased the 'steady state' potassium current to -913 ± 137 pA from -733 ± 116 pA. Furthermore, RES-701, in contrast to PD155080, failed to sustain this inhibitory effect as, in the presence of endothelin-1 and acetylcholine, the 'steady state' potassium current returned to a value of -768 ± 96 pA, at a step potential of -100 mV.

6 In conclusion, endothelin-1 clearly inhibits the effects of acetylcholine on $I_{K(ACh)}$ in rabbit atrial cardiomyocytes. This effect is primarily mediated by an ET_A receptor-subtype, but is transiently and partially mediated by a RES-701-sensitive ET_B receptor subtype. Inhibition of the $I_{K(ACh)}$ may account for the positive chronotropic properties of endothelin-1.

Keywords: Patch-clamp; endothelin; potassium current; PD155080; RES-701; acetylcholine; cardiomyocyte

Introduction

Endothelin-1 is a 21 amino acid peptide that was originally isolated and purified from the supernatant of porcine cultured aortic endothelial cells (Yanagisawa *et al.*, 1988). Within the cardiovascular system, endothelin-1 contracts arterial and venous smooth muscle (Clarke *et al.*, 1989; Cocks *et al.*, 1989), increases the force of contraction of the heart (Ishikawa *et al.*, 1988a; Moravec *et al.*, 1989; Kelly *et al.*, 1990) and accelerates heart rate (Ishikawa *et al.*, 1988b). The physiological effects of endothelin-1 are mediated by at least two pharmacologically distinct receptor subtypes, ET_A and ET_B, which have been cloned from mammalian tissue (Arai *et al.*, 1990; Sukurai *et al*, 1990; Sakamoto *et al.*, 1991; Masaki *et al.*, 1994).

The electrophysiological effects of endothelin-1 are not well understood, and there is a lack of clarity with respect to its effects on cardiac potassium currents. In guinea-pig isolated ventricular cardiomyocytes, endothelin-1 enhances the delayed rectifier K^+ current ($I_{K(V)}$) (Habuchi *et al.*, 1992) and partially inhibits the ATP activated K^+ current ($I_{K(ATP)}$) (Kobayashi *et al*, 1996). However, endothelin-1 has no effect on the $I_{K(V)}$ from chick isolated cardiomyocytes (Bkaily *et al.*, 1995). In atrial cardiomyocytes from rat (Kim, 1991) and adult guinea-pig hearts (Ono *et al.*, 1994), endothelin-1 has been shown to have a negative modulating effect on heart rate, which is attributed to activation of the acetylcholine-activated potassium current ($I_{K(ACh)}$) (Kim, 1991; Ono *et al.*, 1994). However, the effects of endothelin on heart rate are complex. Intravenous administration of endothelin-1 can cause a biphasic response *in vivo*. In the initial phase, there is a fall in mean blood pressure, total peripheral resistance and contractility (dP/dt), which is accompanied by an increase in heart rate and cardiac output. In the second phase, mean blood pressure and total peripheral resistance increase, while contractility, cardiac output and sometimes heart rate decrease (Yang *et al.*, 1991). In rat isolated atria, endothelin-1 has been shown to both increase and decrease the rate of spontaneous contraction in a concentration-dependent manner. At a concentration of 10 nM, en-

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dothelin-1 maximally increased the rate of contraction, while at a concentration of 100 nM, the rate of contraction was reduced (Ono *et al.*, 1995).

It has been observed that endothelin-1 elicits a negative chronotropic response by activation of the $I_{K(ACh)}$ in spontaneously beating cells (Kim, 1991), while a positive chronotropic response is evident in isolated tissues (Ishikawa *et al.*, 1988b; Reid *et al.*, 1989; Ono *et al.*, 1995). As such, there is a distinct lack of clarity with regard to the underlying mechanism. Endothelin-1 has been shown to block the $I_{K(ATP)}$ in porcine coronary artery smooth muscle cells (Miyoshi *et al.*, 1992), and recently, ventricular cardiomyocytes (Kobayashi *et al.*, 1996). Muscarinic-activated K^+ channels (K_{ACh}) and ATP-sensitive K^+ channels (K_{ATP}) can be activated by both M_2-muscarinic cholinoceptors and P_1-purinoceptors (Kurachi, 1995). Since endothelin-1 blocks K_{ATP} channels in smooth muscle and cardiac cells, it is feasible that endothelin-1 may inhibit the $I_{K(ACh)}$ in cardiomyocytes.

The aims of the present study were to investigate the effects of endothelin-1 on the $I_{K(ACh)}$ and 'steady state' potassium current in rabbit isolated atrial and ventricular cardiomyocytes and to identify which receptor-subtype mediates the response.

Methods

Cell preparation

New Zealand White rabbits (2.5–3 kg) were anaesthetized with sodium pentabarbitone (50 mg kg^{-1}, i.v.) following heparin administration (400 iu kg^{-1}). The heart was removed and suspended on a Langendorff apparatus via the ascending aorta, before being perfused with oxygenated (95% O_2, 5% CO_2) modified Krebs Ringer Buffer (KRB) at 37°C. The KRB contained (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). Enzymatic digestion followed with KRB supplemented with 0.12% (w/v) collagenase. The heart was removed from the Langendorff apparatus and the atria and ventricles were separated. Cells were released by mechanical chopping and gentle trituration (Kelso *et al.*, 1995). Upon restoration of Ca^{2+} tolerance, the cells were placed in M199 medium with Earle's salts, supplemented with: creatine 5 mM, taurine 2 mM, streptomycin 100 iu ml^{-1}, penicillin 100 μg ml^{-1}, at pH 7.4 and 25°C.

Recording technique

An aliquot of the cell suspension was placed in a transparent recording chamber and allowed to settle for 10 min before being bathed with a Tyrode solution of the following composition (in mM): NaCl 137, KCl 5.4, $CaCl_2$ 3, $MgCl_2$ 1.2, HEPES 5, glucose 10, pH 7.4. Voltage clamp experiments were performed with the whole cell voltage-clamp configuration of the patch clamp technique (Hamill *et al.*, 1981). Patch pipettes, fabricated from thin walled borosilicate glass (1.5 mm o.d., Clarke Electrochemical, U.K.), had resistances of 1–3 MΩ when filled with an internal solution, composition (in mM): K-gluconate 170, $MgCl_2$ 2, Na_2GTP 0.1, creatine phosphate 2.5, HEPES 10, KCl 20, EGTA 11, $CaCl_2$ 1 (pH 7.2; pCa of approximately 8; MaxChelator, v6.63). Solution junction potentials were adjusted to zero and the series resistance electronically compensated (<80%).

Experimental protocol

Currents were recorded with a patch-clamp amplifier (Axopatch 1D, Axon Instruments, Foster City, California, U.S.A.), and low pass filtered at 5 kHz (Bessel filter). The currents were digitised (2.5 kHz) and stored on computer for subsequent analysis by use of customised software. Potassium currents were elicited by voltage pulses of 400 ms duration from a holding potential of −40 mV to test potentials of −100 to +60 mV at a frequency of 0.2 Hz. The magnitude of the 'steady state' potassium current was determined before the end of the voltage pulse in relation to the baseline holding current. Acetylcholine-activated potassium currents were evoked by the same pulse protocol as described for the 'steady state' potassium currents, but in the presence of 1 μM acetylcholine. Holding currents (I_{hold}) were measured relative to 'true zero', by averaging over a 20 ms epoch before the voltage pulse. All potassium currents were recorded in the presence of 5 μM nifedipine. Drugs were applied locally to the cell by a gravity fed microperfusion system at approximately 150 μl min^{-1}, which allowed the solution bathing the cell to be changed in approximately 2 s. Current-voltage relationships were determined at 90 s intervals following drug application.

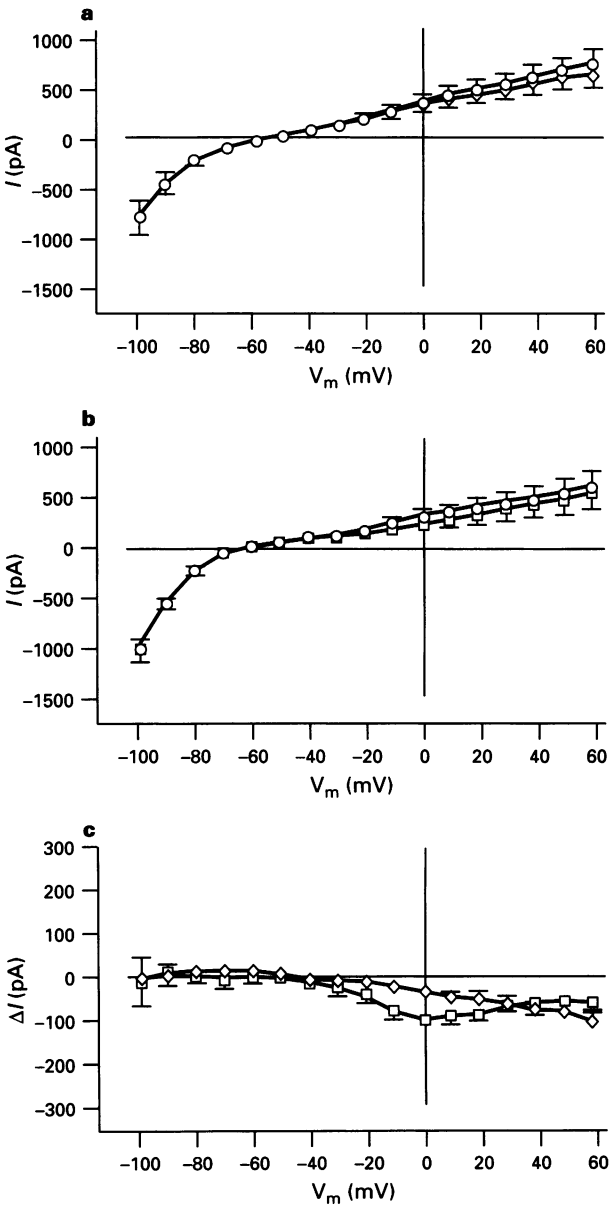

Figure 1 Current-voltage relations for the 'steady state' potassium current in rabbit atrial cardiomyocytes. The 'steady state' potassium current (I) is plotted as a function of the membrane potential (V_m). Holding potential −40 mV. (a) In control conditions (○) and following a 90 s time period (◇; $n=6$). (b) In control conditions (○) and following exposure to endothelin-1 (□; 10 nM; $n=5$). (c) The voltage-dependency of the difference current (△ I) obtained by subtracting the current under control conditions from the current following a 90 s time period (◇) and exposure to endothelin-1 (□). Data shown represent mean ± s.e.

Table 1 A summary of the time courses for the interventions as a function of the difference current from rabbit isolated atrial cardiomyocytes

	Membrane potential											
	−100 mV				−20 mV				+50 mV			
	Time (s)											
	0	90	180	270	0	90	180	270	0	90	180	270
Control (pA)	0	−7±51	9±55	−47±44	0	−17±7	−41±13	−57±18	0	−86±23	−151±42	−160±51
ET-1 (pA)	0	−12±58	3±62	68±61	0	−49±17	−76±18	−82±15	0	−64±21	−84±18	−107±28
ACh (pA)	0	−366±112	−248±131	−165±84	0	171±42	57±30	27±20	0	76±71	−50±54	−91±32
ACh+ET-1 (pA)	0	11±33	51±21	98±20	0	−20±24	−45±24	−46±18	0	−99±68	−104±68	−105±74
ACh+ET-1+PD155 (pA)	0	−225±52	−169±32	ND	0	151±41	90±35	ND	0	105±46	37±41	ND
Ach+ET-1+RES (pA)	0	−232±65	−38±54	ND	0	65±26	33±16	ND	0	47±38	25±28	ND

The difference current was obtained by subtracting the current under control conditions (time '0') from the current in the presence of, acetylcholine (ACh), endothelin-1 (ET-1), PD155080 (PD155) and RES-701 (RES) and the combinations at the times indicated. Data represent the mean±s.e. of 4–6 experiments. ND, - not determined.

Data analysis

Difference current-voltage relationships were determined by subtracting the control current-voltage relationship data from that obtained following application of drug(s). Data are expressed as mean±s.e. and were analysed by Friedman's ANOVA and Wilcoxons' Signed Ranks tests for matched pairs or Kruskall-Wallis ANOVA for unmatched groups, where appropriate. Statistical analysis of current-voltage relationships were made at key points on the curves, at −100 mV, −20 mV and +50 mV for atrial cells and at −100 mV, −50 mV and +50 mV for ventricular cells. $P<0.05$ was taken as indicating statistical significance.

Materials

Endothelin-1 was purchased from Bachem Inc. (California, U.S.A.). PD155080 (sodium 2-benzo[1,3]dioxol-5-yl-3-benzyl-4(4-methoxy-phenyl)-4-oxobut-2-enoate) was a gift from Parke-Davis Pharmaceuticals (Ann Arbour, Michigan, U.S.A.). RES-701 (Gly-Asn-Trp-His-Gly-Thr-Ala-Pro-Asp-Trp-Phe-Phe-Asn-Tyr-Tyr-Trp) was purchased from the American Peptide Co. (California, U.S.A.). Collagenase (type II) was acquired from Serva (West Germany). All other chemicals were obtained from Sigma-Aldrich Chemical Co. (Poole, Dorset, UK). Nifedipine, endothelin-1, PD155080 and RES-701 were dissolved in dimethyl sulphoxide (<0.05% v/v). All other compounds were dissolved in de-ionised water.

Results

Atrial cells

'Steady state' potassium current The inward component of the 'steady state' potassium current was stable over the time course of the experiments, although there was a small decrease ($P<0.05$) in the outward component of this current with time (Figure 1a, Table 1); at +50 mV, the current was decreased by 85±23 pA from a control value of 659±127 pA over a 90 s epoch. The voltage-dependence of the 'steady state' potassium current shows inward rectification for potentials negative to the reversal potential of −55 mV, and outward rectification for clamp potentials positive to −55 mV. Endothelin-1, at a concentration of 10 nM, did not affect the amplitude of the 'steady state' potassium current or its voltage-dependence (Figure 1b). Endothelin-1 appeared to decrease the current amplitude between potentials of −20 mV and +20 mV as expressed in the different current-voltage relationships (Figure 1c). PD155080 (1 μM), an ET_A receptor-selective antagonist, and RES-701 (1 μM), an ET_B receptor-selective antagonist, did not alter the amplitude or shift the current-voltage relationship of the 'steady state' potassium current over the voltage range of −80 mV to +60 mV (PD155080: 77±14 pA compared to a control value of 90±17 pA at a potential of −20 mV; RES-701: 31±8 pA compared to a control value of 37±9 pA at a potential of −20 mV). At a potential of −100 mV, there appeared to be a change in the current, which is highlighted in the difference current-voltage profile as an outward deflection of 79±41 pA and 42±26 pA for PD155080 and RES-701 respectively; this did not occur in the time control experiments (Figure 1c). In all experiments, there was a small change ($P<0.05$) in the recorded current at potentials positive to −30 mV (PD155080: 307±31 pA compared to a control value of 350±35 pA at a potential of +50 mV; RES-701: 212±33 pA from a control value of 233±37 pA at a potential of +50 mV); which is consistent with the time effect found in control experiments (Figures 1a,c).

Effect of acetylcholine and endothelin-1 on the 'steady state' potassium current Acetylcholine-activated potassium currents were elicited upon exposing the cells to 1 μM acetylcholine (Figure 2). Acetylcholine increased ($P<0.05$) the amplitude of

both the inward (-1321 ± 291 pA compared to a control value of -955 ± 191 pA, at -100 mV) and outward components (354 ± 53 pA compared to a control value of 183 ± 19 pA, at -20 mV) of the 'steady state' potassium current (Figure 2a,b), and displaced the reversal potential by -6 mV from a control value of -61 mV (Figure 2b). The difference current-voltage relationship for the acetylcholine-induced change in potassium conductance, $I_{K(ACh)}$, showed inward rectification at potentials negative to the reversal potential of -67 mV, and outward rectification at more positive clamp potentials (Figure 2c). With time, the $I_{K(ACh)}$ gradually decreased ($P<0.05$) in amplitude, as typified by the change in the holding current at a holding potential of -40 mV; over a 270 s epoch the acetylcholine-induced change in the I_{hold} decreased from 161 ± 37 pA to 46 ± 16 pA (Figure 2d). Hence, in subsequent experiments data were compared at 90 s, as this was when the maximal effect was observed (Table 1). The $I_{K(ACh)}$ was completely inhibited by the muscarinic cholinoceptor selective antagonist, atropine (10 μM; Figure 3a,b). At potentials negative to -80 mV, there was an apparent decrease in the amplitude of the inward component of the potassium current from -775 ± 134 pA to -650 ± 88 pA, at a step potential of -100 mV (Figure 3b), which is seen as an outward deflection in the difference current-voltage relationship of 125 ± 54 pA (Figure 3c, Table 1).

Endothelin-1 (10 nM) in the presence of acetylcholine (1 μM), had no effect on the amplitude of the 'steady state' potassium current (-871 ± 98 pA compared to a control value of -882 ± 88 pA, at -100 mV; Figure 4a,b) or its voltage-dependence (Figure 4b). Thus, endothelin-1 can be said to inhibit the $I_{K(ACh)}$ in a similar manner to atropine (Figure 3c), as observed from the difference current-voltage relationship (Figure 4c, Table 1).

Effects of receptor-selective antagonist on endothelin-1 mediated inhibition of $I_{K(Ach)}$ The ET_A receptor-selective antagonist, PD155080 (1 μM), in the presence of endothelin-1 and acetylcholine, increased ($P<0.05$) the amplitude of the inward (-1030 ± 210 compared to a control value of -804 ± 224 pA, at -100 mV) and outward (241 ± 47 pA compared to a control value of 90 ± 17 pA, at -20 mV) components of the 'steady state' potassium current (Figure 5a,b). The increase was similar to that produced by acetylcholine alone (Figure 2a,b). Furthermore, PD155080, in combination with endothelin-1 and

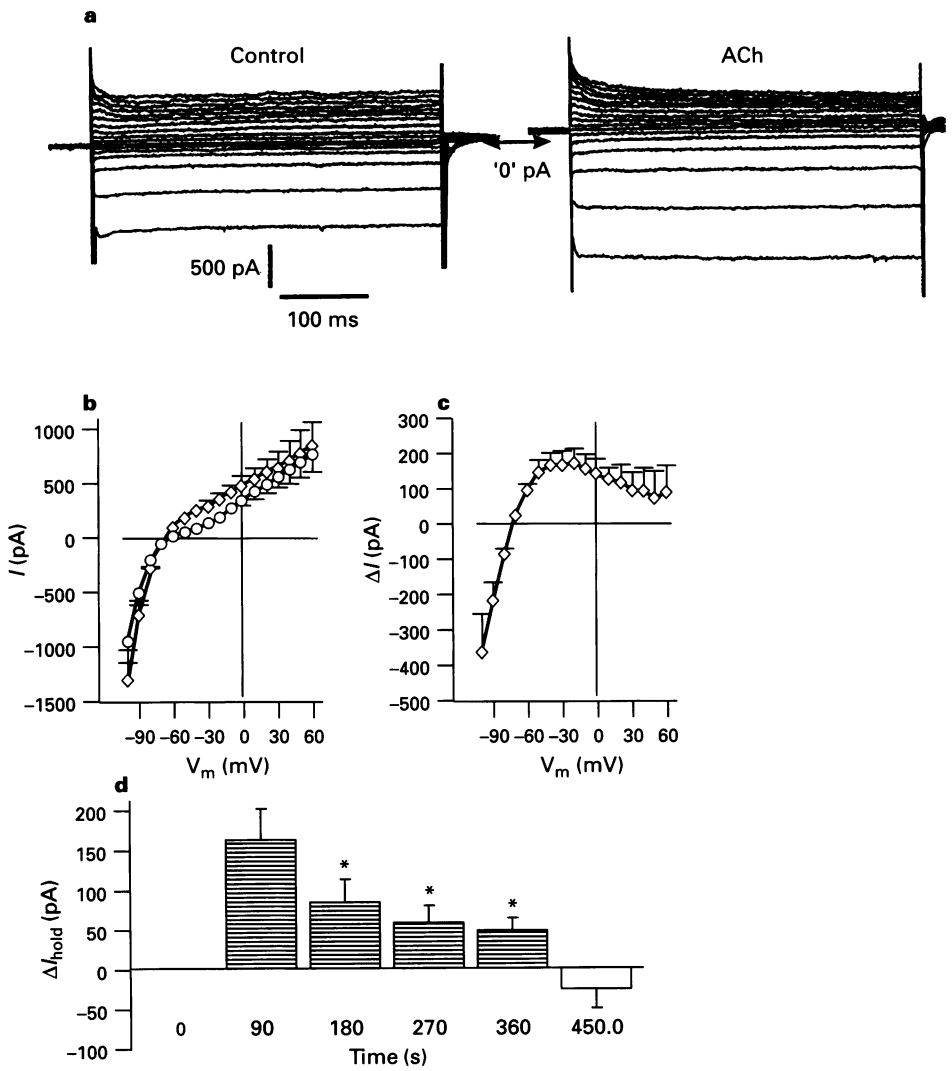

Figure 2 The effects of acetylcholine (1 μM; ACh) on the 'steady state' potassium current from rabbit atrial cardiomyocytes. All recordings were obtained in the presence of 5 μM nifedipine. (a) Voltage-clamp records obtained in the absence and presence of acetylcholine from a holding potential of -40 mV; voltage was stepped from -100 mV to $+60$ mV in 10 mV increments. (b) Current-voltage relationship of the 'steady state' potassium current (I) is plotted as a function of the membrane potential (V_m) in the absence (○) and presence of acetylcholine (◇; $n=6$). (c) The voltage-dependency of the difference current (△ I) obtained by subtracting the current under control conditions from the current in the presence of acetylcholine (◇). (d) The effect of continued exposure to acetylcholine (hatched columns; $n=6$) on the acetylcholine induced change in holding current (△ I_{hold}) as a function of time; washout (open column). Data shown represent mean ± s.e. $^*P<0.05$ with respect to 90 s exposure to acetylcholine.

acetylcholine, shifted the reversal potential from a control value of −56 mV, to −68 mV (Figure 5b). The difference current-voltage profile is similar to that for acetylcholine (Figure 2c); in other words, PD155080 blocked the inhibitory effect of endothelin-1. The increase in the 'steady state' potassium current was sustained upon continued exposure to PD155080 in the presence of endothelin-1 and acetylcholine; after a further 90 s exposing to the interventions, the current was still increased ($P<0.05$; −973±214 pA compared to a pre-drug control value of −804±224 pA, at −100 mV, Table 1).

RES-701 (1 μM), an ET_B receptor-selective antagonist, in the presence of endothelin-1 and acetylcholine, increased ($P<0.05$) the inward component of the 'steady state' potassium current to −914±137 pA from a control of −733±116 pA, at −100 mV, and shifted the reversal potential by −9 mV to −63 mV from a control value of −54 mV (Figure 6a,b). However, RES-701 did not affect the outward component of the potassium current (257±46 pA compared to a control value of 233±37 pA, at +50 mV). The difference current-voltage relationship in the presence of RES-701 was similar in profile to that for acetylcholine (Figure 6c). However, the amplitude of this current, $I_{K(ACh)}$, was smaller than that obtained in the presence of acetylcholine, at all potentials (Figure 6c). In addition, RES-701, in the presence of endothelin-1 and acetylcholine, only transiently increased the 'steady state' potassium current to −914±137 pA (at −100 mV); upon a further 90 s exposure to the drug combination, the current decreased to −769±96 pA, which is comparable to the pre-drug control current amplitude of −733±116 pA (Table 1).

Background current (I_{hold}) at a holding potential of −40 mV Acetylcholine (1 μM) shifted the I_{hold} by 161.3±37.4 pA ($P<0.05$) in a depolarizing direction from a control value of 80±9 pA to 242±38 pA (Figure 7). Atropine antagonized ($P<0.05$) the acetylcholine-induced shift in the I_{hold} (Figure 7). Endothelin-1 (10 nM) prevented ($P<0.05$) the acetylcholine-induced shift in I_{hold} (Figure 7). While PD155080 (1 μM) prevented ($P<0.05$) the endothelin-1 mediated inhibition of the acetylcholine-induced change in I_{hold}, RES-701 (1 μM) did not (Figure 7).

Ventricular cells

Potassium currents Endothelin-1 (10 nM) did not alter the amplitude (−2.37±0.11 nA compared to a control value of −2.46±0.13 nA at −100 mV) or current-voltage relationship of the 'steady state' potassium current (data not shown). Acetylcholine (1 μM) did not affect the holding current or

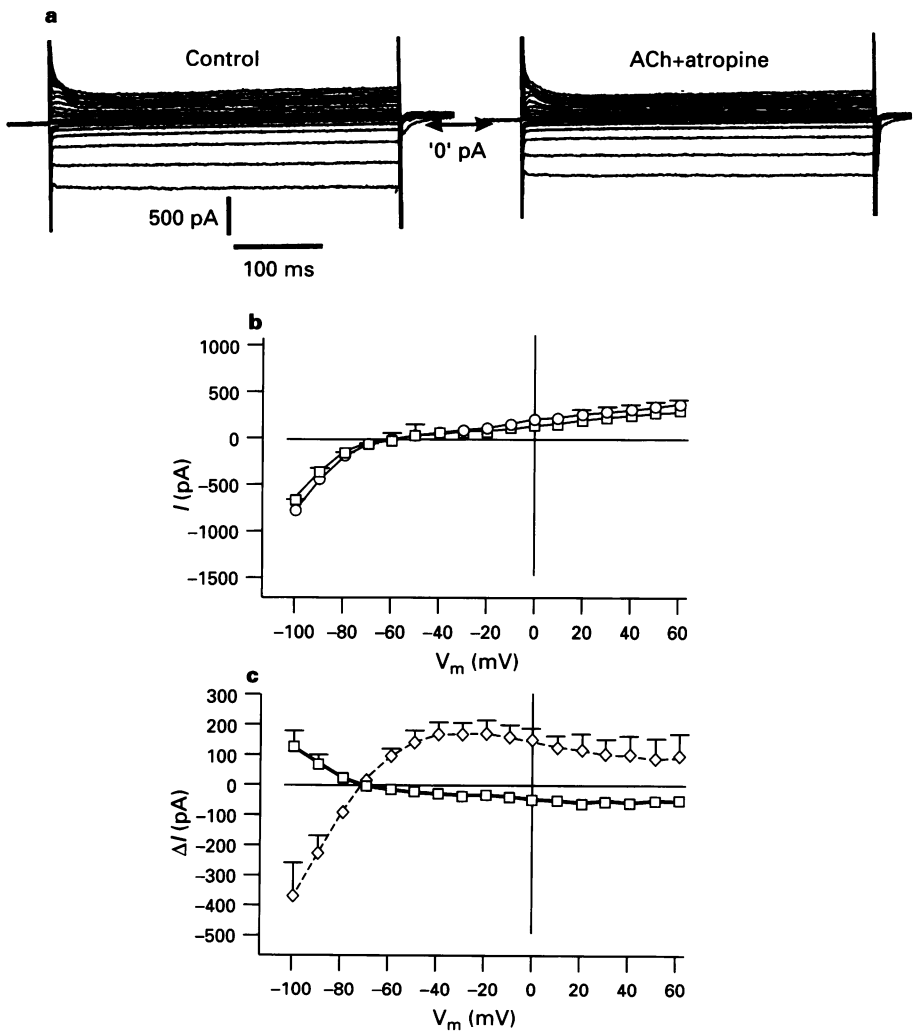

Figure 3 The effects of acetylcholine (1 μM) and atropine (10 μM) on the 'steady state' potassium current from rabbit atrial cardiomyocytes showing the 'steady state' potassium current. All recordings were obtained in the presence of 5 μM nifedipine. (a) Voltage-clamp records obtained in the absence and presence of acetylcholine and atropine from a holding potential of −40 mV; voltage was stepped from −100 mV to +60 mV in 10 mV increments. (b) Current-voltage relationship of the 'steady state' potassium (I) is plotted as a function of the membrane potential (V_m) in the absence (○) and presence of acetylcholine and atropine (□; $n=4$). (c) The voltage-dependency of the difference current (△ I) obtained by subtracting the current under control conditions from the current in the presence of acetylcholine and atropine (□), and for comparison, acetylcholine (◇; 1 μM; $n=6$). Data shown represent mean±s.e.

'steady state' potassium current (-2.7 ± 0.09 nA compared to a control value of -2.62 ± 0.05 nA at -100 mV) in ventricular cells; hence the $I_{K(ACh)}$ was not elicited in these cells.

Discussion

In the present study we showed that, (1) endothelin-1 inhibits the $I_{K(ACh)}$ in atrial cardiomyocytes isolated from rabbit myocardium; (2) this response is primarily mediated by the ET_A receptor-subtype and only partially mediated by an ET_B receptor-subtype; and (3), endothelin-1 does not have any effect on the 'steady state' potassium current of ventricular cardiomyocytes isolated from rabbit myocardium.

Endothelin-1 *per se* did not alter the 'steady state' potassium current in atrial or ventricular cardiomyocytes isolated from rabbit myocardium, which is in agreement with the findings of Ono *et al.* (1994). On the other hand, Habuchi *et al.* (1992), found that endothelin-1 enhanced the $I_{K(V)}$ in guinea-pig isolated ventricular cardiomyocytes. Such difference can be accounted for by variation between species. In rabbit cardiomyocytes, the $I_{K(V)}$ is either absent or of small amplitude, while in guinea-pig cardiomyocytes the $I_{K(V)}$ is a prominent characteristic (Varro *et al.*, 1991).

Our data, which showed that endothelin-1 inhibited the $I_{K(ACh)}$, are in contrast to the findings of Kim (1991), who found that endothelin-1 activated the $I_{K(ACh)}$ in spontaneously contracting cultured atrial cardiomyocytes of the rat. Furthermore, under conditions whereby the $I_{K(ACh)}$ was maximally stimulated, endothelin-1 did not attenuate the response (Kim, 1991). This activation of the $I_{K(ACh)}$, was not associated with a shift in either the reversal potential or I_{hold}. The latter findings are remarkable, as we and others (Belardinelli & Isenberg, 1983; Ono *et al.*, 1994) have shown that acetylcholine-mediated activation of $I_{K(ACh)}$ shifts the reversal potential to a more negative value and displaces the I_{hold} in an outward direction. The differences between these studies, in which either an increase or decrease of the $I_{K(ACh)}$ is observed, may occur as a consequence of the different preparations used, such as quiescent versus spontaneously active cells, cultured versus non-cultured cells, neonate versus adult preparation and species differences. Ono *et al.* (1994) also found that endothelin-1 activates the $I_{K(ACh)}$ and displaces the I_{hold} (at a holding potential of -40 mV) in the outward direction in quiescent atrial cardiomyocytes from guinea-pig. Endothelin-1 also increased the I_{hold} in rabbit atrial cardiomyocytes, although, unfortunately the data relating to the current-voltage relationship were not published.

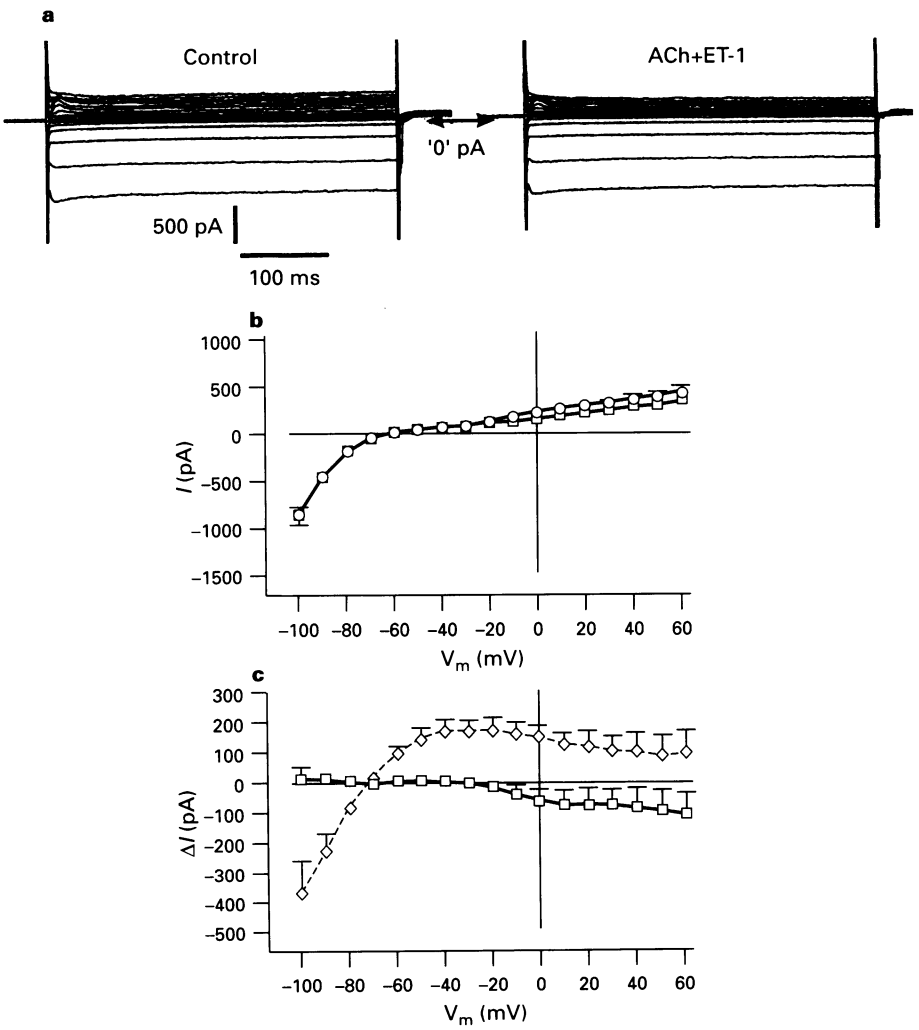

Figure 4 The effects of endothelin-1 (10 nM; ET-1) in the presence of acetylcholine (1 μM) on the 'steady state' potassium current from rabbit atrial cardiomyocytes. All recordings were obtained in the presence of 5 μM nifedipine. (a) Voltage-clamp records obtained in the absence and presence of endothelin-1 and acetylcholine, from a holding potential of -40 mV; voltage was stepped from -100 mV to $+60$ mV in 10 mV increments. (b) Current-voltage relationship of the 'steady state' potassium current (I) is plotted as a function of the membrane potential (V_m) in the absence (○) and presence of endothelin-1 and acetylcholine (□; $n=5$). (c) The voltage-dependency of the difference current (△ I) obtained by subtracting the current under control conditions from the current in the presence of acetylcholine and atropine (□), and for comparison, acetylcholine (◇; 1 μM; $n=6$). Data shown represent mean $\pm$ s.e.

The difference between our data and those of Ono *et al.* (1994) may be due to the experimental conditions under which the current recordings were obtained, in particular the composition of the patch electrode solution. Unlike Ono *et al.* (1994), we included GTP in our electrode solution, as it is intrinsically involved in the signal transduction mechanisms associated with endothelin-1, and in an effort to minimise the problem of intracellular dialysis when using the whole cell ruptured patch-clamp technique. A previous study investigating the effects of endothelin-1 on the I_{Ca} showed endothelin-1 to increase the I_{Ca} when GTP was included in the electrode solution and to decrease the I_{Ca} when GTP was omitted from the electrode solution (Lauer *et al.*, 1992).

In guinea-pig ventricular cardiomyoctes, endothelin-1 partially inhibited the nicorandil and cromakalim-induced increase in the $I_{K(ATP)}$, an effect which could be blocked by the ET_A receptor-selective antagonist, BQ-485 (Kobayashi *et al.*, 1996). This has particular relevance to findings from our study as the $K_{(ACh)}$ and K_{ATP} can both be activated by M_2-muscarinic cholinoceptors and P_1-purinoceptors (Kurachi, 1995). Furthermore, it was suggested that a pertussis toxin sensitive G-protein may exert an inhibitory influence on essential step(s) of the subcellular mechanism(s) of the ET_A-receptor-mediated inhibition of the $I_{K(ATP)}$ (Kobayashi *et al.*, 1996).

The inhibitory effect of endothelin-1 on the $I_{K(ACh)}$ is prevented by the ET_A receptor-selective antagonist, PD155080 (Doherty *et al.*, 1995), but is only partially and transiently prevented by the ET_B receptor-selective antagonist, RES-701 (Tanaka *et al.*, 1994). Similarly, the ET_A receptor-selective antagonist, BQ-123, was shown to block the endothelin-1-induced increase in $I_{K(ACh)}$, whereas the ET_B receptor-selective antagonist, RES-701, had no effect in atrial cells (Ono *et al.*, 1994). From our data, it was determined that the endothelin-1-mediated effect on $I_{K(ACh)}$ was determined to be primarily coupled to the ET_A receptor. However, the ET_B receptor is also involved in this response, but probably to a lesser extent given the transient, as well as the partial, nature of the inhibition. The endothelin-1-mediated inhibition of the $I_{K(ACh)}$ may explain the positive chronotropic effect of this peptide *in vivo*. It was previously speculated, based upon the rank order of potency of endothelin-1 and endothelin-3, that the chronotropic effect of this peptide was mediated by the ET_B receptor (Ishikawa *et al.*, 1988b; Moravec *et al.*, 1989; Ishikawa *et al.*, 1991).

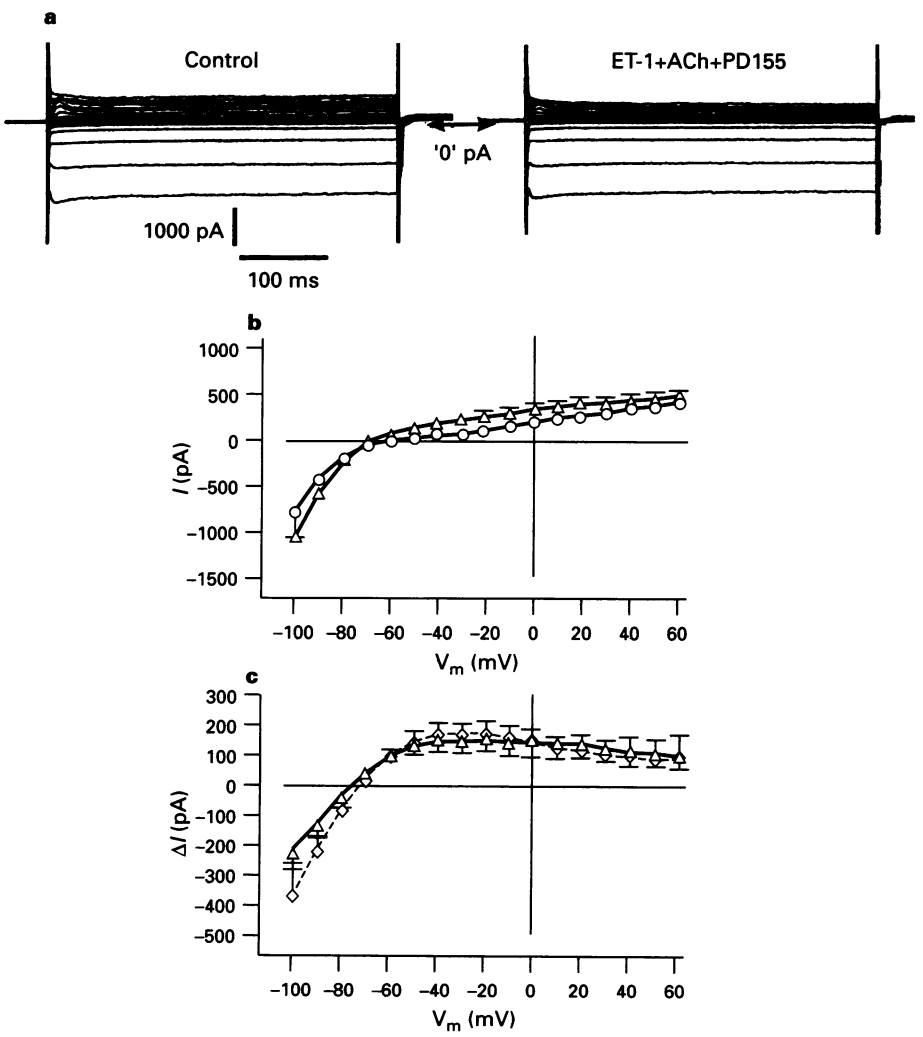

Figure 5 The effects of endothelin-1 (10 nM; ET-1) in the presence of acetylcholine (1 μM; ACh) and the ET_A receptor-selective antagonist, PD155080 (1 μM; PD155), on the 'steady state' potassium current from rabbit atrial cardiomyocytes. All recordings were obtained in the presence of 5 μM nifedipine. (a) Voltage-clamp records obtained in the absence and presence of endothelin-1, acetylcholine and PD155080 from a holding potential of −40 mV; voltage was stepped from −100 mV to +60 mV in 10 mV increments. (b) Current-voltage relationship for the 'steady state' potassium current (*I*) is plotted as a function of the membrane potential (V_m) in the absence (○) and presence of endothelin-1, acetylcholine and PD155080 (△; $n=6$). (c) The voltage dependency of the difference current (△ *I*) obtained by subtracting the current under control conditions from the current in the presence of endothelin-1, acetylcholine and PD155080 (△) and for comparison acetylcholine (◇; 1 μM; $n=6$). Data shown represent mean ± s.e.

Furthermore, while endothelin-1 and endothelin-3 were equipotent chronotropic agents, endothelin-1 was found to be a more potent inotrope than endothelin-3, suggesting that the latter response is mediated by the ET_A receptor. However, the use of receptor-selective antagonists in examining the functional responses to these peptides, has indicated greater complexity with regard to receptor influence. For example, the finding of similar potencies of endothelin-1 and endothelin-3 to increase ventricular contractile function led to speculation that this response was mediated by ET_B receptors (Takanashi & Endoh, 1991). However, further pharmacological analysis with the ET_A receptor-selective antagonist, BQ-123, indicated that contractile function was mediated by at least three receptor-subtypes which were thought to be either 'typical' or 'atypical' ET_A receptors, rather than the ET_B receptor.

A recognised technical difficulty in many studies is instability of the variable being recorded; for example, rundown of the I_{Ca}, and/or desensitization can arise from exposure to a drug (acetylcholine). This makes it difficult to distinguish the drug-induced change from a change in the variable being recorded with time. We reduced this problem by shortening the time taken to change the micro-environment surrounding the cell (approximately 2 s), by use of a multi-outlet gravity fed microperfusion system, so, reducing the duration of the experiments and potential time effects. To enhance the temporal performance of the microperfusion system, all outlets were allowed to flow simultaneously (zero 'dead' space). Care was taken in the design of the bath and perfusion system to ensure parallel uncontaminated streams were formed.

The acetylcholine-induced increase in the 'steady state' potassium current in atrial cardiomyocytes was inhibited by atropine, substantiating that the current was $I_{K(ACh)}$. The $I_{K(ACh)}$ rapidly decreased in the continuous presence of acetylcholine, indicating the desensitizing nature of this current. Similar effects with acetylcholine have been obtained in rabbit sinoatrial node cells (Honjo *et al.*, 1992), and rat and guinea-pig atrial cardiomyocytes (Kim, 1993). Desensitization of the $I_{K(ACh)}$ is biphasic (Boyett *et al.*, 1995), the initial rapid phase may be due to dephosphorylation of the K^+ channel or the G-protein, which can decrease the mean open time of the channel. The second, more slow phase could arise from phosphorylation of the muscarinic cholinoceptor resulting in a reduction in

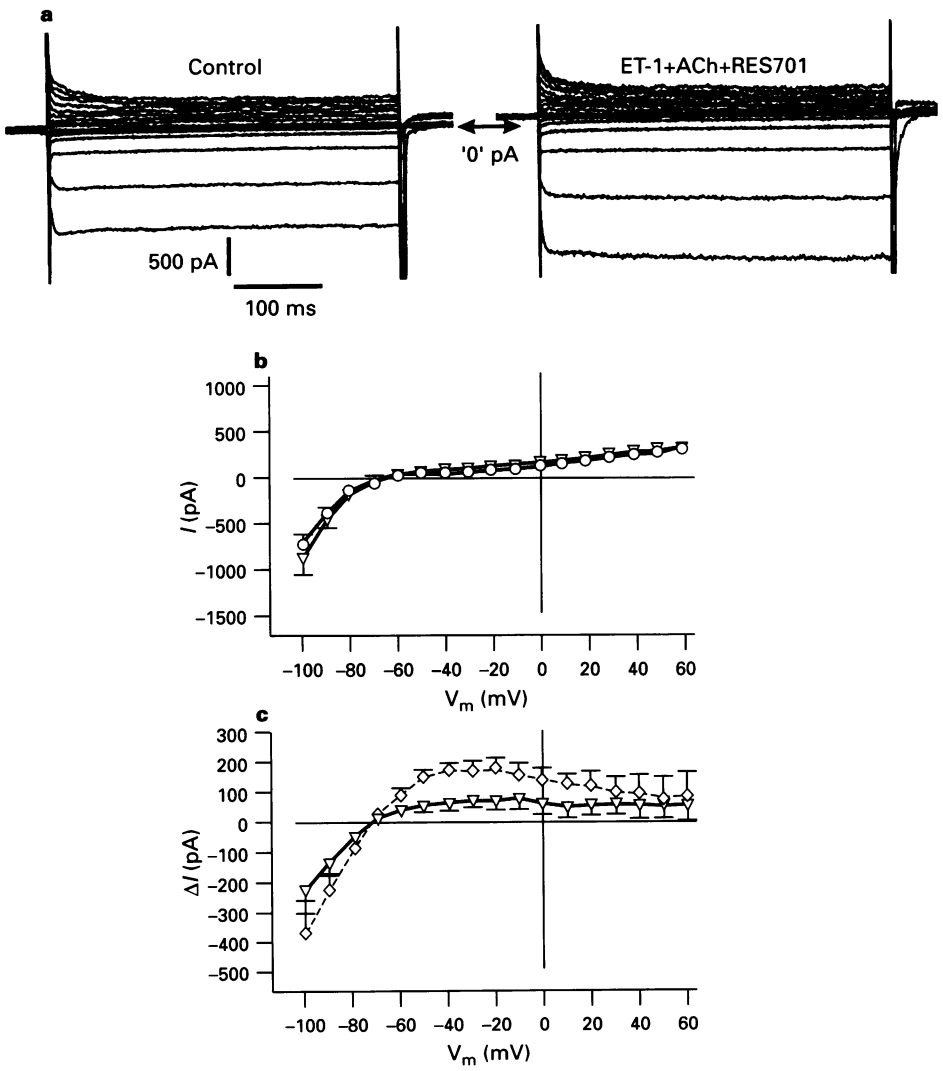

Figure 6 The effects of endothelin-1 (ET-1, 10 nM) in the presence of acetylcholine (ACh, 1 μM) and the ET_B receptor-selective antagonist, RES-701 (1 μM), on the 'steady state' potassium current from rabbit atrial cardiomyocytes. All recordings were obtained in the presence of 5 μM nifedipine. (a) Voltage-clamp records obtained in the absence and presence of endothelin-1, acetylcholine and RES-701 from a holding potential of −40 mV; voltage was stepped from −100 mV to +60 mV in 10 mV increments. (b)Current-voltage relationship for the 'steady state' potassium current (I) is plotted as a function of the membrane potential (V_m) in the absence (○) and presence of endothelin-1, acetylcholine and RES-701 (▽; $n=6$). (c) The voltage dependency of the difference current (△ I) obtained by subtracting the current under control conditions from the current in the presence of endothelin-1, acetylcholine and RES-701 (▽), and for comparison acetylcholine (◇; 1 μM; $n=6$). Data shown represent mean ± s.e.

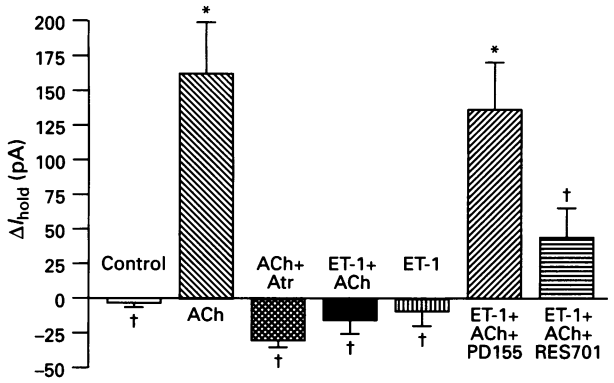

Figure 7 The effect of control (open column; $n=5$), acetylcholine (ACh; 1 μM; $n=6$), acetylcholine (1 μM) in the presence of atropine (Atr; 10 μM; $n=4$), endothelin-1 (ET-1, 10 nM) in the presence of acetylcholine (1 μM $n=5$), endothelin-1 (10 nM; $n=4$), endothelin-1 (10 nM) in the presence of acetylcholine (1 μM) and the ET_A receptor-selective antagonist, PD155080 (PD155; 1 μM; $n=6$), and endothelin-1 (10 nM) in the presence of acetylcholine (1 μM) and the ET_B receptor-selective antagonist, RES-701 (1 μM; $n=6$) on the change in holding current ($\triangle I_{hold}$) at a holding potential of −40 mV. Data shown represent mean ± s.e. $^*P<0.05$ with respect to control; $\dagger P<0.05$ with respect to the acetylcholine response.

high affinity agonist binding (Boyett *et al.*, 1995). At potentials negative to −80 mV, atropine, in the presence of acetylcholine, tended to decrease the 'steady state' potassium current relative to the control recording. This trend may indicate the presence of an acetylcholine-induced current which is insensitive to atropine, or an inhibition of a tonal component to the 'steady state' potassium current. Acetylcholine did not affect the 'steady state' potassium current from isolated ventricular cardiomyocytes, suggesting the absence of an $I_{K(ACh)}$ in ventricular cells, which is in agreement with results from other studies (Soejima & Noma, 1984; Pfaffinger *et al.*, 1985; Carmeliet & Mubagwa, 1986; Hartzell & Simmons, 1987).

The cholinoceptor is coupled by a G-protein (G_K) to the associated potassium channel either directly, or indirectly via the actions of phospholipase A_2 (Kim *et al.*, 1989; Kurachi, 1989). As such, the potential targets for modification in response to endothelin-1 are, the cholinoceptor, G_K and the K^+ channel. It is possible that endothelin-1 could modify these targets through one or more of its multiple signal transduction mechanisms. Endothelin activates phospholipase C (Galron *et al.*, 1990; Bogoyevitch *et al.*, 1993b), inhibits adenylate cyclase activity (Vogelsang *et al.*, 1994), raises intracellular pH (Kramer *et al.*, 1991), and stimulates myelin basic protein kinase p42 and p44 mitogenic activated protein kinase (Bogoyevitch *et al.*, 1993a). Therefore, possibilities exist whereby an effector of the endothelin-1 signal transduction pathway may phosphorylate the cholinoceptor or the G-proteins, G_K, or dephosphorylate the associated K^+ channel; all of which are known to modulate the response to acetylcholine (Boyett *et al.*, 1995). Alternatively, an intracellular effector arising from the endothelin-1 signal transduction pathway may influence coupling of the G-protein to the K^+ channel. Recently, a complex between the small GTP-binding protein, $p21^{ras}$ and its GTPase-activating protein has been found to impair coupling of the cholinoceptor to the K^+ channel (Yatani *et al.*, 1990; 1991).

Endothelin-1-mediated inhibition of the $I_{K(ACh)}$ may account for the well established positive chronotropic properties of the peptide (Ishikawa *et al.*, 1988b; Reid *et al.*, 1989; Ono *et al.*, 1995). The K_{ACh} channel is coupled to the M_2-muscarinic cholinoceptor (Kurachi *et al.*, 1986). When acetylcholine binds to the receptor there is an influx of potassium which hyperpolarizes the cell; this increases the driving force required to reach the threshold potential and reduces the electrical excitability of the cell. By preventing the acetylcholine-induced hyperpolarization of the cell, endothelin-1 could elicit a positive chronotropic effect *in vivo*. However, further electrophysiological studies investigating the effects of endothelin-1 on the hyperpolarization-activated current (DiFrancesco, 1991) and T-type Ca^{2+} current (Hagiwara *et al.*, 1988) are required to understand fully the ionic mechanisms underlying the direct chronotropic properties of endothelin-1

As plasma levels of endothelin-1 are elevated following myocardial ischaemia (Yasuda *et al.*, 1990; Miyauchi *et al.*, 1992), endothelin-1-mediated inhibition of $I_{K(ACh)}$ could modulate the arrhythmogenic environment. Several studies suggest that endothelin-1 is an arrhythmogenic agent, by virtue of direct effects or indirectly as a result of vasoconstriction (Yorkane *et al.*, 1990; Nichols *et al.*, 1990; Salvati *et al.*, 1991; Hom *et al.*, 1992). However, whether inhibition of the $I_{K(ACh)}$ is beneficial or deleterious will depend upon the causative effects.

In conclusion, endothelin-1 clearly inhibits the $I_{K(ACh)}$ in rabbit isolated atrial cardiomyocytes, an effect which is primarily mediated by an ET_A receptor-subtype. Inhibition of the $I_{K(ACh)}$ may account for the well established positive chronotropic properties of endothelin-1.

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