β-Adrenergic and cholinergic modulation of the inwardly rectifying K^+ current in guinea-pig ventricular myocytes

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1. Whole-cell patch-clamp technique was used to study the β-adrenergic and cholinergic regulation of the inwardly rectifying K^+ conductance (g_{K1}) in isolated guinea-pig ventricular myocytes.

2. In Cl^--free solutions or in the presence of 9-anthracenecarboxylic acid or Co^{2+}, bath-applied isoprenaline (Iso) partially inhibited the steady-state whole-cell conductance (g_{ss}) calculated from the steady-state current (I_{ss})–voltage (I_{ss}–V) curve at membrane voltages (V_m) negative to the equilibrium potential for potassium (E_K). I_{ss} was also inhibited at V_m positive to E_K when the extracellular [K^+] was 20 mM. The Iso-sensitive component of g_{ss} exhibited the characteristics of the inwardly rectifying K^+ conductance (g_{K1}).

3. The Iso-induced inhibition of g_{K1} was reversible, concentration dependent, blocked by propranolol, mimicked by both forskolin and dibutyryl cAMP, and prevented by including a cAMP-dependent protein kinase (PKA) inhibitor in the pipette solution. These findings suggest that PKA mediates the Iso-induced inhibition of g_{K1}.

4. The apparent dissociation constant (K_D) for the concentration dependence of Iso-induced inhibition was 0·035 μM and the Hill coefficient was ~1·0. A maximal Iso concentration (1 μM) inhibited g_{K1} by 40 ± 4·1% (mean ± S.E.M.; $n = 13$).

5. Bath application of acetylcholine (ACh, 0·1 μM or more) antagonized the Iso-induced (1 μM) inhibition of g_{K1}; [ACh] > 1·0 μM antagonized 88 ± 2·1% ($n = 10$) of the inhibition. ACh increased the K_D for Iso to inhibit Iso-sensitive g_{K1} and also reduced the maximal Iso-induced inhibition.

6. ACh-induced antagonism could be abolished by pre-incubating myocytes with pertussis toxin (PTX), suggesting that a muscarinic receptor-coupled, PTX-sensitive G protein, G_i, is involved.

7. ACh (10 μM) also antagonized ~70% of the dibutyryl cyclic AMP (1 mM)-induced inhibition of g_{K1} ($n = 3$), suggesting that the ACh-induced antagonism involves more than simply inhibiting the Iso-mediated activation of adenylyl cyclase via the activated G_i.

8. Intracellularly applied okadaic acid (OkA, 1 μM) did not alter g_{K1} (control = 134 ± 5·1 nS *vs.* OkA = 136 ± 6·1 nS), but the Iso-induced decrease in g_{K1} was less ($P < 0·001$) with OkA present (42·1 ± 2·4 nS, $n = 5$) than when absent (54·0 ± 2·2 nS, $n = 10$). However, ACh (10 μM) failed to antagonize Iso-induced inhibition with OkA present, suggesting involvement of a protein phosphatase.

Activation of β-adrenergic receptors can modulate several ion channels in cardiac myocytes via a direct coupling of the receptors to the G protein, G_s, to activate adenylyl cyclase and thereby increase cAMP production. The increased level of cAMP in turn enhances cAMP-dependent protein kinase (PKA) activity to enable phosphorylation of several channel proteins involved in producing the positive inotropic and electrophysiological effects associated with β-adrenergic receptor activation in ventricular tissue (see Hartzell, 1988 and Susanni, Vatner & Homcy, 1992, for

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review) including voltage-dependent Ca^{2+} channels (Reuter & Scholz, 1977; Kameyama, Hofmann & Trautwein, 1985), delayed rectifier K^+ channels (Bennett, McKinney, Begenisich & Kass, 1986; Giles, Nakajima, Ono & Shibata, 1989; Yazawa & Kameyama, 1990), Na^+ channels (Tytgat, Vereecke & Carmeliet, 1990; Matsuda, Lee & Shibata, 1992) and Cl^- channels (Harvey & Hume, 1989; Bahinski, Nairn, Greengard & Gadsby, 1989; Tareen, Ono, Noma & Ehara, 1991).

In contrast to the many reports concerned with autonomic influences on these ionic current systems, information regarding the effects of adrenergic and cholinergic agonists on cardiac inwardly rectifying K^+ current (I_{K1}) channels is limited to a very few reports. Two studies, using indirect evidence, have suggested that the background K^+ conductances of canine cardiac Purkinje fibres (Gadsby, 1983) and coronary sinus tissues (Boyden, Cranefield & Gadsby, 1983) can increase during exposure to isoprenaline (Iso). Another study suggested that Iso can inactivate I_{K1} channels in canine cardiac Purkinje cells via a non-β-adrenoreceptor-mediated mechanism (Tromba & Cohen, 1990). Because of this lack of consensus and the paucity of investigations into the underlying mechanisms, the present study was undertaken to determine if β-adrenergic and cholinergic receptors have a role in the regulation of ventricular I_{K1} channels.

METHODS

Cell preparation

Guinea-pig ventricular myocytes were isolated by an enzymatic dissociation method similar to that developed by Mitra & Morad (1985). Briefly, guinea-pigs weighing 150–200 g were anaesthetized with pentobarbitone (60 mg kg^{-1}, I.P.) and their hearts were quickly excised. After cannulating the aorta, hearts were mounted on a Langendorff-type apparatus and perfused retrogradely for 5 min via the aortic cannula with Ca^{2+}-free Tyrode solution under a hydrostatic pressure of about 100 cmH_2O. All perfusates were bubbled with 100% O_2 and warmed to 37 °C. The perfusion was then changed to a Ca^{2+}-free Tyrode solution containing collagenase (Type I; 1·5 mg ml^{-1}), albumin (BSA; 1 mg ml^{-1}) and protease (Type XIV; 1 mg ml^{-1}) for 3–4 min. Next the collagenase-containing solution was washed out with 50 ml of a high-K^+, low-Cl^- Kraftbrühe (KB) solution (composition below; Isenberg & Klockner, 1982) and the heart was minced; the mince was gently agitated in this KB solution for about 15 min and then filtered through a 200 μm nylon mesh to remove undigested tissue chunks. The filtrate was centrifuged at 50 g for 2 min to pellet the surviving myocytes; the cell pellet was then resuspended in KB medium and stored at room temperature for an hour before electrophysiological study commenced.

Solutions and chemicals

KB solution contained (mM): KOH, 115·9; glutamic acid, 80·0; taurine, 10·0; oxalic acid, 14·0; potassium phosphate, 10·0; KCl, 25·0; Hepes, 10·0; glucose, 11·0; EGTA, 0·5; pH was adjusted to 7·2 with KOH (Isenberg & Klockner, 1982). Two different control external solutions were employed. One contained Cl^- and the other was Cl^--free. The Cl^--containing control external solution was a modified Tyrode solution containing (mM): NaCl, 140; KCl, 5·4; $CaCl_2$, 1·8; $MgCl_2$, 0·5; Hepes, 5·0; glucose, 10; and either $CoCl_2$ (2·0 mM; Zhang, Koumi, Wasserstrom & Ten Eick, 1993) or 9-anthracenecarboxylic acid (9-AC; 0·1 mM; Harvey & Hume, 1989) to minimize the contribution of Iso-induced, cAMP and protein kinase A (PKA)-dependent Cl^- current (I_{Cl}) to the cellular membrane current (I_m) and thus prevent I_{Cl} from masking or obscuring an effect of Iso on I_{K1}. The pH of the solution was adjusted to 7·4 with NaOH. Cl^--free external solution contained (mM): sodium glutamate, 140; potassium glutamate, 5·4; $CaSO_4$, 1·8; $MgSO_4$, 0·5; Hepes, 5·0; glucose, 10 (pH adjusted to 7·4 with NaOH). Ca^{2+}-free modified Tyrode solution was made by omitting $CaCl_2$. Cl^--containing Tyrode solutions with low (2 mM) or high (20 mM) K^+ concentrations were prepared by adding the required amount of 1 M KCl to a K^+-free stock Tyrode solution. L-type Ca^{2+} current was blocked with nifedipine (1–5 μM) added to Cl^--free control external solution and by the 2 mM Co^{2+} that had been added to the Cl^--containing solution to partially block I_{Cl}. When desired, Na^+ current (I_{Na}) was inhibited with tetrodotoxin (TTX; 10 μM) and I_{K1} was blocked with Ba^{2+} (1·0 mM). Dihydroouabain (DHO; 300 μM) was added to Cl^--free external solution in some experiments to eliminate current that might be generated by the sarcolemmal Na^+ pump (Mogul, Rasmussen, Singer & Ten Eick, 1989). Similarly, patch pipettes were filled with either a Cl^--containing or a Cl^--free internal solution. The composition of the Cl^--containing internal pipette solution was (mM): potassium aspartate, 110; KCl, 20; KH_2PO_4, 1·0; $MgCl_2$, 1·0; K_2-ATP, 3·0; Hepes, 5·0; and EGTA, 5·0 (pH adjusted to 7·2 with KOH). The Cl^--free pipette solution contained (mM): potassium glutamate, 130; KH_2PO_4, 1·0; $MgSO_4$, 1·0; K_2-ATP, 3·0; Hepes, 5·0; and EGTA, 5·0 (pH adjusted to 7·2 with KOH).

Iso (0·001–10 μM), propranolol (1 μM), forskolin dissolved in ethanol (5–10 μM), dibutyryl cAMP (1–5 mM), and 9-AC (0·1 mM) dissolved in dimethyl sulphoxide (DMSO) were added to the external solution when and as required from stock solutions immediately before use. Acetylcholine (ACh; 0·01–10 μM) was added to the external solution for some experiments. The synthetic peptide inhibitor of PKA, PKI(5–24)amide, was prepared as described previously (Cheng *et al.* 1986) and added to the pipette solution at a concentration of 50 μM when needed. Pertussis toxin (PTX) was dissolved in KB solution at a final concentration of 5 μg ml^{-1} with albumin (3 mg ml^{-1}); myocytes to be pretreated were incubated in this PTX solution for up to 90 min at 35 °C. Okadaic acid (OkA) was dissolved in DMSO and added (1 μM) to either the internal or external solution, also when needed. Rapid exchange of test solutions was accomplished by delivering a stream to the patched myocytes as a jet from the mouth of a fine-bore polyethylene tube positioned within 1 mm from the myocyte. Test solutions were perfused at a rate of 3–6 ml min^{-1}, and complete solution exchanges could be achieved within 20–40 s. Unless otherwise stated, the experimentally employed reagent chemicals and biologicals were obtained from Sigma Chemical Co.

Electrophysiological recordings

Whole-cell currents were recorded using the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The amplifier and head stage were designed by M. Yoshii (Narahashi, Tsunoo & Yoshii, 1987). The feedback resistance of the head stage was 100 MΩ. Electrodes were fabricated from 1·0 mm o.d. glass capillary tubes (Kimax-51; Kimble Products, Toledo, OH, USA), using a programmable horizontal micropipette puller (Flaming/Brown, Model P-87; Sutter Instrument Co., San

Rafael, CA, USA). Electrodes filled with internal solution had tip resistances of 1·5–2·5 MΩ.

The series resistance attributed to the pipette tip and the cell interior was compensated by approximately 85% to minimize the time course of the capacitative surge by summing a fraction of the converted current signal to the command potential and feeding it back to the positive input of the operational amplifier. The capacitative transient that remained after the series resistance had been compensated to the maximal extent possible without causing ringing was constant throughout the course of the experiments. The output of the voltage-clamp amplifier was adjusted to give zero current when the tip of the patch pipette (filled with internal solution) was immersed in the bath containing control external solution, the bath being connected to virtual earth by a 3 M KCl–agar bridge inserted into a 3 M KCl–AgCl–Ag junction. This caused a voltage bias of +7 ± 2 mV (mean ± S.D., $n = 30$) positive to the voltage measured in a earthed puddle of internal solution due to the liquid junction potential that occurs between the internal and external solutions; this voltage bias is not accounted for in the reported data. Therefore, the voltages measured during voltage clamps and then used to plot current–voltage relationships (I–V curves) are 7 ± 2 mV positive to the 'true' transmembrane voltage because the biasing liquid junction does not exist after intracellular access and an effective dialysis is achieved (Sheets & Ten Eick, 1994).

After gaining access in the whole-cell patch-clamp configuration, myocytes and junction potentials were allowed to 'stabilize' electrophysiologically for 10–15 min before collecting data. Following this period, 300 ms duration voltage-clamp steps, ranging typically between −120 and +40 mV, were applied from a holding potential (V_h) of −40 mV. The whole-cell membrane currents were filtered at 10 kHz with a two-pole active filter, digitized at a sampling rate of 40 kHz, and stored on the Winchester drive of an LSI 11/73 computer (Digital Equipment Corp., Maynard, MA, USA) for subsequent analysis. All data were collected at room temperature (20–22 °C).

Data analysis and statistics

The steady-state currents (I_{ss}) elicited during voltage-clamp steps applied in 10 mV increments from a V_h of −40 mV to voltages ranging between −120 and +40 mV for 300 ms were used to construct current–voltage (I–V) curves. I_{ss} was taken as the current measured at the end of the 300 ms pulses and normalized to the cellular membrane capacity. The I_{ss}–V curves were then used to estimate the value of the slope conductance for the inward rectifier (g_{K1}) to determine if selected experimental interventions had changed g_{K1}. Slope conductances for the voltage range between approximately 10 and 40 mV negative to the reversal potential (V_{rev}) for I_{ss} were calculated from least-squares linear regressions fitted to the data defining the capacity-normalized I_{ss}–V curves. For example, in Fig. 1 the values of I_{ss} for voltages between (and including) −80 and − 120 mV were used to calculate g_{K1} for each of the experimental conditions represented in the figure. For all cases reported, the mean I_{ss}–V curves were linear in this range of voltages, exhibiting coefficients of linearity (r) of $> 0{\cdot}98 \pm < 0{\cdot}016$. The assumption that the Iso-induced change in the thus measured slope conductance during our experiments reflects change in g_{K1} will be assessed subsequently in the Results section.

The Iso and ACh concentration–response curves were fitted to the equation: $Y = 1/\{1 + ([M]^n/K_D)\}$, where Y is the relative inhibition of g_{K1}, [M] is the concentration of Iso, n is the Hill coefficient, and K_D is the apparent dissociation constant.

K^+ equilibrium potentials (E_K) for 22 °C were calculated using the Nernst equation. Results are expressed as means ± S.E.M. Statistical analyses were performed using Student's t test or one-way analysis of variance as appropriate; $P < 0{\cdot}05$ was considered statistically significant.

RESULTS

β-Adrenergic inhibition of whole-cell I_{ss}

When the solutions perfusing the bath chamber and filling the patch electrode were Cl^- free, bath-applied Iso (1 μM) caused a mean decrease in steady-state whole-cell conductance (g_{ss}) of 40 ± 4·1% ($n = 13$) at voltages negative to E_K. Figure 1 shows families of currents obtained from a myocyte before (Fig. 1*A*) and during (Fig. 1*B*) exposure to 1 μM Iso, and ~10 min after Iso had been washed out of the bath chamber (Fig. 1*C*). In each family, hyperpolarizing steps negative to −80 mV elicited inward currents with rapidly activating transients and steps positive to −70 mV evoked outward currents. The families of currents yielded I–V curves (Fig. 1*D*) demonstrating that bath (i.e. external) application of Iso decreased I_{ss} without changing V_{rev} which, being ~−74 mV, was approximately 7 mV positive to E_K (i.e. ~−81 mV), a difference approximately equal to the zero offset potential of +7 ± 2 mV (see Methods). Although Iso at least partially inhibited I_{ss} at all voltages within the range routinely scanned (i.e. −120 to +40 mV), the inhibition was most prominent at voltages negative to the reversal potential in the linear portion of the I–V curve where I_{ss} should overwhelmingly reflect I_{K1} during Cl^--free conditions. Iso-sensitive current (i.e. the current obtained during control conditions minus the current obtained in the presence of Iso) could also be detected at voltages positive to E_K (see inset, Fig. 1*D*). As suggested above, the I–V curve derived from the family of Iso-sensitive currents (not shown) resembled a typical I_{K1}–V curve showing strong inward rectification positive to E_K.

An analysis of the mean ($n = 5$) I_{ss}–V curves (at voltages negative to V_{rev}) obtained during the control condition and in the presence of 1 μM Iso (Fig. 1*D*) reveals that Iso reduced the mean g_{K1} by ~44% from 139 ± 5·4 nS during control to 78 ± 2·9 nS when Iso was present; g_{K1} recovered almost completely from the Iso-induced inhibition during an ~10 min washout period (137 ± 5·3 nS), the difference between g_{K1} during the control and washout periods not being significant ($P > 0{\cdot}5$). However, the differences between the value of g_{K1} obtained in the presence of Iso and those obtained during both the control and washout periods were highly significant ($P < 0{\cdot}001$).

In experiments which were identical to those illustrated in Fig. 1 except that Cl^--containing internal and external solutions with either Co^{2+} or 9-AC added to the external

solution were used, bath-applied Iso (1 μM) again reversibly decreased I_{ss} by 30 ± 2% ($n = 17$; data not shown). Analysis of the mean I_{ss}–V curves to assess the slope conductances at voltages negative to V_{rev} during control, in the presence of Iso and after washout of Iso, gave values of 131 ± 5·6, 86 ± 3·2 and 128 ± 5·1 nS, respectively. Mean slope conductances ($n = 6$) for control and washout were not statistically different ($P > 0·5$) but that obtained in the presence of Iso was statistically different from the values for both the control and washout periods ($P < 0·001$).

Iso-sensitive I_{ss} is Iso-sensitive I_{K1}

The Iso-induced decrease in g_{K1} was unaffected when the Na^+–K^+ pump was inhibited by the presence of 300 μM DHO in the bath solution. Mean g_{K1} in Cl^--free external solutions during control was 141 ± 5·6 nS; during exposure to Iso it was 87 ± 3·8 nS in the absence of DHO and 88 ± 3·9 nS in the presence of DHO ($n = 5$), indicating that the DHO-sensitive, ATP-dependent Na^+–K^+ pump has no significant role in the Iso-induced change in the I_{ss}–V curve. If the Iso-induced change in the slope conductance of I_{ss} negative to E_K is the reflection of an Iso-sensitive I_{K1}, addition of Ba^{2+} (1 mM) to a Cl^--free external solution to block I_{K1} should eliminate the effect of Iso in altering the slope conductance. Indeed, in the presence of Ba^{2+} the effect of Iso on the slope conductance of I_{ss} was abolished (not shown) and the Ba^{2+}-sensitive currents (not shown) were indistinguishable from Iso-sensitive currents such as those shown in the inset of Fig. 1*D*. These findings suggest that the observed change in the slope conductance of I_{ss} is most probably the reflection of an Iso-sensitive component of g_{K1}.

To obtain support for the idea that the Iso-sensitive I_{ss} was an Iso-sensitive component of I_{K1} even in the presence of Cl^--containing solutions, several characteristics of the Iso-sensitive I_{ss} were examined to determine if they resembled

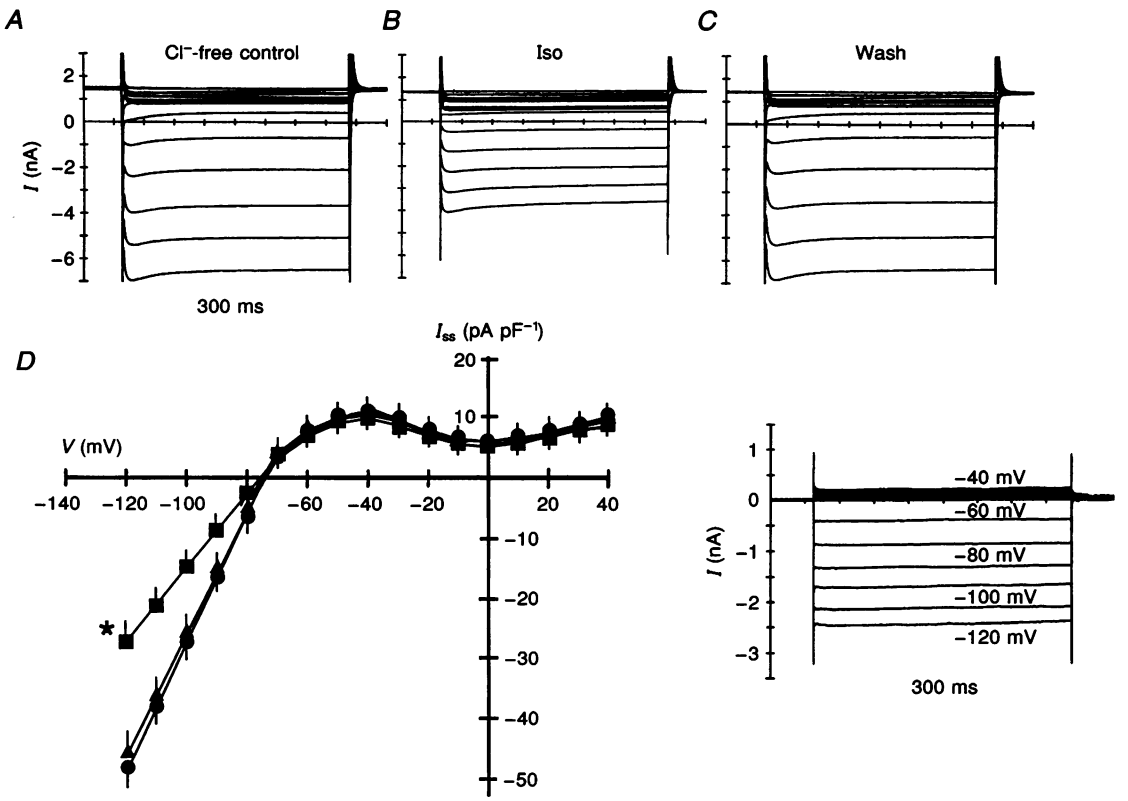

Figure 1. Isoprenaline inhibits whole-cell I_{K1} in chloride-free solutions

A, a representative family of control whole-cell I_{K1} currents recorded in Cl^--free solutions from a guinea-pig myocyte. Voltage clamps from a holding potential of −40 mV to test potentials ranging from −120 to +40 mV in 10 mV steps were 300 ms in duration. *B*, a family of I_{K1} currents recorded after exposure to 1 μM Iso for 10 min (same whole-cell patch as *A*). *C*, a family of I_{K1} currents recorded after washing Iso from the bath for 10 min (same patch as *A*). *D*, steady-state current–voltage (I_{ss}–V) relationships for I_{K1} obtained during control (●), during exposure to Iso (■) and after washout of Iso (▲). Current amplitudes are normalized to cell capacitance. Vertical bars indicate the standard error ($n = 5$). * Slope conductance is significantly different ($P < 0·05$) from the control value. The inset at the right shows a representative family of Iso-sensitive currents and indicates that Iso inhibits both inward and outward I_{ss}. Except for the current recorded at −40 mV, the voltages used to obtain the currents are given below the traces; that for −40 mV, the most outward trace, is given above. Temperature, 22 °C.

those expected for I_{K1}. Figure 2*A* (inset) shows a family of Iso-sensitive currents obtained in external solutions containing 20 mM K^+ and the mean *I–V* curves obtained from myocytes exposed to 2·0, 5·4 and 20 mM external K^+ concentration ($[K^+]_o$). In each $[K^+]_o$, the *I–V* curve for the Iso-sensitive current qualitatively resembles the I_{ss}–*V* curves depicted in Fig. 1. When $[K^+]_o$ increased from 2·0 to 5·4 and then to 20 mM, g_{K1} increased from 13·9 ± 0·9 to 29·5 ± 1·4 and 58·9 ± 3·4 nS, respectively, and the $\log_{10}$ of the slope conductances for the Iso-sensitive *I–V* curves in the voltage range negative to the approximate E_K value were linearly proportional ($r = 0{\cdot}998$) to the $\log_{10}$ of the $[K^+]_o$, with a slope of 0·63. Reversal potential shifted linearly with the $\log_{10}$ of the $[K^+]_o$ (Fig. 2*B*), changing ~58 mV for a tenfold change in external K^+ concentration. The mean reversal potentials of the Iso-sensitive current were approximately −40, −71 and −97 mV when the $[K^+]_o$ was 20, 5·4 and 2·0 mM respectively, values which, when the +7 ± 2 mV bias in the zero voltage level is accounted for (see Methods), are within ~3 mV of the calculated values for E_K (i.e. ~−49, −82 and −107 mV, respectively). Because the change in reversal potential calculated with the Nernst equation for room temperature is 58·0 mV, these findings suggest that the Iso-sensitive current was highly selective for K^+. These findings are consistent with the hypothesis that the Iso-sensitive I_{ss} is a component of I_{K1} and support the idea that the Iso-sensitive I_{ss} was dominated by I_{K1} even when the experiments were done in Cl^--containing solutions. Iso-sensitive current was observed at a membrane voltage (V_m) positive to E_K when $[K^+]_o$ was 20 mM (inset, Fig. 2*A*), confirming the finding in 5·4 mM $[K^+]_o$ (inset, Fig. 1*D*) that the Iso-induced decrease in g_{K1} occurs at voltages thought to be relevant to normal cellular electrophysiological function.

Iso-induced inhibition of I_{K1} involves β-adrenoreceptors

To confirm that inhibition of I_{K1} by Iso is mediated by stimulation of β-adrenoreceptors, the effect of the β-blocker propranolol on the Iso-induced inhibition of g_{K1} was

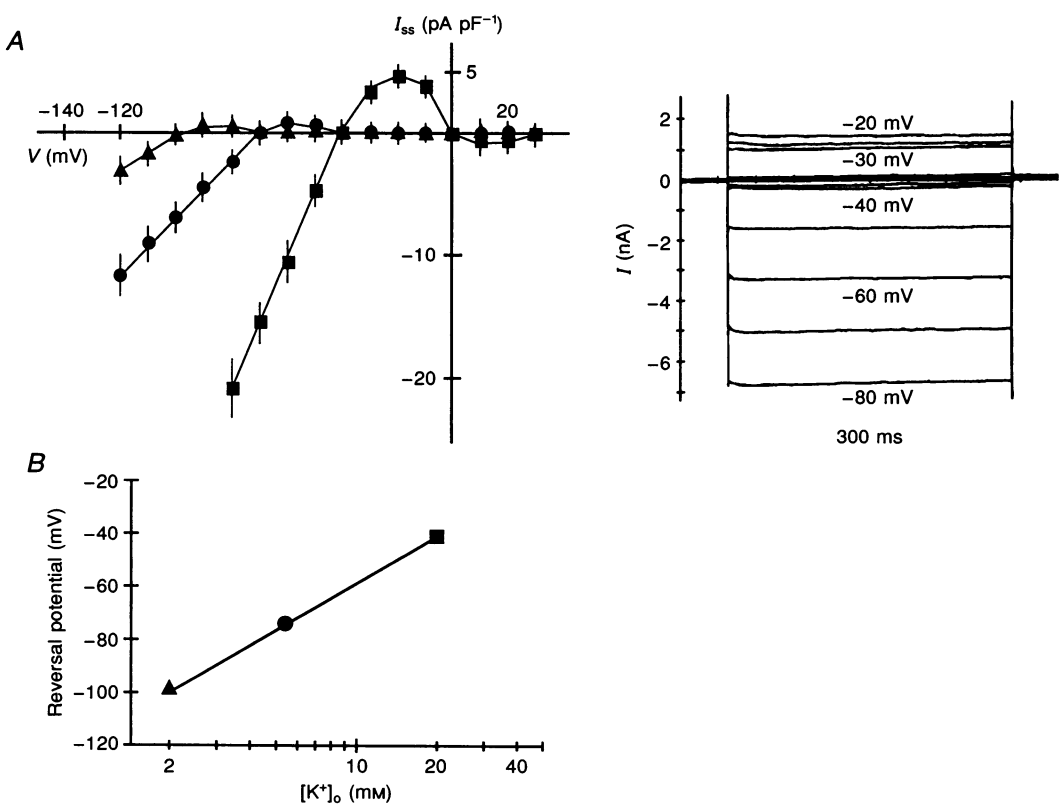

Figure 2. Effect of external K^+ concentration on isoprenaline-sensitive current

A, mean steady-state normalized *I–V* relationships of Iso-sensitive currents obtained by subtracting the current in the presence of Iso (1 μM) from the control when the external K^+ concentrations ($[K^+]_o$) were 2·0 (▲, $n = 3$), 5·4 (●, $n = 6$) and 20 mM (■, $n = 3$). Data plotted as means ± S.E.M. (Cl^- present, 2 mM $CoCl_2$ in the external solutions). The inset shows an example family of Iso-sensitive currents obtained in $[K^+]_o$ of 20 mM demonstrating that Iso-sensitive I_{ss} was observed positive to E_K (voltages are given below the traces except for −20 mV, which is given above). *B*, the reversal potentials of Iso-sensitive current (from *A*) plotted semilogarithmically as a function of the $[K^+]_o$ (symbols the same as for *A*); reversal potentials (uncorrected for the +7 ± 2 mV junction potential between the internal and external solutions, see Methods) were approximately −97, −71 and −40 mV in 2·0, 5·4 and 20 mM $[K^+]_o$, respectively.

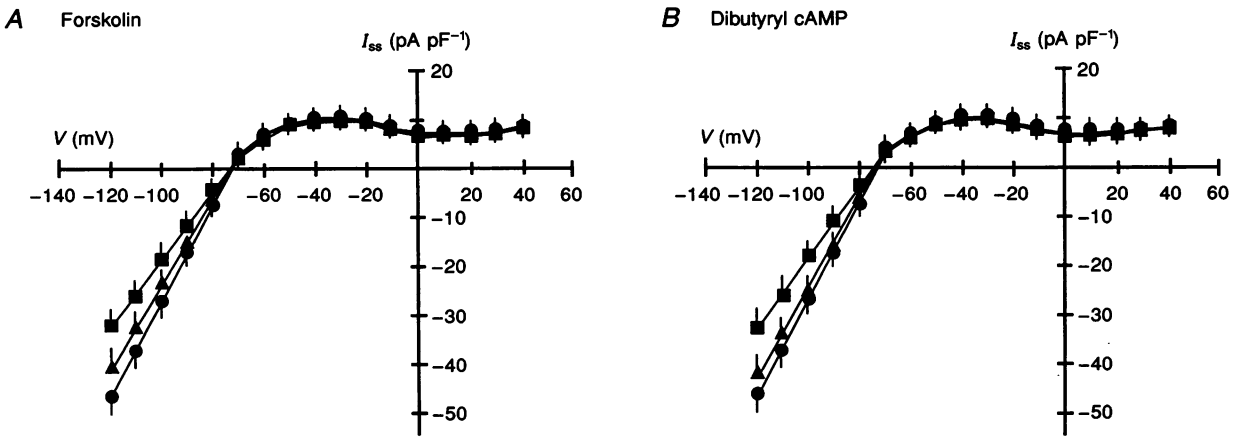

Figure 3. The effect of forskolin and dibutyryl cAMP on I_{K1}

A, normalized steady-state *I–V* relationships for I_{K1} obtained during the control period (●), during exposure to forskolin (10 μM, ■) and after washout (▲) recorded in normal Tyrode solution containing 2 mM $CoCl_2$. Each *I–V* curve was averaged from five different cells and the current magnitude during exposure to forskolin was significantly different from those from the two others ($P < 0{\cdot}05$). *B*, normalized steady-state *I–V* relationships for I_{K1} obtained during the control period (●), during exposure to dibutyryl cAMP (5 mM, ■) and after washout (▲) recorded in normal Tyrode solution containing 2 mM $CoCl_2$. Each *I–V* curve was averaged from five different cells and the current magnitude during exposure to dibutyryl cAMP was significantly different from those from the other two curves ($P < 0{\cdot}05$). Data plotted as means ± S.E.M.

characterized in Cl^--containing external solutions. After Iso had decreased mean g_{K1} from 136 ± 7·1 to 92 ± 6·0 nS, the addition of propranolol (1 μM) to the solution that already contained Iso returned g_{K1} to 127 ± 7·1 nS, a value that was not significantly different from control ($P > 0{\cdot}6$, $n = 3$) but was different from that in Iso alone ($P < 0{\cdot}001$). In addition, after propranolol (1 μM) had been present in the bath perfusate for 5 min, subsequent exposure to Iso (1 μM) did not inhibit I_{K1}, as the *I–V* curves and values of g_{K1} obtained in the presence and absence of Iso were virtually identical ($n = 4$; data not shown). These results indicate that Iso inhibits I_{K1} by a

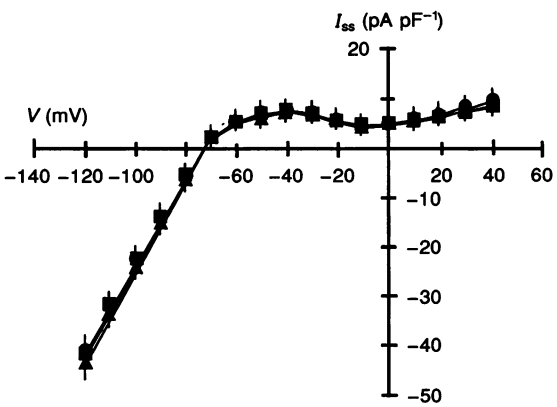

Figure 4. Inhibiting cAMP-dependent protein kinase abolished isoprenaline and forskolin-induced inhibition of I_{K1}

Effects of Iso and forskolin on the mean (± S.E.M.; $n = 5$) normalized steady-state *I–V* relationships derived from families of whole-cell I_{K1} recorded in Cl^--containing solutions when 2 mM $CoCl_2$ was included in the external solution and the synthetic peptide inhibitor PKI (50 μM) was present in the patch pipette (internal) solution. Controls (●) were recorded 15 min after gaining intracellular access to allow PKI to diffuse into the cell. Currents recorded when PKI was present in the patch pipette exhibited time- and voltage-dependent properties that were similar to those recorded in the absence of PKI. The *I–V* curves for currents obtained after exposure to Iso (1 μM, ■) in the bath for 10 min and for currents recorded after washing Iso out and then exposing the cell to forskolin (10 μM, ▲) in the bath for 10 min were not statistically different from the control or from one another ($P > 0{\cdot}9$).

mechanism directly involving β-adrenoreceptors. They also indicate that propranolol itself had no detectable effect on I_{K1}.

Effects of cyclic AMP and forskolin

To address the question 'does the inhibitory effect of Iso on I_{K1} involve activation of adenylyl cyclase and a consequent change in intracellular cAMP level?', the effect of forskolin (dissolved in ethanol) on whole-cell g_{K1} was examined using Cl^--containing internal and external solutions. Although ethanol alone at its final solvent concentration had no effect on I_{K1}, forskolin (10 μM) added to the external solution decreased g_{K1} significantly from 133 ± 5·8 to 92 ± 3·9 nS ($P < 0·01$, $n = 5$; see Fig. 3*A*). Upon washout for ~20 min, g_{K1} returned to 128 ± 5·5 nS, a value that was different from that in the presence of forskolin ($P < 0·02$) but not different from the control ($P > 0·5$). The forskolin-induced decrease to ~69% of the control was similar to that caused by 1 μM Iso (i.e. to ~66% of control).

Bath application of a membrane permeable surrogate for cAMP, dibutyryl cAMP (5 mM, a concentration which can increase cardiac I_{Ca} maximally (Kameyama, Hofmann & Trautwein, 1985)) also reversibly reduced whole-cell g_{K1} significantly from 132 ± 5·4 to 95 ± 3·9 nS ($P < 0·01$, $n = 5$) or to ~63% of control (see Fig. 3*B*). Additionally, after maximally inhibiting I_{K1} with either 10 μM forskolin or 5 mM dibutyryl cAMP, 1 μM Iso failed to suppress I_{K1} further ($n = 3$; data not shown), suggesting that the β-adrenoreceptor-coupled G protein, G_s, is not directly coupled to I_{K1} channels or operating via a cAMP-independent pathway.

Effect of inhibiting protein kinase A

To investigate whether PKA was involved in the decrease in g_{K1} induced by the β-agonist, the effects of Iso, forskolin and dibutyryl cAMP on I_{K1} were examined after PKA was selectively inhibited with PKI, applied internally via the pipette solution (Cheng *et al.* 1986). Figure 4 shows that, when PKI (50 μM) was present in the pipette solution during experiments performed in Cl^--containing solutions, bath application of either 1 μM Iso or 10 μM forskolin did not reduce g_{K1}, which was 121 ± 6·5 nS during control, 122 ± 6·1 nS in the presence of Iso ($n = 5$) and 124 ± 6·6 nS in the presence of forskolin ($n = 5$). In further support of this notion, bath-applied dibutyryl cAMP (5 mM) also had no detectable effect on g_{K1} when PKI was present internally ($n = 3$; data not shown). The finding that I_{K1} was not inhibited by Iso when PKA had been inhibited by PKI is

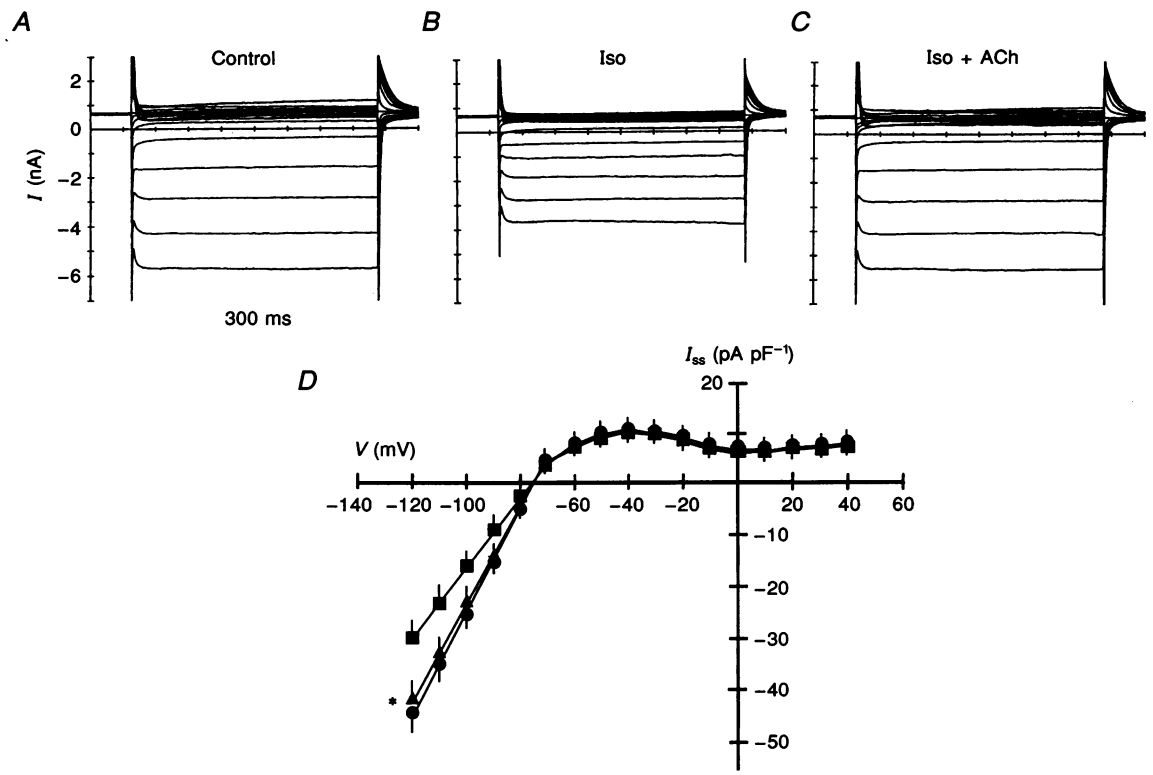

Figure 5. ACh antagonized the effect of isoprenaline on I_{K1}

A, a representative family of control whole-cell I_{K1} currents recorded in Cl^--free solutions. *B*, a family of I_{K1} currents recorded during exposure to 1 μM Iso for 10 min (same cell as *A*). *C*, a family of I_{K1} currents recorded after addition of ACh (10 μM) in the continued presence of Iso (same cell as *A*). *D*, mean (± S.E.M.; $n = 5$) normalized steady-state *I–V* relationships for I_{K1} obtained during control conditions (●), during exposure to 1 μM Iso (■) and subsequent exposure to 10 μM ACh in the presence of Iso (▲). * $P < 0·05$ for Iso *vs.* Iso + ACh.

also consistent with the idea that the β-adrenoreceptor-coupled G_s is not directly coupled to I_{K1} channels or operating via a PKA-independent pathway.

Acetylcholine antagonism of Iso-induced I_{K1} inhibition

Generally, ACh can antagonize cAMP-mediated cardiac effects of β-agonists. Therefore, we addressed the question of whether ACh could antagonize the Iso-induced inhibition of whole-cell I_{K1}. During experiments in which Cl^--free solutions were used (Fig. 5; $n = 5$), when ACh (10 μM) was added to external solution that already contained Iso, the Iso-induced inhibition of g_{K1} (from 125 ± 5·6 nS during control (Fig. 5*A*) to 75 ± 3·4 nS with Iso present (Fig. 5*B*)) was 88 ± 4% antagonized, g_{K1} returning to 119 ± 5·2 nS (Fig. 5*C*). The mean *I–V* curves obtained during control and subsequent to the addition of ACh to the bath are almost superimposed (Fig. 5*D*), whereas g_{K1} determined in the presence of Iso (1 μM) alone was statistically different from the values obtained both during control and during exposure to Iso plus ACh ($P < 0·05$ or less, $n = 5$). Furthermore, when a maximally effective concentration of Iso (1 μM) was added to the bath perfusate in which ACh (10 μM) was already present, no Iso-induced change in g_{K1} could be detected ($n = 3$; data not shown). However, after ACh had been washed from the bath, Iso was again able to inhibit whole-cell I_{K1} ($n = 5$; not shown) indicating that this effect of ACh was reversible. When Cl^--containing solutions were used, ACh (10 μM) also reversed 76 ± 5% ($n = 4$; data not shown) of the Iso-induced decrease in the slope conductance of I_{ss} and ~75% of a forskolin (10 μM)-induced inhibition in a single experiment.

Concentration dependence of Iso-induced inhibition altered by ACh

The concentration dependence of the inhibitory effect of Iso on Iso-sensitive I_{K1} elicited by pulses from −40 to −120 mV was defined in Cl^--free external and internal solutions (Fig. 6*A*). Significant Iso-induced inhibition could be detected at an Iso concentration as low as 10 nM; maximal inhibition was obtained at ~1–10 μM. The half-maximal inhibition (apparent dissociation constant, K_D) occurred at 0·035 μM and the Hill coefficient was ~1. When the concentration dependence of the inhibition of Iso-sensitive I_{K1} was assessed in the presence of either partially (0·1 μM) or nearly completely antagonistic (10 μM) concentrations of ACh, the curves were altered so that the K_D for Iso was increased in an ACh concentration-dependent manner (see legend to Fig. 6) and the maximal efficacy of Iso to inhibit the Iso-sensitive I_{K1} was reduced, also in an ACh concentration-dependent manner (Fig. 6*A* and *B*). The half-maximal concentration of ACh for antagonizing the inhibition of I_{K1} produced by 1 μM Iso (i.e. the apparent IC_{50}) was ~110 nM and the maximal concentration was ~1–10 μM. However, even 10 μM ACh did not appear to prevent the inhibitory effect of 1 μM Iso completely, antagonizing only 86 ± 3% ($n = 5$) of the Iso-induced inhibition (Fig. 6*B*).

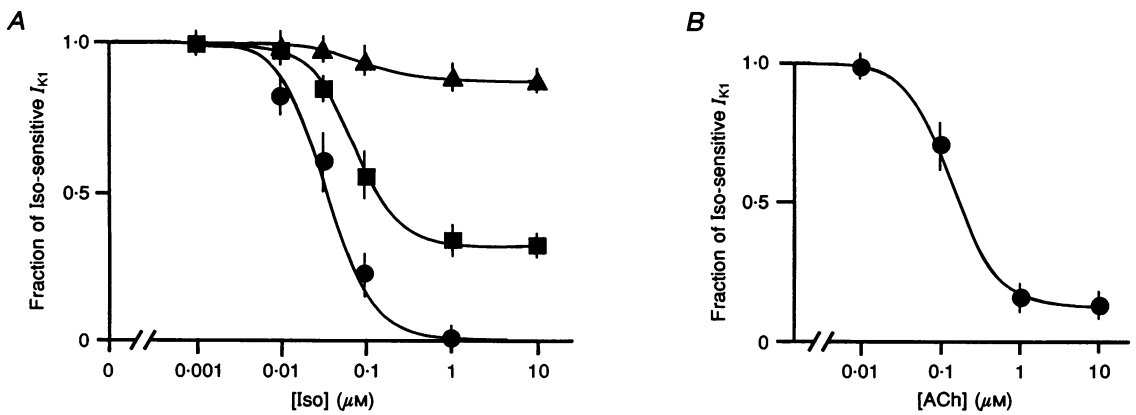

Figure 6. Concentration dependencies of the inhibition of whole-cell I_{K1} by isoprenaline and of its antagonism by ACh

A, the normalized Iso concentration dependence of the inhibition of the steady-state whole-cell I_{K1} current elicited with voltage clamps from a holding potential of −40 to −120 mV during Cl^--free conditions plotted as means ± S.E.M. ($n = 4–6$). The uninhibited fraction of the Iso-sensitive I_{K1} (obtained by normalizing to the maximally inhibited value) was plotted against Iso concentration. Analysis of the concentration dependence yielded an apparent dissociation constant (K_D) of 0·035 μM and a Hill coefficient of ~1 in the absence of ACh (control, ●). The K_D was 0·064 and 0·089 μM in the presence of 0·1 (■) and 10 μM (▲) ACh, respectively. The Hill coefficient was unchanged in the presence of ACh. *B*, mean (± S.E.M.; $n = 4–5$) normalized ACh concentration dependence of the antagonizing effect of ACh on the Iso-induced inhibition of the steady-state I_{K1} current in Cl^--free conditions. The fraction of the inhibited Iso-sensitive I_{K1} (normalized to the maximally inhibited value) was plotted against the ACh concentration (1 μM Iso present). The IC_{50} for ACh was 0·11 μM with a Hill coefficient of ~2.

Antagonism by ACh involves G_i but not adenylyl cyclase

The question of whether the effect of ACh in antagonizing the Iso-induced inhibition of I_{K1} involves the G protein, G_i, was addressed using myocytes pretreated with PTX. Iso-induced inhibition of whole-cell g_{K1} still occurred in PTX-pretreated myocytes, g_{K1} being reduced from 135 ± 6·0 nS during control to 91 ± 3·9 nS in Iso (1 μM, $n = 5$). Neither of these values for g_{K1} in PTX-treated myocytes were statistically different from those obtained during control or in the presence of Iso in untreated myocytes. However, 10 μM ACh did not even partially antagonize the Iso-induced inhibition in PTX-pretreated myocytes, g_{K1} remaining at 92 ± 4·5 nS when studied in either Cl^--free ($n = 3$) or Cl^--containing solutions ($n = 3$). The g_{K1} determined in each of these conditions was significantly less than that for the control (i.e. Iso absent; $P < 0·001$), suggesting that G_i is involved in the ACh-induced antagonism of the β-adrenergically mediated reduction in g_{K1}.

If the ACh-induced antagonism of the Iso-induced inhibition of I_{K1} results primarily from a G_i-mediated effect on adenylyl cyclase to reduce the production of cAMP ordinarily obtained in response to β-receptor activation, an inhibition of I_{K1} produced by bath exposure to dibutyryl cAMP, acting as a cAMP surrogate, should not be substantially altered when the myocyte is exposed to ACh. In contrast to this prediction, under Cl^--free conditions ACh (10 μM) was able to antagonize ~70% ($n = 3$) of the inhibition of g_{K1} produced by 1 mM dibutyryl cAMP (e.g. from 138 ± 8·2 nS during control to 91 ± 5·8 nS in dibutyryl cAMP; see Fig. 7), an amount which was slightly less than that obtained when the inhibition produced by bath-applied Iso (1 μM) was antagonized by ACh (10 μM; i.e. ~88%). Therefore these findings suggest that, although a portion of the antagonism may involve an ACh-induced effect of G_i on cyclase activity, the full extent of the antagonism of the Iso-induced inhibition of I_{K1} does not appear to be accounted for by this mechanism alone.

Okadaic acid prevents ACh-induced antagonism of Iso-induced inhibition

Recently, evidence suggesting that muscarinic cholinergic agonists can directly activate type 1 protein phosphatase in guinea-pig ventricular muscle has appeared (Ahmad, Green, Subuhi & Watanabe, 1989; Neumann, Gupta, Schmitz, Scholz, Nairn & Watanabe, 1991). As a test of the

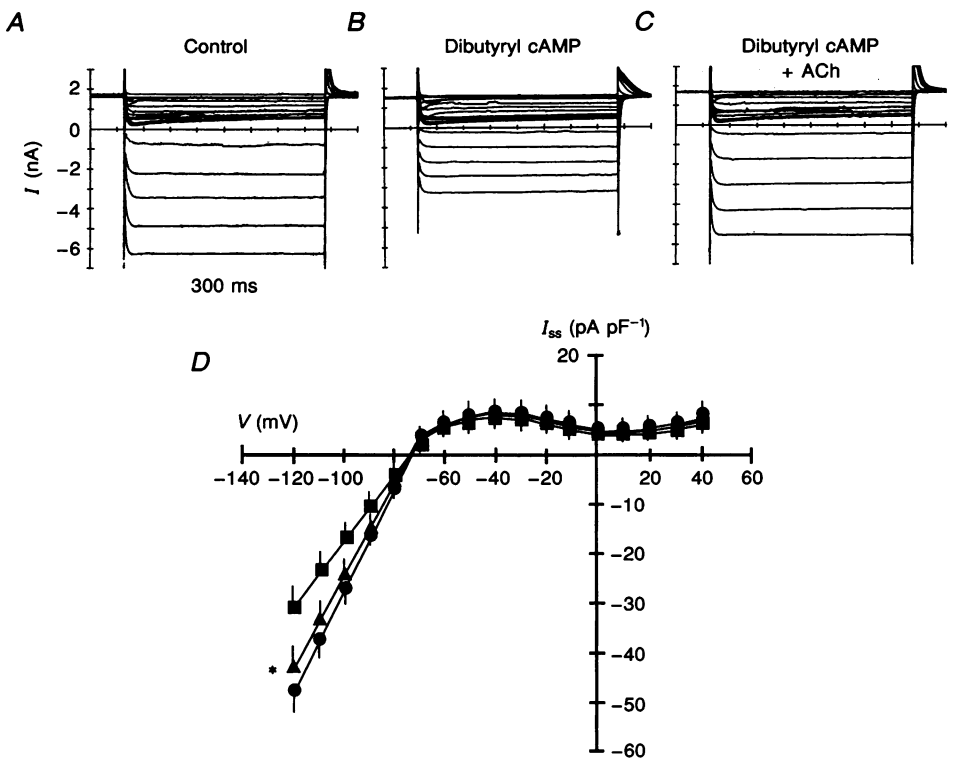

Figure 7. ACh antagonizes the inhibition of I_{K1} induced by dibutyryl cAMP

A, a representative family of control whole-cell I_{K1} currents recorded in Cl^--free solutions. *B*, a family of I_{K1} currents recorded during exposure to dibutyryl cAMP (1 mM) for 10 min (same whole-cell patch as *A*). *C*, a family of I_{K1} currents during exposure to ACh (10 μM) in the continued presence of dibutyryl cAMP (also same cell as *A*). *D*, mean (± S.E.M.; $n = 3$) normalized steady-state *I–V* relationships for I_{K1} obtained during control (●), during exposure to 1 mM dibutyryl cAMP (■) and subsequent exposure to ACh in the presence of dibutyryl cAMP (▲). * $P < 0·05$ for dibutyryl cAMP *vs.* dibutyryl cAMP + ACh.

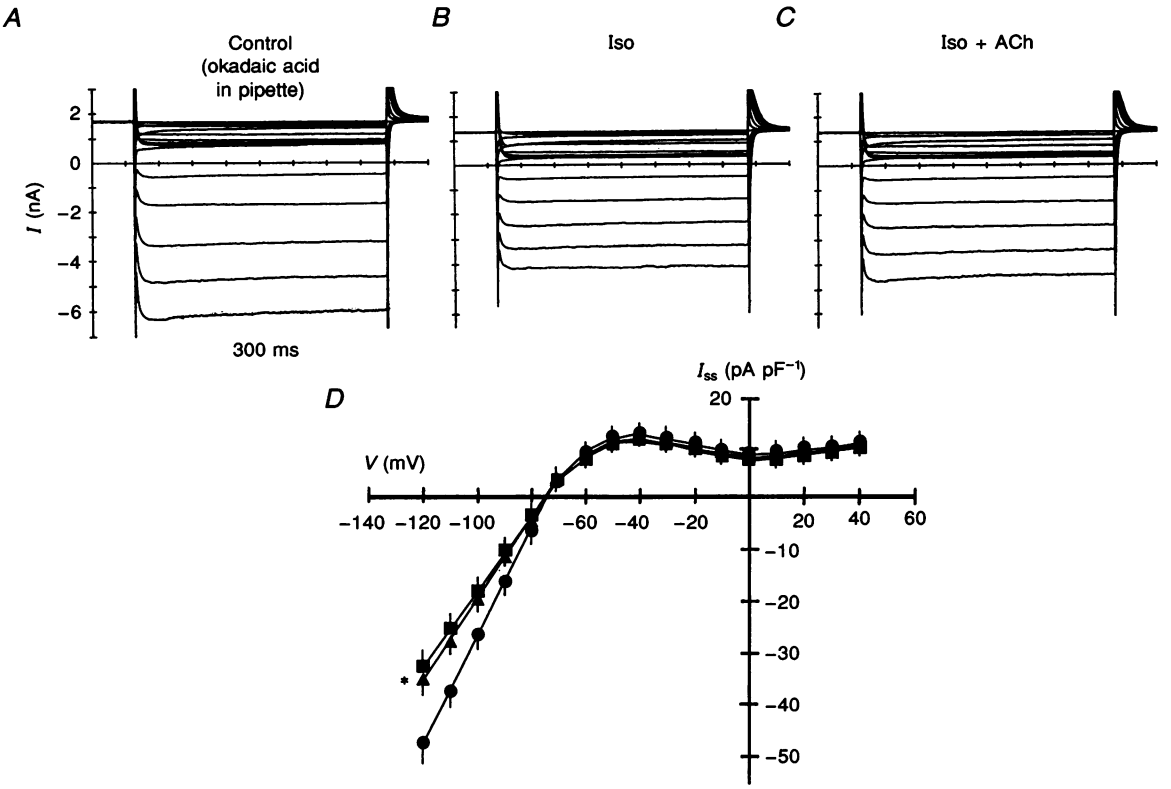

Figure 8. Okadaic acid prevents ACh-induced antagonism of the inhibition of I_{K1} induced by isoprenaline

A, a representative family of control whole-cell I_{K1} currents recorded in Cl^--free solutions with okadaic acid (1 μM) included in the pipette solution. *B*, a family of I_{K1} currents recorded during exposure to 1 μM Iso for 10 min (same whole-cell patch as *A*). *C*, a family of I_{K1} currents recorded during exposure to ACh (10 μM) in the continued presence of Iso (also same cell as *A*). *D*, mean ($\pm$ S.E.M.; $n = 5$) normalized steady-state *I–V* relationships for I_{K1} obtained during control (●), during exposure to 1 μM Iso (■) and subsequent exposure to 10 μM ACh in the presence of Iso (▲). * $P < 0{\cdot}05$ for Iso *vs.* control.

hypothesis that ACh antagonizes the effects of Iso on I_{K1} through an action involving protein phosphatase, the effect of OkA, a potent inhibitor of protein phosphatase 1 and 2A (Bialojan & Takai, 1988), was examined to determine if OkA could diminish or abolish the ACh-induced antagonism of the Iso-induced inhibition of I_{K1}. Figure 8 shows families of currents recorded in Cl^--free solutions with OkA (1 μM) added to the internal solution prior to (i.e. control; Fig. 8*A*) and during bath exposure to Iso (1 μM; Fig. 8*B*) and after addition of ACh (10 μM; Fig. 8*C*) to the bath solution already containing Iso. OkA decreased the extent to which ACh antagonized the Iso-induced inhibition. There were no statistically significant differences between the g_{K1} in the presence of Iso alone (95 $\pm$ 4·4 nS) and in the presence of Iso and 10 μM ACh (97 $\pm$ 4·5 nS) when OkA was present, indicating that OkA had significantly diminished the antagonism by bath-applied ACh of the Iso-induced inhibition of g_{K1}.

Neither the control g_{K1} nor the Iso-induced inhibition of g_{K1} were enhanced in the presence of OkA internally. In fact, the decrease in g_{K1} caused by Iso (1 μM) was significantly greater ($P < 0{\cdot}01$) in the absence of OkA (54·0 $\pm$ 2·2 nS; $n = 10$) than in the presence of OkA internally (42·1 $\pm$ 2·4 nS; $n = 5$), despite there being no difference in the control values for g_{K1} obtained in the absence (134 $\pm$ 5·1 nS) or presence (136 $\pm$ 6·1 nS) of OkA. These findings are in contrast to the report of Hescheler, Mieskes, Ruegg, Takai & Trautwein (1988) showing that OkA enhanced the effects of Iso on guinea-pig L-type I_{Ca} and the delayed rectifying K^+ current.

DISCUSSION

Our purpose was to determine if g_{K1} could be modified by agonists mimicking autonomic neurotransmitters. Using Iso and ACh to activate β-adrenergic and M_2-cholinergic receptors, the results indicate that Iso and its intracellular surrogates can decrease g_{K1} and that this effect can be antagonized by ACh. The findings suggest that ACh modulates channel dephosphorylation by a mechanism involving an OkA-inhibitable protein phosphatase in addition to its effect on adenylyl cyclase to inhibit cAMP-dependent, PKA-mediated phosphorylation of inwardly

rectifying K^+ channels caused by β-receptor activation. The idea that protein phosphatase has an important role in governing the balance between cardiac ion channels in a phosphorylated state and those in a dephosphorylated state is not new, having been suggested by Hescheler *et al.* (1988) based on their findings on L-type Ca^{2+} current and the delayed rectifying K^+ current in the guinea-pig using OkA.

Iso-sensitive current composed predominantly of I_{K1}

Although the experimental solutions and conditions used in the present study were selected specifically to minimize contributions from other current systems, we considered it important to demonstrate that the component of the whole-cell membrane current principally affected by Iso and ACh was in fact I_{K1}. The characteristics of the Iso-sensitive I_{ss} in both Cl^--free and Cl^--containing solutions were found to resemble the signature characteristics of I_{K1} closely. The time courses of the Iso-sensitive currents and the inwardly rectifying shape of the I–V curve emulated that expected for guinea-pig Ba^{2+}-sensitive I_{K1}. The relationship between the log of its slope conductance and the log of $[K^+]_o$ was linear, with a slope of 0·63. The reversal potentials were within 3–4 mV of E_K and tracked E_K at essentially the theoretical rate of 58 mV for a tenfold change in $[K^+]_o$. Thus the weight of evidence, including the results from our single channel studies on inwardly rectifying K^+ channels (Koumi, Wasserstrom & Ten Eick, 1995), support the conclusions that Iso-sensitive I_{ss} was composed predominantly of I_{K1} and that Iso can inhibit g_{K1}.

It was necessary to block at least part of the Iso-activated, cAMP-dependent Cl^- current (Harvey & Hume, 1989; Bahinski *et al.* 1989) to observe the Iso-induced inhibition of g_{K1}. Although the extent to which I_{Cl} had been inhibited when Cl^--containing solutions were used was not determined, it appears that a substantial portion of the cAMP-dependent g_{Cl} must have been blocked or that initially g_{Cl} must have been rather small compared to g_{K1} because (a) the values of V_{rev} for the I–V curves obtained in Cl^--containing solutions were not different from those obtained when Cl^--free solutions were used, and (b) the change in V_{rev} was ~58 mV per tenfold change in $[K^+]_o$ in Cl^--containing solutions, as predicted by the Nernst equation for a pure K^+ current at 22 °C.

Iso-induced inhibition involves G_s-linked, cAMP-dependent, PKA-mediated channel phosphorylation

Iso inhibited whole-cell I_{K1} in a concentration-dependent manner with the concentration for half-maximal inhibition being 0·035 μM; the Hill coefficient was ~1·0. This value for the K_D is similar to that for the half-maximal concentration for the Iso-induced enhancement of I_{Ca} (0·038 μM from Kameyama *et al.* 1985) and to that for the effect of Iso on the delayed rectifier K^+ current (0·018 μM from Yazawa & Kameyama, 1990). The notion that Iso also inhibits g_{K1} by a process involving an increase in cAMP is consistent with reports that cytosolic cAMP in guinea-pig and rat ventricular myocytes increases during exposure to Iso with a half-maximal concentration of 0·01–0·1 μM (Watanabe & Besch, 1975; Powell & Twist, 1976). Although we have not studied the involved second messenger signalling pathway in detail, it seems likely that the classical G_s-linked, cAMP-dependent, PKA-mediated pathway is involved. We were able to mimic the Iso-induced inhibition with forskolin and dibutyryl cAMP and to prevent it, regardless of whether Iso or a surrogate agonist had produced it, by adding PKI to the internal solution. These results are those predicted if the inhibition involves phosphorylating channels conducting inwardly rectifying K^+ current (i_{K1}) via the classical β-agonist-activated, intracellular second messenger system governing PKA activity. Because neither Iso, forskolin nor cAMP inhibited g_{K1} when PKI had been added to the internal solution, it is unlikely that the inhibition significantly involved either a β-receptor-coupled G_s directly linked to i_{K1} channels or a cAMP- and PKA-independent pathway.

Basis for the interaction between Iso and ACh

In 1975, Watanabe & Besch developed the idea that ACh antagonized the cardiac effects of β-adrenergic agonists via an action which inhibited their effect to activate adenylyl cyclase, and thereby inhibited the extent to which Iso can increase intracellular [cAMP] (Watanabe & Besch, 1975). This early view of the intracellularly located pathway involving adenylyl cyclase has been extended; G_i, having been activated via ACh-stimulated M_2-cholinergic receptors, is said to inhibit the G_s-mediated activation of adenylyl cyclase to produce the ACh-induced antagonism of the effects of β-agonists (Katada, Bokoch, Smigel, Ui & Gilman, 1984; Gilman, 1987 for review). In the present study, the ACh-induced antagonism was PTX sensitive suggesting that G_i is indeed involved in the antagonism. However, the finding that the control g_{K1} and the extent of the Iso-induced inhibition of g_{K1} were not significantly altered in PTX-treated myocytes (compared with untreated myocytes) suggests that, ordinarily, the basal level of g_{K1} is not significantly influenced by G_i-mediated mechanisms. Consistent with this idea is the finding that the basal (control) g_{K1} was also unaffected by the presence or absence of OkA.

The present results suggest that, in addition to the effect of ACh on cyclase activity, at least one other PTX-sensitive mechanism has an important role in the ACh-induced antagonism of the Iso-induced inhibition of g_{K1}. If the antagonism involved solely a G_i-mediated modulation of the extent to which G_s can activate adenylyl cyclase, the inhibition of g_{K1} by the dibutyryl surrogate for cAMP should have been essentially insensitive to ACh. In fact, more than two-thirds of the dibutyryl cAMP-induced inhibition was antagonized by ACh (Fig. 7). The finding that ACh applied directly to a cell-attached patch antagonized the Iso-induced decrease in the open probability of the i_{K1} channels in the patch much more effectively than did bath-

applied ACh (Koumi *et al* 1995) also supports this idea. It is difficult to reconcile the findings on single i_{K1} channels recorded from cell-attached patches with a postulation that an ACh-induced inhibition of cyclase is the sole mechanism of the antagonism.

There are at least three conceivable ways in which ACh could antagonize dibutyryl cAMP-induced inhibition that could also operate to antagonize at least some of an Iso-induced inhibition. The first is by inhibiting the cAMP-dependent activation of PKA, the second is by directly inhibiting the PKA-mediated phosphorylation, and the third is by enhancing the dephosphorylation process. The finding that OkA prevented the ACh-induced antagonism irrespective of whether ACh was applied before or after Iso provides a clue regarding which possibility is the more likely candidate. If ACh directly inhibits either the activation of PKA or the phosphorylation event, inhibition of protein phosphatase should not be expected to affect the ACh-induced antagonism when ACh is applied before either Iso or dibutyryl cAMP. This is because, in the face of this putative ACh-induced inhibition, little phosphorylation would be expected to occur; when rather few channels are phosphorylated, inhibiting channel dephosphorylation can have little effect on the number of phosphorylated channels. In contrast, if Iso or dibutyryl cAMP were applied before ACh, a full-blown Iso or dibutyryl cAMP-induced channel phosphorylation would have already occurred prior to the ACh exposure. However, a fully phosphorylated state should not be maintained if either of these two postulated inhibitory effects of ACh operate because protein phosphatase would constantly dephosphorylate the phosphorylated channels. Therefore, it is only in the instance when ACh is applied after Iso that OkA should abolish the ACh-induced antagonism if the action of ACh involves inhibiting PKA-mediated phosphorylation. However, ACh was found to both prevent and reverse Iso and dibutyryl cAMP-induced inhibition of g_{K1}, and both effects of ACh were prevented by OkA. This finding can be explained if the effect of ACh involves enhancing some process associated with the OkA-sensitive dephosphorylation of channels, rather than inhibiting a process involved with phosphorylation.

The findings indicating that the ACh-induced antagonism was prevented by OkA are consistent with the idea that an OkA-inhibitable protein phosphatase is involved in the antagonism. OkA is said to inhibit both protein phosphatase 1 and 2A (Nishiwaki *et al.* 1990). Protein phosphatase 1 has been found in guinea-pig heart and it has recently been shown that it can be inhibited by a PKA-mediated mechanism involving phosphorylation of a phosphatase inhibitor-1 (Neumann *et al.* 1991). The latter finding has led to the suggestion that this decrease in protein phosphatase activity could result in an enhancement of other phosphorylation-dependent effects of Iso (Ahmad *et al.* 1989; Neumann *et al.* 1991); ACh antagonism could then involve a reversal or inhibition of the process that leads to the decrease in protein phosphatase activity. This would allow i_{K1} channels to again dephosphorylate at a normal rate and enable the normal balance between phosphorylated and dephosphorylated channels to be restored.

Such a mechanism is consistent with the finding that both the Iso- and dibutyryl cAMP-induced inhibition of g_{K1} were antagonized by an OkA-sensitive effect of ACh. Therefore, one reasonable explanation for the ACh-induced antagonism of the Iso-induced inhibition of g_{K1} is that it involves an M_2-cholinergic receptor-linked, PTX-sensitive G_i protein coupled to a pathway involving an OkA-sensitive protein phosphatase whose role is to dephosphorylate Iso-inhibited, phosphorylated i_{K1} channels. The results of our single channel studies (Koumi *et al.* 1995) support this idea and further suggest that the involved OkA-sensitive protein phosphatase molecules reside in close proximity to the channels they service.

Significance of protein phosphatase in modulating activity of phosphorylated channels

When the cAMP level rises in response to β-adrenoreceptor activation, many cellular processes are enhanced in addition to fostering conditions in which the number of sarcolemmal ion channels in a phosphorylated state would ordinarily be expected to increase. It is conceivable that having all cAMP-driven cellular processes always regulated in lock-step with the cAMP level is not in a cell's best interest. This raises the question, can the rates of phosphorylating and dephosphorylating ion channels be modulated at least semi-independently of other cAMP-driven processes so that the channels do not always respond slavishly to the intracellular cAMP level? For example, if a cellular need for enhanced glycolysis arises in a situation in which it would be detrimental to the heart as an organ or to the organism to phosphorylate i_{K1} channels (thus reducing g_{K1}), it would be advantageous if cells could regulate their protein phosphatase activity and increase the rate of channel dephosphorylation. Such a mechanism, being partially insulated from the demands on the myocyte for a high cellular level of cAMP, would allow the extent of channel phosphorylation to be modulated at least somewhat independently of the level of PKA activity. The finding that an ACh-induced, OkA-sensitive mechanism exists which can modulate the effects of a PKA-mediated phosphorylation of g_{K1} channels, suggests that the organism can exert a regulatory influence on the phosphorylation state of its cardiac ion channels somewhat independently of other cellular functions.

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