Effects of acetylcholine on the Na^+–K^+ pump current in guinea-pig ventricular myocytes

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1. The whole-cell patch clamp technique was used to study the effects of acetylcholine (ACh) on Na^+–K^+ pump current (I_p) in acutely isolated guinea-pig ventricular myocytes. Studies were performed in the absence and presence of the β-agonist isoprenaline (Iso).

2. ACh had no effect on I_p at low or high $[Ca^{2+}]_i$ at any voltage in the absence of Iso. Iso alone inhibited I_p at low $[Ca^{2+}]_i$ and shifted the I_p–V relationship at high $[Ca^{2+}]_i$ in a negative direction. Addition of 1 μM ACh reversed these effects of Iso. $K_{0.5}$ for the effects of ACh was about 16 nM, regardless of $[Ca^{2+}]_i$.

3. The actions of ACh on the heart are usually mediated via muscarinic receptors. Atropine, a muscarinic antagonist, blocked the effects of ACh on I_p in the presence of Iso, suggesting that these effects are also mediated by muscarinic receptors.

4. Muscarinic receptors are usually coupled to a G_i protein, leading to inhibition of adenylyl cyclase and a reduction of cAMP levels. We have shown previously that basal levels of cAMP are very low in guinea-pig ventricular myocytes, and that a membrane-permeant cAMP analogue, chlorophenylthio-cAMP (CPTcAMP), mimics the effects of Iso. ACh did not reverse the effects of CPTcAMP, supporting the hypothesis that the effects of ACh on I_p are also mediated via inhibition of adenylyl cyclase.

5. The present results suggest that a high level of parasympathetic tone alone does not affect the activity of ventricular Na^+–K^+ pumps. However, if sympathetic tone is high, then muscarinic stimulation can reciprocally modulate Na^+–K^+ pump activity.

Cardiac membrane currents are modulated by the autonomic nervous system. For example, sympathetic stimulation and consequent activation of β-receptors is known to increase the amplitude of Ca^{2+} current, shift the delayed rectifier K^+ current and the hyperpolarization-activated current, i_f (see reviews by Hartzell, 1988; Gadsby, 1990*a*; Clapham, 1990; DiFrancesco, 1990), and activate a time- and voltage-independent Cl^- conductance (Tareen, Ono, Noma & Ehara, 1991; Hwang, Horie, Nairn & Gadsby, 1992; Iyadomi, Hirahara & Ehara, 1995). Parasympathetic stimulation, and consequent activation of muscarinic receptors by acetylcholine (ACh), in general reverses the effects of β-stimulation. In some cardiac cell types, the action of ACh occurs even in the absence of concurrent β-receptor activation, e.g. the negative shift of i_f activation in sinus node (DiFrancesco & Tromba, 1987). In other cardiac preparations, ACh has no apparent effect when applied alone but can reverse the actions of concurrent β-activation, e.g. the ACh-induced negative shift of i_f activation in canine Purkinje fibres occurs only in the presence of isoprenaline (Iso) (Chang, Gao, Tromba, Cohen & DiFrancesco, 1990).

We have previously demonstrated that β-receptor activation by Iso alters the Na^+–K^+ pump current (I_p) in guinea-pig ventricular myocytes (Gao, Mathias, Cohen & Baldo, 1992). This action of Iso is dependent on the internal Ca^{2+} concentration ($[Ca^{2+}]_i$). At a $[Ca^{2+}]_i$ of 1·4 μM, Iso shifts the voltage dependence of the Na^+–K^+ pump current (I_p–V) in a negative direction but does not affect the maximum I_p seen at positive voltages. At a $[Ca^{2+}]_i$ of 15 nM, Iso inhibits I_p at all voltages without any voltage shift. At intermediate $[Ca^{2+}]_i$, both effects are observed (Gao, Mathias, Cohen, Shi & Baldo, 1996). The net result at −60 mV is that Iso increases I_p at $[Ca^{2+}]_i$ greater than 100 nM, and reduces I_p at $[Ca^{2+}]_i$ less than 100 nM (Gao *et al.* 1992). These effects are mediated via the cAMP-dependent cascade, and involve protein kinase A (PKA)-dependent phosphorylation (Gao, Cohen, Mathias & Baldo, 1994; Gao, Mathias, Cohen & Baldo, 1995*b*). In the present study, we have investigated the actions of the parasympathetic transmitter ACh on I_p. Hasuo, Koketsu & Minota (1988) reported that ACh indirectly stimulated the electrogenic Na^+–K^+ pump mechanism in bullfrog atrial muscle fibres via extracellular

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K^+ accumulation. To minimize this indirect action on the pump, we used isolated single guinea-pig ventricular myocytes to reduce concerns about narrow extracellular spaces and we added blockers to reduce the K^+ conductances present in the sarcolemma and the T-tubular system. Our results demonstrate that ACh alone has no measurable effect on I_p but can reverse the effects of the β-agonist Iso.

METHODS

Single ventricular myocytes were enzymatically isolated from guinea-pig hearts as first described by Isenberg & Klöckner (1982) and subsequently by Gao *et al.* (1992). Guinea-pigs, weighing 300–500 g, were killed with an overdose of sodium pentobarbitone (1 ml of 360 mg ml^{-1}, I.P.). The isolated cells were stored in Kraft-Brühe solution containing (mM): KCl, 83; K_2HPO_4, 30; $MgSO_4$, 5; sodium pyruvic acid, 5; β-hydroxybutyric acid, 5; creatine, 5; taurine, 20; glucose, 10; EGTA, 0·5; KOH, 2; Na_2-ATP, 5; pH 7·2.

The pipette solution contained (mM): sodium aspartic acid, 50; CsOH, 100; TEACl, 20; NaH_2PO_4, 10; Hepes, 5; EGTA, 11; $CaCl_2$, 1 or 10 (giving a free $[Ca^{2+}]_i$ of 15 nM or 1·4 μM, respectively); glucose, 10; Mg-ATP, 5; pH 7·2. The external Tyrode solution contained (mM): NaCl, 137·7; KCl, 5·4; NaOH, 2·3; $MgCl_2$, 1; glucose, 10; Hepes, 5; $BaCl_2$, 2; $CdCl_2$, 1; pH 7·4. The 60 mM pipette $[Na^+]$ was used to saturate the intracellular Na^+-binding sites of the Na^+–K^+ pump (reviewed by Gadsby, 1984; Nakao & Gadsby, 1989; Gao, Mathias, Cohen & Baldo, 1995*a*). The replacement of K^+ by Cs^+ and TEA^+ in the pipette solution and the addition of Ba^{2+} to the external solution was to eliminate the K^+ conductances. Cd^{2+} in the external solution was used to block Ca^{2+} channel current (carried by Ba^{2+}) and the Na^+–Ca^{2+} exchanger.

Cells were placed in a temperature-controlled lucite bath (32 ± 0·5 °C), with a solution flow rate of 3 ml min^{-1} and a volume of 0·65 ml. Solutions were changed with a manual valve prior to entering the bath. The dead space was 0·15 ml. An Axopatch-1A amplifier (Axon Instruments) and the whole-cell patch clamp technique were employed to observe cell membrane current. Patch pipette resistances were 1–3 MΩ prior to sealing. I_p was defined as the inward shift in holding current observed during bath application of Tyrode solution containing 1 mM dihydro-ouabain (DHO; Sigma). The cells were clamped at −60 mV for this measurement. The voltage dependence of I_p, the I_p–V relationship, was observed using hyperpolarizing voltage ramps. Cells were initially clamped at +20 mV, then hyperpolarized to −100 mV over a 4 s period. The complete experimental protocol is shown in Fig. 1. Voltage ramps were applied before, during and after DHO application. The I_p–V relationship was calculated by subtracting the I–V curve in the presence of DHO from that recorded prior to DHO application. To check for stability of the membrane conductance during the experiment, we routinely compared the I–V curves before and after DHO application. Only experiments in which the two I–V curves were almost identical were used. The I_p–V relationships were fitted with the equation:

$$I_p = I_\infty/(1 + \exp(-(\sigma(V - V_{½})F/RT)), \quad (1)$$

where I_∞ is the maximal value of I_p (at $V = +\infty$), V is membrane potential, $V_{½}$ is the membrane potential at which I_p is 50% of I_∞, σ is a factor between 0 and 1, representing the slope of the voltage dependence of I_p at $V_{½}$, and F, R and T have their usual meanings.

External solution containing 1 mM DHO, a concentration that almost totally blocks the Na^+–K^+ pumps in guinea-pig ventricular myocytes (Daut & Rüdel, 1982; Gao *et al.* 1992), was washed into the tissue bath to block the Na^+–K^+ pump current. With the exception of the dose-dependent studies, external solutions containing 1 μM Iso (Sigma) and/or 1 μM ACh (Sigma) were used to study the effects of Iso and ACh on I_p. Atropine (1 μM; Sigma) was used to block muscarinic receptors. Chlorophenylthio-cAMP (CPTcAMP; 0·5 mM; Sigma) was used to examine the role of cAMP in mediating the effects of ACh on I_p. All patch clamp data were displayed on a Norland digital storage oscilloscope (Hi-Techniques, Madison, WI, USA) and recorded by a PC for later analysis. The sampling rate was 500 ms per point and the data were low-pass filtered at 10 Hz during steady-state recording. The I_p–V relationship was constructed from data sampled at a rate of 40 ms per point, filtered at 2 kHz, and analysed at 5 mV averaging points within a ±2·5 mV window.

Results are given as means ± S.D.

RESULTS

ACh has no effect on I_p at either low or high $[Ca^{2+}]_i$

We investigated the effects of ACh on I_p when cells were held at −60 mV using pipette solutions containing either low (15 nM) or high (1·4 μM) free Ca^{2+}. I_p was measured as the inward shift in holding current caused by application of 1 mM DHO. At 15 nM free Ca^{2+}, the ratio of I_p in the presence of ACh to that in the absence (control (Con); $I_{p(ACh)}/I_{p(Con)}$) was 0·97 ± 0·03 ($n = 4$). At 1·4 μM free Ca^{2+}, $I_{p(ACh)}/I_{p(Con)}$ was 1·01 ± 0·09 ($n = 5$). Thus at −60 mV, ACh had no effect on I_p at either low or high $[Ca^{2+}]_i$.

Since I_p is voltage dependent (reviewed by DeWeer, Gadsby & Rakowski, 1988; Gadsby, 1990*b*), we next examined the effects of ACh on the I_p–V relationship. These results together with the ramp protocol employed are shown in Fig. 1. Figure 1*A*–*C* illustrates how the I–V relationships were obtained from a series of ramps before, during and after exposure to 1 mM DHO in the absence (Fig. 1*B*) or in the presence (Fig. 1*C*) of ACh. Figure 1*D* and *E* illustrates the results obtained using this protocol in five cells at low (15 nM) and high (1·4 μM) pipette Ca^{2+}. ACh had no effect on the I_p–V relationship in either condition.

ACh reverses the effects of Iso on I_p at −60 mV at either low or high $[Ca^{2+}]_i$

Bath solution containing Iso was first applied to activate the cAMP-dependent PKA cascade via β-adrenergic receptors, and change the activity of the Na^+–K^+ pump (Gao *et al.* 1992, 1994, 1995*b*). The effects of ACh on I_p at low or high $[Ca^{2+}]_i$ were then investigated in the presence of Iso.

Figure 2*A* shows data from an experiment at low $[Ca^{2+}]_i$. The cell was held at −60 mV. On application of Iso, I_p declined from 181 to 143 pA, illustrating the inhibition of I_p by Iso at low $[Ca^{2+}]_i$ (Gao *et al.* 1992). Application of Tyrode solution containing ACh and Iso shifted the holding

current in an outward direction. This is probably due to ACh-induced inhibition of inwardly directed Cl^- current activated by Iso at −60 mV (Tareen *et al.* 1991; Hwang *et al.* 1992) as well as to an increase in I_p (see below). When the holding current had stabilized, I_p was measured in the presence of both Iso and ACh ($I_{p(Iso+ACh)}$). In this example, $I_{p(Iso+ACh)}$ was 175 pA. On washout of ACh, I_p declined to 145 pA. Similar results were observed in five other cells, and the mean ratio $I_{p(Iso+ACh)}/I_{p(Iso)}$ was 1·26 ± 0·06. These results suggest that ACh reverses the inhibitory effect of Iso on I_p at low $[Ca^{2+}]_i$.

Figure 2*B* shows data recorded from a myocyte at high $[Ca^{2+}]_i$ at −60 mV. On application of Iso, I_p increased from 193 to 233 pA (Gao *et al.* 1992). Application of ACh and Iso induced an outward shift of the holding current, but reduced the pump current ($I_{p(Iso+ACh)}$). In this cell, $I_{p(Iso+ACh)}$ was 189 pA. Similar results were observed in a total of eight cells, and the mean ratio $I_{p(Iso+ACh)}/I_{p(Iso)}$ was 0·80 ± 0·08.

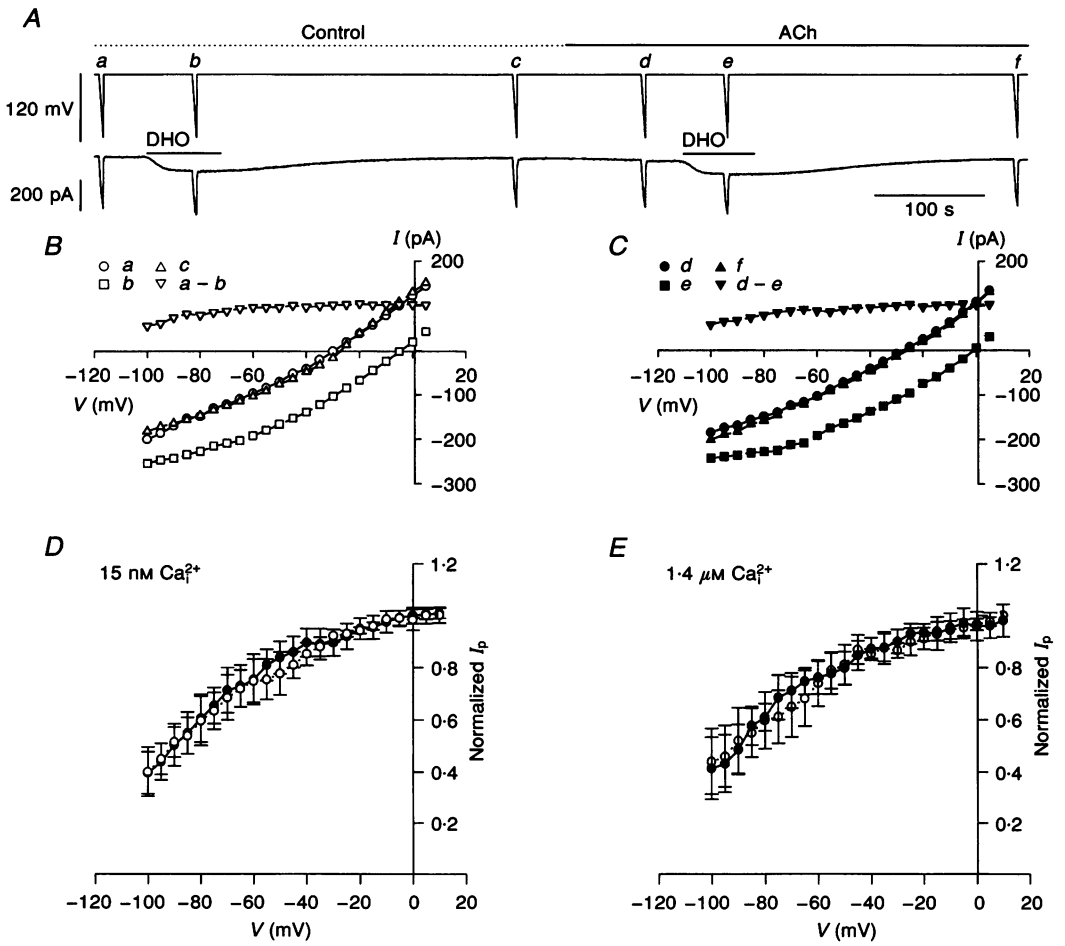

Figure 1. ACh has no effect on the voltage dependence of I_p (I_p–V)

A, voltage (upper trace) and current (lower trace) recordings from a single ventricular myocyte. Voltage ramps were delivered before, during and after DHO (1 mM) application in both normal Tyrode solution (*a*, *b* and *c*, respectively) and Tyrode solution containing 1 μM ACh (*d*, *e* and *f*, respectively). *B*, *I–V* relationships of membrane currents under control conditions. *a–c*, *I–V* curves before, during and after DHO application, respectively. The control I_p–V relationship was calculated as the difference between curves *a* and *b* (*a* − *b*). *C*, *I–V* relationships of membrane currents in the presence of 1 μM ACh. *d–f*, *I–V* curves before, during and after DHO application, respectively. The I_p–V relationship in the presence of ACh was calculated as the difference between *d* and *e* (*d* − *e*). *D*, normalized I_p–V curves (normalized to the maximum measured I_p) at low $[Ca^{2+}]_i$ (15 nM). The I_p–V relationships in the control conditions (○) and in the presence of ACh (●) were averaged from 5 cells; error bars indicate S.D. The two *I–V* curves almost overlap. *E*, normalized I_p–V curves at high $[Ca^{2+}]_i$ (1·4 μM). I_p–V curves in the control conditions (○) and in the presence of ACh (●) were averaged from 5 cells and appear almost identical. Error bars indicate S.D.

These results suggest that ACh reverses the stimulatory effect of Iso on I_p at −60 mV at high $[Ca^{2+}]_i$.

Dose–response relationship of the effect of ACh on the Iso-induced change in I_p

We next examined the dose–response relationship of the ability of ACh to reverse the actions of Iso on I_p. Iso (1 μM) was present throughout the experiment. The cell was held at −60 mV and I_p was measured first in Iso alone, and then in the presence of Iso plus some test concentration of ACh. Sample results at low pipette $[Ca^{2+}]$ (15 nM) are shown in Fig. 3*A*. The mean data from a range of ACh concentrations at 15 nM pipette Ca^{2+} are shown in Fig. 3*B*. The continuous line shows the best fit to the standard 1 : 1 Langmuir binding curve with a $K_{0.5}$ value of 17·3 nM.

We performed a similar set of experiments at 1·4 μM pipette Ca^{2+}. Sample results are shown in Fig. 3*C*. The mean data from all experiments at 1·4 μM pipette Ca^{2+} are shown in Fig. 3*D*. $K_{0.5}$ was 14·6 nM.

ACh reverses the effects of Iso on the I_p–V relationship at either low or high $[Ca^{2+}]_i$

The activation of β-adrenergic receptors by Iso reduces I_p by 20–25% at all physiologically relevant membrane potentials at low $[Ca^{2+}]_i$ (Gao *et al.* 1996). We tested whether ACh could reverse this effect using the protocol shown in Fig. 1, except that Iso was present in the external solution throughout the experiment.

Figure 4*A* and *B* essentially shows previously published findings that Iso reduced I_p by 20% at all test potentials (Gao *et al.* 1996). In Fig. 4*A*, the normalized voltage dependence of I_p in the control conditions and in Iso was averaged from three cells. The smooth curves are the best fits to eqn (1). The $V_{½}$ values obtained by curve fitting in the control conditions and in Iso were −93·6 and −89·1 mV, respectively. The I_p–V relationship in the presence of Iso did not show a significant shift along the voltage axis, but I_p was reduced at all test potentials. The ratios $I_{p(Iso)}/I_{p(Con)}$

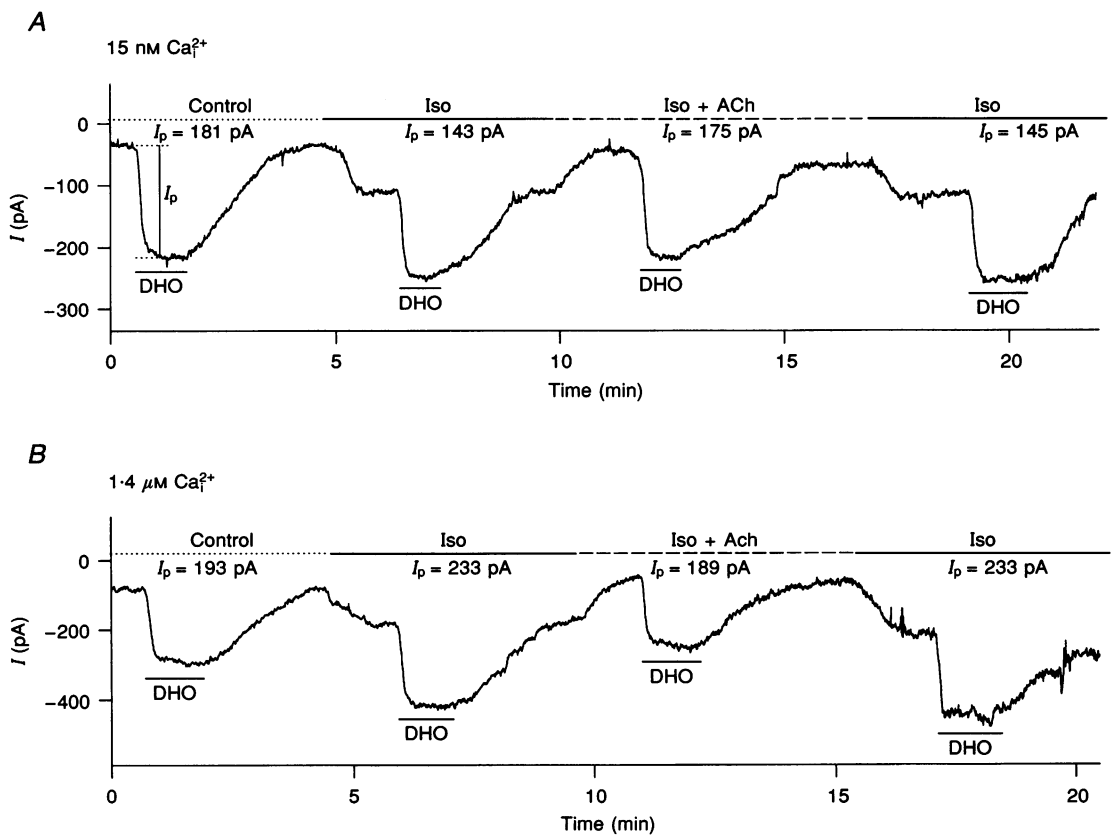

Figure 2. ACh reverses the effects of Iso on I_p at low and high $[Ca^{2+}]_i$ at −60 mV

A, low $[Ca^{2+}]_i$. The myocyte was held at −60 mV and the pipette solution contained 15 nM Ca^{2+}. The continuous line indicates the application of Iso (1 μM) and the dashed line indicates the application of Iso plus 1 μM ACh (Iso + ACh). The vertical line labelled I_p indicates the size of I_p and how it was measured. The effect of ACh on I_p was reversible. *B*, ACh reverses the stimulatory effect of Iso on I_p at high $[Ca^{2+}]_i$. The same protocol as that in *A* was used, except that $[Ca^{2+}]_i$ was 1·4 μM. Again the effect of ACh was reversible. Note that the holding current change induced by ACh application in *A* and *B* may indicate the inhibition of a Cl^- conductance activated by Iso. Data were recorded from 2 different cells. In this and subsequent figures, horizontal bars below the traces indicate the period of application of DHO (1 mM).

plotted against membrane potential in Fig. 4*B* were calculated using the I_p–V data in Fig. 4*A*, and indicate about a 20% inhibition by Iso. Figure 4*C* and *D* shows that ACh reverses the Iso-induced inhibition of I_p–V. The normalized I_p–V curves at low $[Ca^{2+}]_i$ in Iso and in Iso plus ACh were averaged from five cells. The $V_{½}$ values in Iso and in Iso plus ACh were −88 and −92 mV, respectively. Again, there was no significant shift along the voltage axis; however, I_p recorded in ACh plus Iso was enhanced at each test potential, compared with I_p in Iso alone. The ratio $I_{p(Iso)}/I_{p(Iso+ACh)}$, calculated from the I_p–V data in Fig. 4*C*, is roughly identical to $I_{p(Iso)}/I_{p(Con)}$ in Fig. 4*B*, which suggests that ACh reverses the inhibitory effect of Iso on I_p at low $[Ca^{2+}]_i$ at all potentials tested.

We have previously shown that Iso shifts the I_p–V relationship by about 25 mV in the negative direction at high $[Ca^{2+}]_i$ (Gao *et al.* 1996). We tested whether ACh could reverse this voltage shift using the same protocol as in Fig. 4, except that $[Ca^{2+}]_i$ was 1·4 μM.

Figure 5*A* and *B* reproduces our previous findings (Gao *et al.* 1996). The I_p–V curves at high $[Ca^{2+}]_i$ in the control conditions and in Iso were averaged from three cells. The smooth curves are the best fits to eqn (1). The $V_{½}$ values in the control conditions and in the presence of Iso, obtained by curve fitting, were −85 and −111 mV, respectively. These results suggest that the I_p–V relationship in the presence of Iso was shifted by 26 mV in the negative

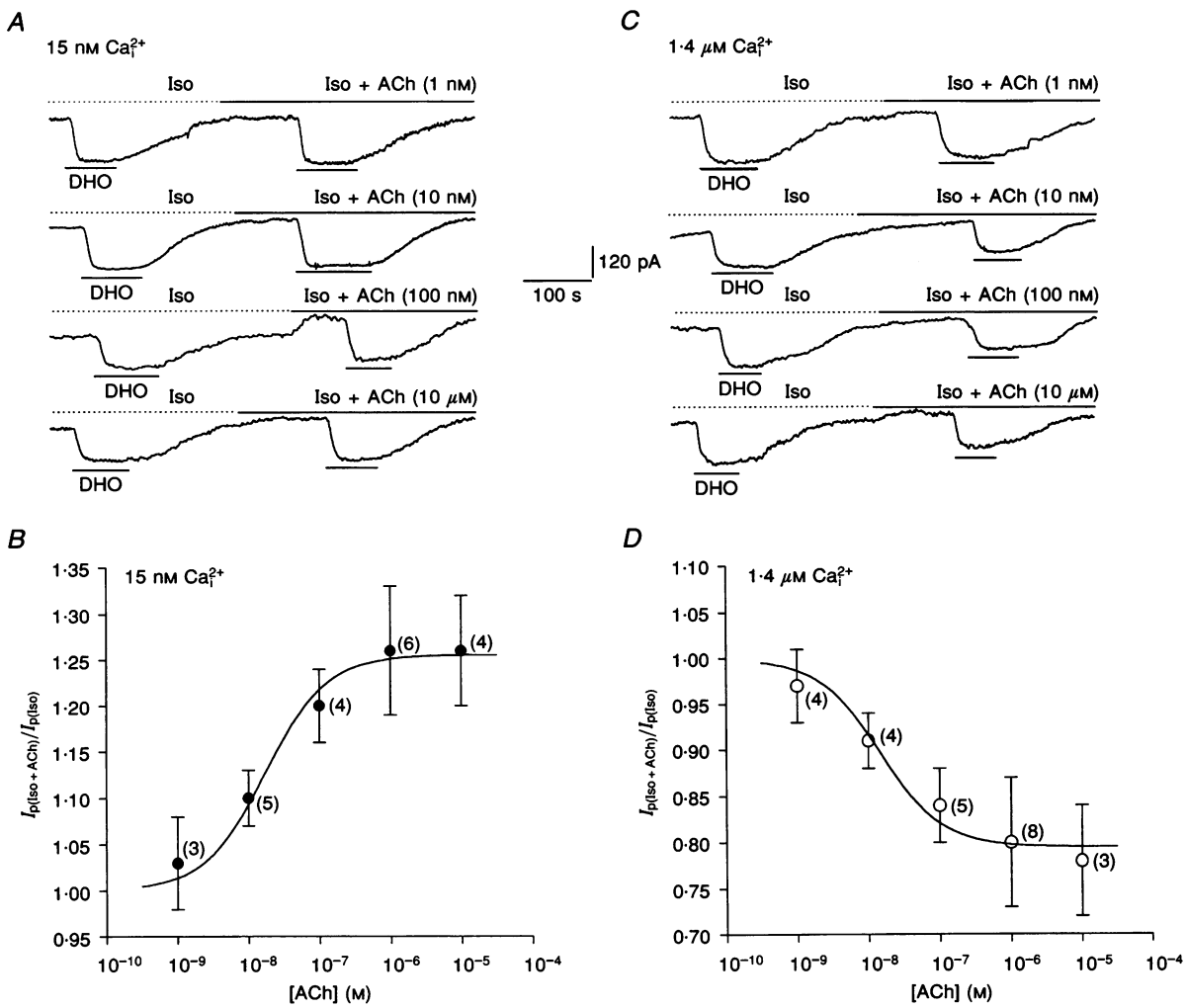

Figure 3. Dose–response relationship for the effects of ACh on I_p

A, raw data illustrating the protocol at low $[Ca^{2+}]_i$. The myocyte was held at −60 mV in Tyrode solution containing 1 μM Iso. Tyrode solution containing 1 μM Iso plus the indicated [ACh] was then added. The amplitude of I_p increased. *B*, dose–response curve for the effects of ACh at 15 nM Ca_i^{2+} obtained using the same protocol as in *A* for a variety of ACh concentrations normalized to I_p in Iso alone ($I_{p(Iso+ACh)}/I_{p(Iso)}$). Numbers in parentheses indicate the number of cells. Error bars indicate S.D. The non-linear curve fit indicates that the maximum pump current, I_{max}, is 1·26 of control and $K_{0·5}$ is 17·3 nM. *C*, raw data at 1·4 μM Ca_i^{2+}, obtained using the same protocol as in *A*. *D*, dose–response relationship at 1·4 μM Ca_i^{2+}. The minimum pump current, I_{min}, is 0·79 of control and $K_{0·5}$ is 14·6 nM. Error bars indicate S.D.

direction, relative to control. The ratios $I_{p(Iso)}/I_{p(Con)}$ were calculated from the I_p–V data in *A*, and are plotted against membrane potential in Fig. 5*B*. The resultant graph suggests that the stimulatory effect of Iso on I_p at high $[Ca^{2+}]_i$ is voltage dependent, increasing with hyperpolarization, and having almost no effect at 0 mV.

Figure 5*C* shows the I_p–V relationships in Iso and in Iso plus ACh. Data were averaged from five cells, and the smooth curves were obtained by curve fitting. The $V_{½}$ values in Iso and in Iso plus ACh were −117 and −83 mV, respectively. These results indicate that the I_p–V relationship in Iso was shifted by ACh to a less negative potential. The ratios $I_{p(Iso)}/I_{p(Iso+ACh)}$, calculated from the I_p–V relationships in Fig. 5*C*, are plotted against membrane potential in Fig. 5*D*, and are almost identical to $I_{p(Iso)}/I_{p(Con)}$ in Fig. 5*B*. This strongly suggests that ACh reverses the Iso-induced voltage shift of the I_p–V relationship at high $[Ca^{2+}]_i$.

Pretreatment with atropine blocks the ACh-induced reversal of the effects of Iso on I_p at −60 mV

The muscarinic antagonist atropine was used to determine whether the effects of ACh on I_p are mediated via muscarinic receptors.

We employed a protocol in which the myocyte was held at −60 mV in the presence of Iso plus 1 μM atropine (Atr). I_p was measured and then 1 μM ACh was applied and I_p again measured. At 15 nM pipette Ca^{2+}, $I_{p(Iso+Atr+ACh)}/I_{p(Iso+Atr)}$ was 1·00 ± 0·04 ($n = 5$).

The same protocol was employed at 1·4 μM pipette Ca^{2+}. The mean ratio $I_{p(Iso+Atr+ACh)}/I_{p(Iso+Atr)}$ from a total of five cells was 0·99 ± 0·05.

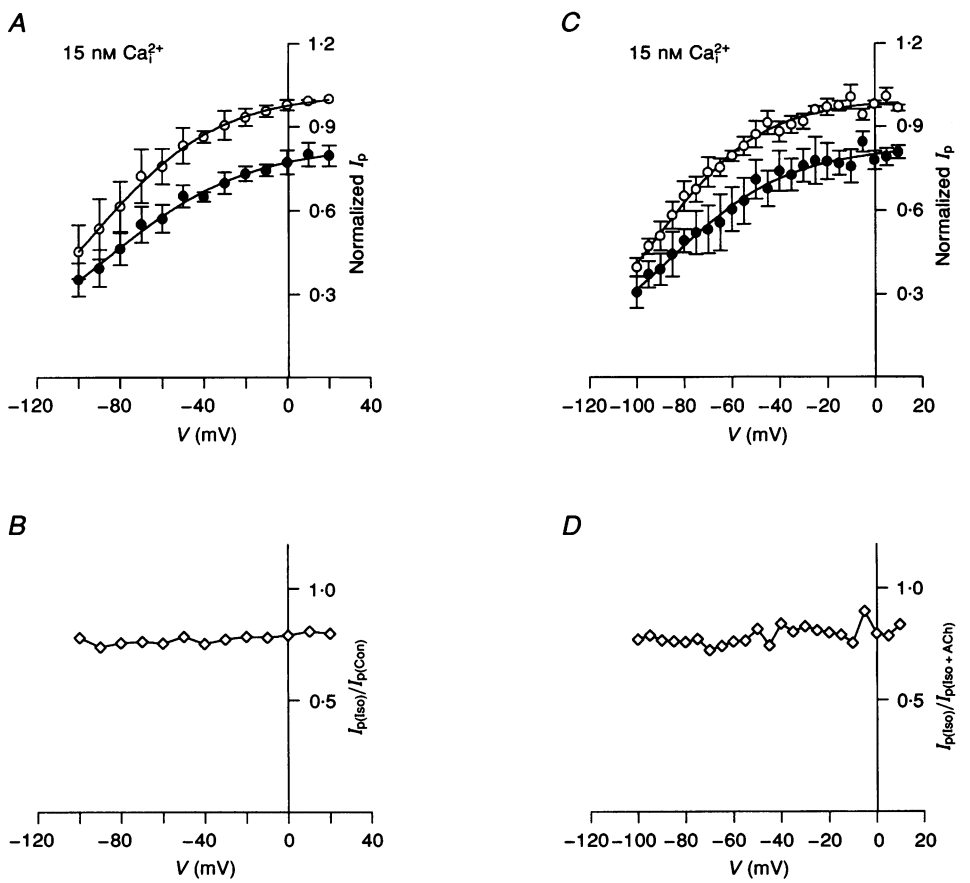

Figure 4. ACh reverses the Iso-induced I_p inhibition at all voltages at low $[Ca^{2+}]_i$ (15 nM)

A, normalized I_p–V relationships in the control conditions (○) and in the presence of 1 μM Iso (●). Data were obtained using the ramp protocol (see Methods and Fig. 1 for details), and were averaged from 3 cells. Error bars indicate S.D. The smooth curves are the best fit to eqn (1). *B*, $I_{p(Iso)}/I_{p(Con)}$, calculated using the I_p–V curves in *A*, is plotted against membrane potential, and indicates that Iso causes about a 20% inhibition of I_p. *C*, normalized I_p–V relationships in Iso (●) and in Iso plus 1 μM ACh (○). Data were averaged from 5 cells. Error bars indicate S.D. The smooth curves are the best fits to eqn (1). *D*, $I_{p(Iso)}/I_{p(Iso+ACh)}$ is plotted against membrane potential. These ratios were calculated using the I_p–V curves in *C*, and are of the same magnitude as $I_{p(Iso)}/I_{p(Con)}$ in *B*, suggesting that ACh reverses the inhibitory effect of Iso on I_p at low $[Ca^{2+}]_i$.

Furthermore, atropine also blocked the ACh-induced change in the holding current at low or high $[Ca^{2+}]_i$ shown in Fig. 2. These results suggest that the effects of ACh on I_p are mediated via muscarinic receptors.

The effects of ACh are mediated by a change in [cAMP]

ACh is known to decrease adenylyl cyclase activity and reduce intracellular [cAMP] (Hartzell, 1988; Lindemann & Watanabe, 1990). This reduction in [cAMP] is the putative cause of the reduction of i_f initiated by muscarinic stimulation (DiFrancesco & Tromba, 1987). We therefore examined the role of cAMP in mediating the effects of ACh on I_p. We have previously demonstrated that a membrane-permeant analogue of cAMP (CPTcAMP) causes changes in I_p that are similar to those induced by β-activation (Gao *et al.* 1994). If ACh acts by reducing cAMP production, there should be no change in I_p when ACh is added after pretreatment with CPTcAMP.

Figure 6*A* shows the results of one experiment at 15 nM Ca_i^{2+}. The control I_p was 94 pA; application of 0·5 mM CPTcAMP reduced I_p to 72 pA, as expected from results of β-stimulation. Addition of 1 μM ACh had little effect ($I_p = 75$ pA). Upon washout of CPTcAMP and ACh, I_p returned to the control value of 94 pA. At 15 nM Ca_i^{2+} the ratio $I_{p(CPTcAMP+ACh)}/I_{p(CPTcAMP)}$ was 1·02 ± 0·06 for five experiments.

Figure 6*B* illustrates a similar test of the hypothesis at 1·4 μM Ca_i^{2+}. The control I_p was 168 pA. Addition of 0·5 mM CPTcAMP to the bath Tyrode solution increased I_p to 224 pA, again expected from our experiments with β-agonists. Addition of 1 μM ACh to the CPTcAMP-containing Tyrode solution had little effect ($I_p = 222$ pA).

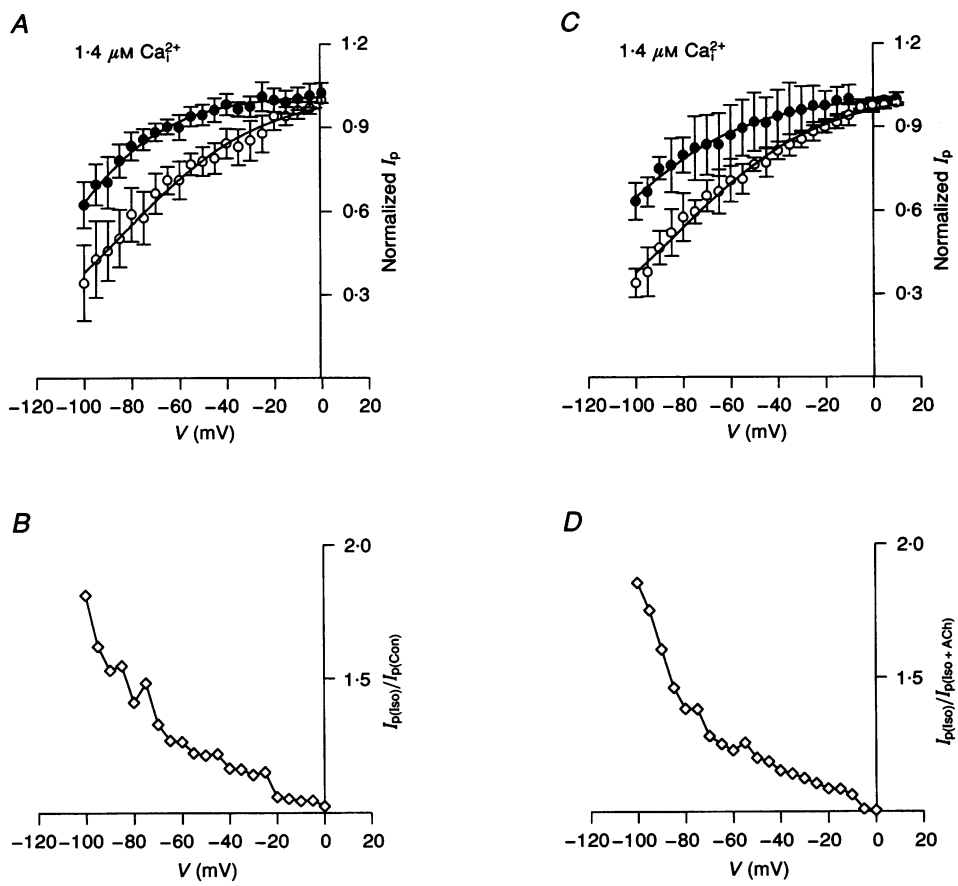

Figure 5. ACh reverses the Iso-induced voltage shift of I_p–V at high $[Ca^{2+}]_i$ (1·4 μM)

A, normalized I_p–V relationships in the control conditions (○) and in 1 μM Iso (●). Data were obtained using the ramp protocol (see Fig. 1) and were averaged from 3 cells. Error bars indicate S.D. The smooth curves are the best fits to eqn (1). *B*, $I_{p(Iso)}/I_{p(Con)}$ is plotted against membrane potential. These ratios were calculated using the I_p–V relationships in *A*, and show that the stimulatory effect of Iso on I_p is voltage dependent. *C*, normalized *I*–*V* relationships of I_p in Iso (●) and in Iso plus 1 μM ACh (○). Data were averaged from 5 cells, and the smooth curves were obtained by curve fitting to eqn (1). Error bars indicate S.D. *D*, $I_{p(Iso)}/I_{p(Iso+ACh)}$ is plotted against membrane potential. These ratios were calculated using the I_p–V relationships in *C*, and are almost equivalent to $I_{p(Iso)}/I_{p(Con)}$ in *B*.

Upon return to normal Tyrode solution, I_p decreased to 163 pA, which is near to the control level. The mean ratio $I_{p(CPTcAMP+ACh)}/I_{p(CPTcAMP)}$ was 0·97 ± 0·02 ($n = 4$).

These experiments demonstrate that high concentrations of exogenous CPTcAMP prevent the actions of ACh and lend support to the hypothesis that ACh works by reducing the cAMP levels raised by β-stimulation.

DISCUSSION

We investigated the effect of the parasympathetic transmitter ACh on I_p. Our data suggest that ACh in the absence of Iso has no effect on I_p regardless of $[Ca^{2+}]_i$; however, ACh reverses all of the Iso-induced effects on I_p. These effects of ACh are eliminated by the muscarinic blocker atropine, therefore they appear to be mediated by an intracellular, second messenger-initiated cascade.

ACh antagonizes the effects of β-stimulation on currents other than I_p. For example, ACh inhibits the Cl^- conductance activated by Iso in guinea-pig ventricular myocytes (Tareen *et al.* 1991; Hwang *et al.* 1992), and reverses the effect of Iso on the pacemaker current, i_f, in canine cardiac Purkinje fibres (Chang *et al.* 1990). The results shown in Fig. 2 not only indicate that ACh reverses the effects of Iso on I_p at either low or high $[Ca^{2+}]_i$, but also suggest that ACh may prevent some of the Iso-induced increase in Cl^- conductance. In the present study, the cells were held at −60 mV, and the equilibrium potential for Cl^-, E_{Cl}, was set at either −49 mV (low pipette $[Ca^{2+}]$) or −34 mV (high pipette $[Ca^{2+}]$). Thus, the inhibition of Cl^- current by ACh would cause an outward current shift.

Although ACh does not have a direct effect on the I_p–V relationship at either low or high $[Ca^{2+}]_i$ (Fig. 1), it does reverse Iso-induced effects on I_p seen at low or high $[Ca^{2+}]_i$ (Figs 2–4). The lack of any action by ACh alone, but the antagonization of the actions of the β-agonist Iso, are very similar to the effects of ACh on i_f in canine Purkinje fibres (Chang *et al.* 1990) and the Cl^- current in guinea-pig ventricular myocytes (Tareen *et al.* 1991; Hwang *et al.* 1992).

It is well established that β-adrenergic activation modulates several cardiac membrane currents by coupling the β-receptors to membrane-bound G-proteins, G_s. The α_s

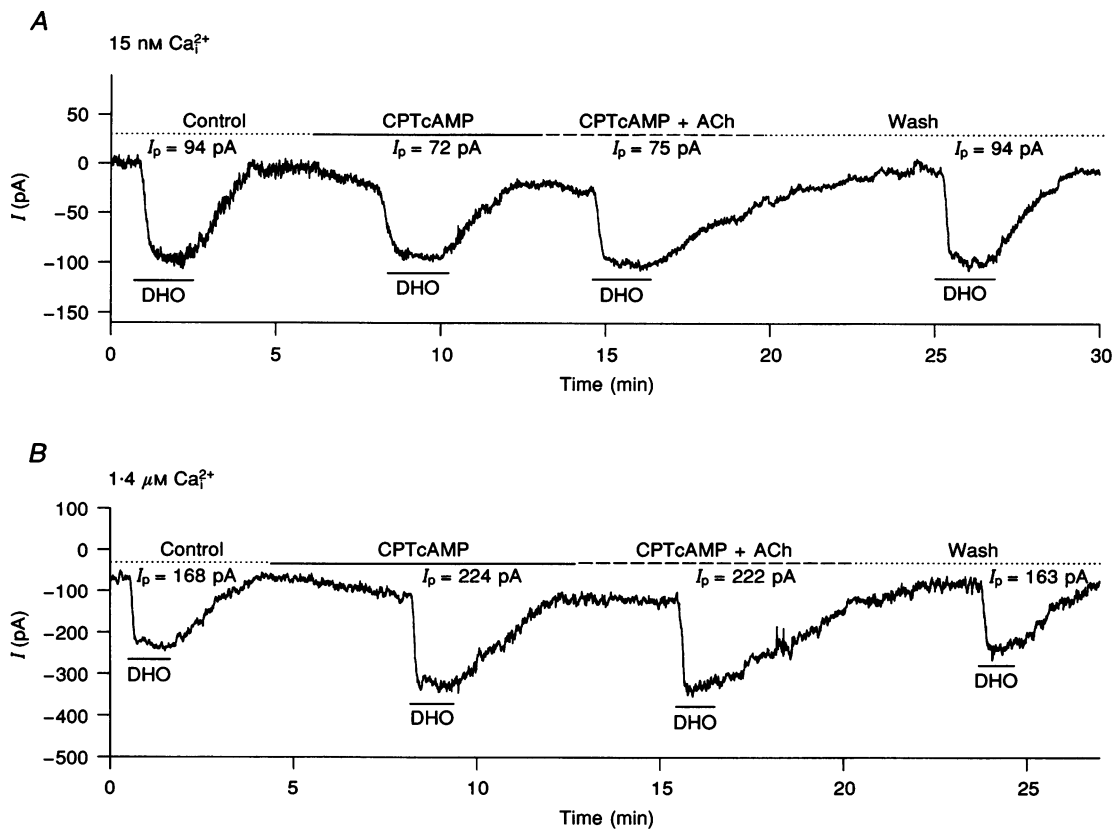

Figure 6. ACh has no effect on CPTcAMP-mediated changes in I_p

A, at 15 nM Ca_i^{2+}, the cell was held at −60 mV and application of CPTcAMP (0·5 mM) reduced I_p. Addition of 1 μM ACh to the CPTcAMP-containing Tyrode solution had no effect on I_p. However, on washout of CPTcAMP and ACh (Wash), I_p returned towards the control value. *B*, same protocol as in *A* but at 1·4 μM Ca_i^{2+}. CPTcAMP augmented I_p, but again addition of ACh to the CPTcAMP-containing Tyrode solution had no effect on I_p. Washout of CPTcAMP and ACh showed that the effects of CPTcAMP on I_p were reversible.

catalytic subunit of G_s dissociates from the $\beta\gamma$ complex by exchanging GDP for GTP, and activates adenylyl cyclase. This increases intracellular cAMP levels, which activates cAMP-dependent PKA, causing phosphorylation of a variety of cellular proteins. The various steps in this pathway induce many functional responses, including alterations of Na^+, Ca^{2+}, Cl^- and K^+ conductances as well as the hyperpolarization-activated current, i_f (see reviews by Hartzell, 1988; Clapham, 1990). In a similar manner, muscarinic activation produces inhibition through a different membrane-bound G-protein, G_i. The α_i subunit dissociates from $\beta\gamma$, and the $\beta\gamma$ complex mediates the inhibition of adenylyl cyclase activity, presumably by complexing with α_s to form an inactive heterotrimer, decreasing intracellular cAMP levels (reviewed by Hartzell, 1988; Lindemann & Watanabe, 1990). We previously demonstrated that the effects of β-adrenergic activation on I_p are mediated by the cAMP-dependent PKA pathway (Gao *et al.* 1994, 1995*b*). In the present study we demonstrate that the action of ACh is also mediated via a change in cAMP. The actions of ACh and Iso on I_p probably share a common mechanism, e.g. ACh reverses the effects of Iso on I_p by reducing the cAMP level elevated by Iso. This mechanism may explain why ACh alone has no effect on I_p; our data (Gao *et al.* 1994) suggest that in the absence of β-stimulation the basal cAMP level is very low in these myocytes. Thus, any effect of ACh on basal cAMP levels would produce an insignificant cellular response.

Functionally, the present results suggest that a high parasympathetic tone alone does not affect the Na^+–K^+ pump. It is only when the sympathetic system is active that the parasympathetic system exerts a modulating influence on Na^+–K^+ pump activity.

Chang, F., Gao, J., Tromba, C., Cohen, I. & DiFrancesco, D. (1990). Acetylcholine reverses effects of β-agonists on pacemaker current in canine cardiac Purkinje fibers but has no direct action. *Circulation Research* **66**, 633–636.

Clapham, D. (1990). Intracellular regulation of ion channels. In *Cardiac Electrophysiology: From Cell to Bedside*, ed. Zipes, D. & Jalife, J., pp. 85–93. W. B. Saunders Company, Philadelphia, PA, USA.

Daut, J. & Rüdel, R. (1982). The electrogenic sodium pump in guinea-pig ventricular muscle: inhibition of pump current by cardiac glycosides. *Journal of Physiology* **330**, 243–264.

DeWeer, P., Gadsby, D. C. & Rakowski, R. F. (1988). Voltage dependence of the Na/K pump. *Annual Review of Physiology* **50**, 225–241.

DiFrancesco, D. (1990). Current i_f and the neuronal modulation of heart rate. In *Cardiac Electrophysiology: From Cell to Bedside*, ed. Zipes, D. & Jalife, J., pp. 28–35. W. B. Saunders Company, Philadelphia, PA, USA.

DiFrancesco, D. & Tromba, C. (1987). Acetylcholine inhibits activation of the cardiac pacemaker current, i_f. *Pflügers Archiv* **409**, 139–142.

Gadsby, D. C. (1984). The Na/K pump of cardiac cells. *Annual Review of Biophysics and Bioengineering* **13**, 373–398.

Gadsby, D. C. (1990*a*). Effects of β adrenergic catecholamines on membrane currents in cardiac cells. In *Cardiac Electrophysiology: A Textbook*, ed. Rosen, M. R., Janse, M. J. & Wit, A. L., pp. 857–876. Futura Publishing Co., New York.

Gadsby, D. C. (1990*b*). The Na/K pump of cardiac myocytes. In *Cardiac Electrophysiology: From Cell to Bedside*, ed. Zipes, D. & Jalife, J., pp. 35–50. W. B. Saunders Company, Philadelphia, PA, USA.

Gao, J., Cohen, I. S., Mathias, R. T. & Baldo, G. J. (1994). Regulation of the β-stimulation of the Na^+–K^+ pump current in guinea-pig ventricular myocytes by a cAMP-dependent PKA pathway. *Journal of Physiology* **477**, 373–380.

Gao, J., Mathias, R. T., Cohen, I. S. & Baldo, G. J. (1992). Isoprenaline, Ca^{2+} and the Na^+–K^+ pump in guinea-pig ventricular myocytes. *Journal of Physiology* **449**, 689–704.

Gao, J., Mathias, R. T., Cohen, I. S. & Baldo, G. J. (1995*a*). Two functionally different Na/K pumps in cardiac ventricular myocytes. *Journal of General Physiology* **106**, 995–1030.

Gao, J., Mathias, R. T., Cohen, I. S. & Baldo, G. J. (1995*b*). A cAMP-dependent PKA pathway mediates the inhibitory effect of β-stimulation on Na/K pump current in guinea pig ventricular myocytes. *Biophysical Journal* **68**, A308.

Gao, J., Mathias, R. T., Cohen, I. S., Shi, J. & Baldo, G. J. (1996). The effects of β-stimulation on the Na^+–K^+ pump current–voltage relationship in guinea-pig ventricular myocytes. *Journal of Physiology* **494**, 697–708.

Hartzell, H. C. (1988). Regulation of cardiac ion channels by catecholamines, acetylcholine and second messenger systems. *Progress in Biophysics and Molecular Biology* **52**, 165–247.

Hasuo, H., Koketsu, K. & Minota, S. (1988). Indirect effects of acetylcholine on the electrogenic sodium pump in bull-frog atrial muscle fibres. *Journal of Physiology* **399**, 519–535.

Hwang, T., Horie, M., Nairn, A. C. & Gadsby, D. C. (1992). Role of GTP-binding proteins in the regulation of mammalian cardiac chloride conductance. *Journal of General Physiology* **99**, 465–489.

Isenberg, G. & Klöckner, V. (1982). Calcium tolerant ventricular myocytes prepared by preincubation in a 'KB-Medium'. *Pflügers Archiv* **395**, 6–18.

Iyadomi, I., Hirahara, K. & Ehara, T. (1995). α-Adrenergic inhibition of the β-adrenoceptor-dependent chloride current in guinea-pig ventricular myocytes. *Journal of Physiology* **489**, 95–104.

Lindemann, J. P. & Watanabe, A. M. (1990). Sympathetic control of cardiac electrical activity. In *Cardiac Electrophysiology: From Cell to Bedside*, ed. Zipes, D. & Jalife, J., pp. 277–283. W. B. Saunders Company, Philadelphia, PA, USA.

Nakao, M. & Gadsby, D. C. (1989). [Na] and [K] dependence of the Na/K pump current–voltage relationship in guinea pig ventricular myocytes. *Journal of General Physiology* **94**, 539–565.

Tareen, F. M., Ono, K., Noma, A. & Ehara, T. (1991). β-Adrenergic and muscarinic regulation of the chloride current in guinea-pig ventricular cells. *Journal of Physiology* **440**, 225–241.

Acknowledgements

This work was supported by grants HL54031, HL20558 and HL28958 from the National Heart, Lung and Blood Institute.

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Received 7 January 1997; accepted 25 March 1997.