Contribution of ATP-sensitive potassium channels to the electrophysiological effects of adenosine in guinea-pig atrial cells

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1. Adenosine caused dose-dependent action potential abbreviation in multicellular guinea-pig atrial preparations, an action antagonized by glyburide (IC_{50}, 31 μM) in both physiological and low-chloride superfusate.

2. When 5 mM ATP was included in pipettes for whole-cell voltage clamp of isolated guinea-pig atrial myocytes, adenosine (10 μM) increased the holding current at −40 mV from 41 ± 8 to 246 ± 31 pA (mean ± S.E.M., $P < 0{\cdot}01$), and glyburide (20 μM) returned the holding current to 69 ± 11 pA ($P < 0{\cdot}01$ *vs.* adenosine alone). Acetylcholine (10 μM) also increased the holding current, but its effects were not altered by glyburide.

3. Both adenosine and acetylcholine induced an additional current component in response to 500 ms voltage steps. Glyburide partially inhibited the adenosine-induced current, but did not alter the effect of acetylcholine. In the presence of maximally effective acetylcholine concentrations, adenosine increased membrane conductance ($P < 0{\cdot}01$), although to a lesser extent than in the absence of acetylcholine.

4. Single K^+ channel activity was seen in only one of eight cell-attached patches in the absence of adenosine or acetylcholine (0·5 mM Ba^{2+} in bath and pipette solutions). With acetylcholine (10 μM) in the pipette, inwardly rectifying channels (conductance, 41 ± 5 pS) were seen in five of six patches. With adenosine (10 μM) in the pipette, single-channel activity was seen in twelve of fourteen patches with two populations of channels, one similar to that induced by acetylcholine and another higher-conductance channel (72 ± 5 pS) that showed less inward rectification. Glyburide (20 μM) suppressed the high-conductance channel (68 ± 2 pS) leaving a single channel type with a conductance of 36 ± 5 pS and strong inward rectification.

5. We conclude that K^+_{ATP} channels contribute to the electrophysiological actions of adenosine on guinea-pig atrium in the presence of physiological intracellular ATP levels, and may therefore play a role in the cardiac electrophysiological effects of adenosine in the absence of myocardial ischaemia.

It is well documented that adenosine can modulate a variety of cardiac cellular functions. Adenosine has a vasodilating action and may play a role in coronary blood flow regulation (Berne, 1963; Bardenheuer & Schrader, 1980), it also suppresses sinoatrial node automaticity and atrioventricular node conduction (Belardinelli, Fenton, West, Linden, Althaus & Berne, 1982; Belardinelli, Giles & West, 1988; Belardinelli, Linden & Berne, 1989), antagonizes the electrophysiological and biochemical effects of β-adrenoceptor stimulation, (Schrader, Baumann & Gerlach, 1977; Dobbson, 1978; Belardinelli & Isenberg, 1983*a*) and may protect against ischaemia-induced cell injury (Ely, Mentzer, Lasley, Lee & Berne, 1985; Mauser, Hoffmeister, Neinaber & Schaper, 1985; Olafsson *et al.* 1987; Homeister, Hoff, Fletcher & Lucchesi, 1990). The cardiac actions of adenosine are related to increased cell membrane K^+ conductance (Drury & Szent-Györgyi, 1929; Hartzell, 1979; Belardinelli & Isenberg, 1983*b*), which appears to occur via the activation of specific membrane adenosine receptors coupled to the inhibitory G-protein (G_i) (Kurachi, Nakajima & Sugimoto, 1986; Song, Thedford, Lerman & Belardinelli, 1992). Similarities

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in the actions of adenosine and acetylcholine (ACh) have long been noted (Hartzell, 1979; Song *et al.* 1992). Kurachi *et al.* (1986) provided evidence to suggest that adenosine and ACh are linked to the same population of K^+ (K^+_{ACh}) channels via different membrane receptors. It is currently widely believed that adenosine produces its cardiac electrophysiological effects by activating K^+_{ACh} channels (Belardinelli *et al.* 1989; Belardinelli, Wu & Visentin, 1990; Lerman & Belardinelli, 1991; Song *et al.* 1992), and the corresponding current has been called $I_{K,ACh,Ado}$ (Lerman & Belardinelli, 1991).

Recent studies of coronary function have suggested that adenosine receptors may also be coupled to ATP-sensitive K^+ (K^+_{ATP}) channels (Daut, Maier-Rudolph, von Breckerath, Merhrke, Gunther & Goedel-Mechen, 1990; Liepert, Becker & Gerlach, 1992; Dart & Standen, 1993). Moreover, in cultured neonatal ventricular myocytes, adenosine has been shown to modulate K^+_{ATP} channels at low $[ATP]_i$ by activating G_i (Kirsch, Codina, Birnbaumer & Brown, 1990). Cardiac adenosine receptors have been implicated in the cardioprotective effects of ischaemic preconditioning (Liu, Thornton, Van Winkle, Stanley, Olsson & Downey, 1991; Thornton, Liu, Olsson & Downey, 1992) and simulated ischaemia and reperfusion (Cordeiro, Howlett & Ferrier, 1992). The K^+_{ATP} channel blocker glyburide antagonizes the protective effect of myocardial preconditioning (Gross & Auchampach, 1992; Thornton *et al.* 1992), suggesting that the cardioprotection associated with adenosine receptor activation during ischaemic preconditioning may be mediated via an effect on $I_{K,ATP}$.

These lines of evidence suggest that cardiac adenosine receptors can be linked to K^+_{ATP} channels, at least in the presence of myocardial ischaemia. The present study was designed to assess the potential role of K^+_{ATP} channels in mediating the electrophysiological effects of adenosine on guinea-pig atrial myocytes under non-ischaemic conditions. We first determined whether the ability of adenosine to reduce action potential duration in multicellular guinea-pig atrial preparations is altered by glyburide. To address underlying ionic mechanisms more directly, we analysed the changes in whole-cell currents and single K^+ channel activity produced by adenosine and their modulation by glyburide. Finally, we compared ionic currents, single-channel activity and glyburide modulation in the presence of adenosine to corresponding phenomena observed with ACh.

METHODS

Multicellular atrial preparation and cell isolation

Atrial tissue preparation. Adult male guinea-pigs weighing 400–500 g were killed by cervical dislocation. The heart was quickly removed and washed in oxygenated Tyrode solution (see Solutions). The left atrium was separated, and the preparation was then pinned to the Sylgard-covered bottom of a 10 ml tissue bath and superfused with Tyrode solution at 10 ml min^{-1}. The bath temperature was maintained at 36 ± 0.5 °C, and the preparation was equilibrated for 60 min before the experiment was begun.

Cell isolation. Single left atrial myocytes were made using a procedure based on the method of Bustamente, Watanabe & McDonald (1981). Briefly, guinea-pigs were killed by cervical dislocation, their hearts were quickly removed and cannulated on a Langendorff column. After the heart was perfused with Ca^{2+}-free Tyrode solution (36 °C; see Solutions) for 8–10 min, it was enzymatically digested for a period of 7–10 min with a solution containing protease (5·2 units ml^{-1}, Type XIV; Sigma) and collagenase (142·6 units ml^{-1}, Type XI; Sigma). The left atrium was then excised from the softened heart, placed in a storage medium (containing (mM): EGTA, 0·5; KCl, 85; K_2HPO_4, 30; K_2ATP, 5; β-hydroxybutyric acid, 5; pyruvate, 5; creatine, 5; taurine, 20; glucose, 20; succinate, 5; $MgSO_4$, 5; and polyvinylpyrrolidine 25 g l^{-1}; pH adjusted to 7·2), minced, and then gently swirled. The myocytes were incubated in the storage medium at room temperature for a minimum of 1 h before exposure to Ca^{2+}-containing solutions. The cells were then placed in a perfusion dish mounted on the stage of an inverted microscope. Myocytes were allowed to adhere to the bottom of the dish for 5–10 min, and were then superfused with Tyrode solution at a flow rate of 2–3 ml min^{-1}. Roughly 40–70% of the myocytes survived after re-exposure to calcium.

Electrophysiological recording

Isolated atrial myocyte experiments were conducted at 33 ± 0.5 °C. The gigaseal patch-clamp technique was applied in the whole-cell and cell-attached patch configurations. Data were sampled at 5 kHz with custom-made software routines and an A/D converter (Neurocorder DR-390; Neuro Data Instruments), and stored on the hard disk of a 386SX/33 computer for subsequent off-line analysis. Recordings were made with an Axopatch 1D amplifier (Axon Instruments, Foster City, CA, USA). All currents were measured relative to the zero-current level. Leak subtraction techniques were not used.

After having been filled with internal solution (see below) the electrodes used for whole-cell voltage clamp had resistances of 4–7 MΩ. Junction potentials were compensated before the pipette touched the cell. The pipette series resistance was compensated to minimize the duration of the capacitive transient. Mean series resistance before and after compensation averaged $11·0 \pm 1·7$ and $5·8 \pm 0·9$ MΩ (means ± S.E.M.), respectively, and the capacitive time constant averaged 562 ± 44 and 216 ± 12 μs, respectively, before and after compensation. Mean cell capacitance averaged $51·0 \pm 2·5$ pF before compensation.

For cell-attached patch experiments, 8–11 MΩ pipettes were used, and recordings were obtained with a low-pass filter (1 kHz, −3 dB). Electrical signals were digitized at 2 kHz using custom-made software (Alenbic Software, Montreal, Canada), and stored on the hard disk of a microcomputer (386SX/33) or recorded on videotape using a videocassette recorder (Sony SL-HF-450).

In studies of multicellular atrial preparations, experiments were conducted at a temperature of 36 ± 0.5 °C. After the preparation had been allowed to equilibrate for 60 min, action potentials were recorded with standard glass microelectrodes filled with 3·0 M KCl and with 10–30 MΩ resistances. Microelectrodes were connected via a Ag–AgCl junction to a high-impedance microelectrode amplifier (KS-700; World Precision Instruments).

A bipolar Teflon-coated platinum electrode was used to deliver square-wave pulses of 2 ms duration and twice diastolic threshold current. Action potentials were displayed on an oscilloscope (model 5115; Tektronix), and transmitted to an analog-to-digital converter (Labmaster; Tecmar). Custom-made software routines (Bascom Consultants, Montreal, Canada) and an IBM PC computer were used to measure action potential characteristics (Nattel & Zeng, 1984).

Solutions

Tyrode solution for the superfusion of isolated atrial preparations contained (mM): NaCl, 129; KCl, 5·4; $CaCl_2$, 1·0; $NaHCO_3$, 20; NaH_2PO_4, 0·9; $MgSO_4$, 0·5; glucose, 10; gassed with 95% O_2–5% CO_2 (pH 7·3–7·4). The cell isolation solution was the same as above except for the omission of Ca^{2+} (nominally calcium free). In some studies of multicellular atrial preparations, superfusate NaCl was replaced by an equivalent molar concentration of sodium methanesulphonate to reduce the external chloride concentration.

In whole-cell voltage clamp experiments, the composition of the external perfusion solution was (mM): NaCl, 135; KCl, 5·4; $CaCl_2$, 1·0; $MgCl_2$, 1·0; Hepes, 10; pH adjusted to 7·4 with NaOH. In all experiments, $CdCl_2$ (100 μM) was added to the superfusate to block Ca^{2+} current (I_{Ca}). The pipette was filled with (mM): potassium aspartate, 120; KCl, 20; $MgCl_2$, 1·0; Hepes, 10; EGTA, 5; Na_2ATP, 5 or 10 (Sigma); GTP, 0·1 (Sigma); adjusted to pH 7·2 with KOH.

In the cell-attached patch experiments, the external and pipette solutions contained (mM): potassium aspartate, 120; KCl, 20; $MgCl_2$, 1·0; Hepes, 10; glucose, 10; $BaCl_2$, 0·5 (to block inward rectifying K^+ current (I_{K1})); adjusted to pH 7·4 with KOH.

Stock solutions of adenosine and ACh (Sigma) were freshly prepared for each experiment in distilled water. In the atrial tissue experiments, adenosine was directly dissolved in Tyrode solution. Glyburide (Sigma) was dissolved in dimethylsulphoxide (DMSO) to create a 100 mM stock solution, and small volumes of the stock solution were added to produce the desired final concentration in the superfusate. The final DMSO concentration in the superfusate was always less than 0·01%, and had no electrophysiological effects at this concentration.

Statistical analysis

Student's paired t test was used to compare single sets of paired data and Student's unpaired t test was used to compare corresponding unpaired data. Group data are represented as means ± S.E.M. Differences with a two-tailed $P < 0·05$ were considered statistically significant. Non-linear curve fitting of concentration–response data was obtained with commercially available software (TableCurve; Jandel Scientific, San Rafael, CA, USA) that uses a Marquardt procedure for parameter estimation.

RESULTS

Effects of adenosine and glyburide on action potential duration in multicellular atrial preparations

Figure 1 shows representative tracings from a cell in a multicellular guinea-pig atrial preparation under control conditions (curve *a*), in the presence of adenosine (100 μM; curve *b*), in the presence of 100 μM adenosine and 50 μM glyburide (*c*), and in the presence of 100 μM of both adenosine and glyburide (*d*). Adenosine substantially reduced atrial action potential duration (APD), and this effect was antagonized by glyburide in a concentration-dependent way. In this cell, 100 μM glyburide almost completely reversed the effect of adenosine on APD.

Figure 2*A* shows concentration–response data for the effects of adenosine on action potential duration at 95% repolarization (APD_{95}), in the presence and absence of a maximally effective concentration (100 μM) of glyburide. Results are shown for experiments performed with standard Tyrode solution (normal Cl^-), and other experiments in which NaCl was replaced by isomolar sodium methanesulphonate in the Tyrode solution (low Cl^-; $[Cl^-]_o$, 7·4 mM). All data were obtained during exposure of each cell to each concentration of adenosine under continuous impalement. In normal Tyrode solution, adenosine decreased APD by a maximum of 71 ± 2% (from 100 ± 6 to 29 ± 2 ms, $P < 0·001$, $n = 6$ cells), with an EC_{50} of 210 μM. Glyburide itself did not alter APD. However, glyburide shifted the adenosine concentration–response curve downwards, reducing the maximum APD reduction from 72 ± 3 to 44 ± 4% ($P < 0·001$), and leaving the adenosine EC_{50} unaffected (average 210 μM without and 200 μM with glyburide), changes typical of a non-competitive antagonist.

The concentration–response relation for the inhibitory action of glyburide was determined by measuring APD

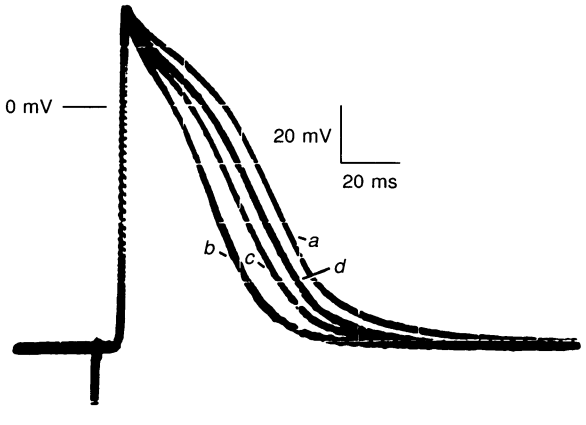

Figure 1. Reversal by glyburide of the effects of adenosine on guinea-pig atrial action potentials

Representative recordings of the effect of adenosine and glyburide on action potentials in an isolated guinea-pig atrial preparation. Action potentials are shown under control conditions (*a*), after 10 min of superfusion with 100 μM adenosine (*b*), and after the addition of 50 μM glyburide (*c*) and 100 μM glyburide (*d*) to the adenosine-containing superfusate.

under control conditions, and then measuring the response to adenosine at its IC_{50} (200 μM) in the absence and presence of a series of increasing glyburide concentrations. The ability of glyburide to oppose adenosine-induced APD shortening was a monotonic function of glyburide concentration (Fig. 2*B*), with a maximum attenuation of just over 50%. The IC_{50} for the action of glyburide was 31 μM, and maximal antagonism was observed at 100 μM.

Activation of purinergic receptors has been shown to induce outward Cl^- currents in guinea-pig atrial cells, an effect which can be reversed by glyburide (Matsuura & Ehara, 1992). This mechanism could explain the effects of adenosine in the multicellular preparation without invoking a role for $I_{K,ATP}$. If the action of adenosine had been due to enhancement of a Cl^- outward current, it should have been clearly altered by reversing the direction of the Cl^- concentration gradient. After replacement of NaCl by sodium methanesulphonate in Tyrode solution, the extracellular Cl^- concentration was reduced 20-fold, to a value (7·1 mM) lower than physiological $[Cl^-]_i$ (Hume & Harvey, 1991). This substantial reduction in $[Cl^-]_o$ did not alter APD_{95}, which averaged 81 ± 4 ms before and 79 ± 4 ms after replacement ($n = 6$). Glyburide alone similarly failed to alter APD after Cl^- replacement, with mean values of 93 ± 7 ms in the absence and 94 ± 8 ms in the presence of 100 μM glyburide ($n = 6$). The triangles in Fig. 2*A* show concentration–response curves for adenosine in the presence and absence of 100 μM glyburide, following extracellular Cl^- replacement. These results are not significantly different from results obtained with standard extracellular $[Cl^-]$, as shown by the circles in Fig. 2*A*.

Glyburide effects on adenosine- and ACh-induced changes in holding current

To evaluate more precisely the ionic mechanisms underlying adenosine-induced APD shortening and its antagonism by glyburide in guinea-pig atrium, we studied changes in ionic currents in isolated guinea-pig atrial myocytes. Figure 3 illustrates the effect of adenosine and the $I_{K,ATP}$ blocker glyburide on holding current at −40 mV, with 5 mM ATP in the pipette. Adenosine (10 μM) rapidly increased the holding current, an effect that showed little time-dependent desensitization (Fig. 3*A*). The addition of glyburide (20 μM) antagonized the effect of adenosine (Fig. 3*B*). Glyburide itself did not alter holding current, but the addition of adenosine in the presence of glyburide resulted in a small increase in holding current (Fig. 3*C*). On the other hand, the addition of ACh (10 μM) during glyburide administration produced a swift increase in holding current. At a holding potential of −40 mV, the holding current averaged 41 ± 8 pA under control conditions ($n = 20$), and adenosine (10 μM) increased the outward holding current to 246 ± 31 pA ($P < 0.01$). The addition of 20 μM glyburide in the presence of adenosine reduced the holding current to 69 ± 11 pA ($n = 10$, $P < 0.01$ *vs.* adenosine alone). The adenosine

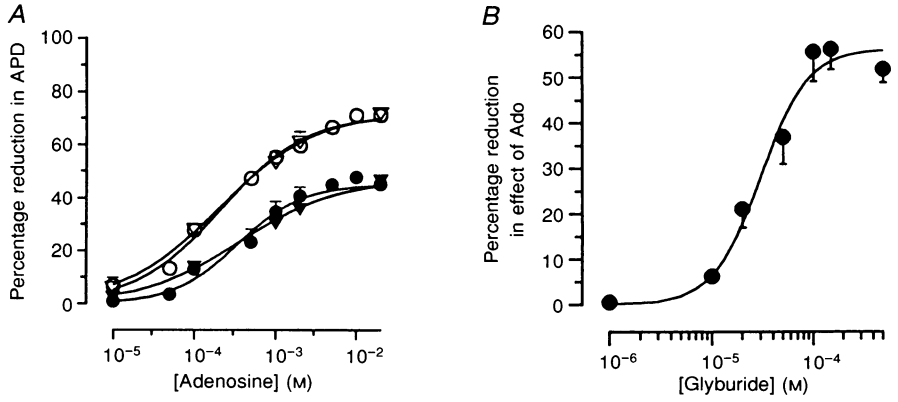

Figure 2. Antagonism by glyburide of concentration-dependent APD abbreviation induced by adenosine

Concentration–response relations for adenosine-induced changes in action potential duration to 95% repolarization (APD_{95}) in the absence and the presence of glyburide. *A*, adenosine decreased APD_{95} in a concentration-dependent manner (○); however, the effect was suppressed by co-administration with 100 μM glyburide in six cells (●). Similar results were obtained when NaCl in the Tyrode solution was replaced by sodium methanesulphonate, reducing superfusate $[Cl^-]$ to 7·4 mM (Low Cl; ▽, control; ▼, with 100 μM glyburide; $n = 6$). *B*, concentration–response curve for glyburide inhibition of the APD_{95}-shortening effect of 200 μM adenosine in multicellular guinea-pig atrial preparations ($n = 11$ for control and 10–100 μM glyburide, 7 for 150 μM glyburide, and 6 for 1 and 500 μM glyburide). Curves shown are best-fit estimates to the equation $E = E_{max}\ (1 + (K/C)^n)$, where E is the effect at a concentration C, E_{max} is the maximum effect, K is the concentration for half-maximal action, and n is the Hill coefficient.

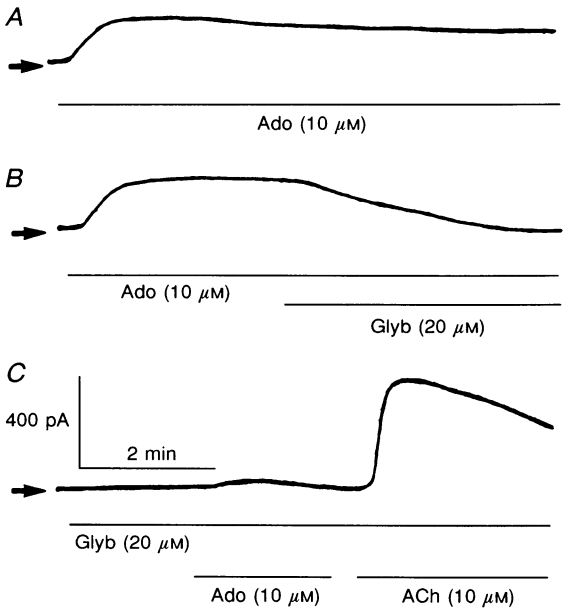

Figure 3. Glyburide antagonizes the effects of adenosine on the holding current

Representative recordings of changes in holding current at −40 mV induced by adenosine (Ado) in the whole-cell voltage clamp configuration. The holding current was recorded as a function of time (scale in *C*), and periods of drug exposure are indicated by the horizontal bars. Here and in subsequent figures arrows indicate zero current levels. *A*, the addition of 10 μM adenosine to the perfusion solution caused a prompt and sustained increase in the holding current. *B*, adenosine-induced current was suppressed by the addition of 20 μM glyburide (Glyb). *C*, adenosine only slightly increased the holding current in the presence of glyburide; however, the subsequent addition of acetylcholine (ACh) induced a prompt outward shift in the holding current. *A*, *B* and *C* were recorded from different cells. Similar results were obtained in six cells for each protocol.

effect on holding current was glyburide sensitive in all cells studied. Holding current after the addition of glyburide alone averaged 48 ± 12 pA, not significantly different from corresponding control values in the same fifteen cells, 59 ± 15 pA. In the presence of glyburide, adenosine increased outward current to a reduced extent, from 57 ± 18 to 91 ± 23 pA ($P < 0{\cdot}05$, $n = 9$).

Figure 4 illustrates the effects of glyburide pretreatment on the action of ACh. A single exposure to ACh produced long-lasting desensitization to the effect of ACh on the holding current. Because we wished to compare ACh action in the absence and presence of glyburide in the same cell, we studied the effects of consecutive 5 min exposure periods to ACh separated by 20 min, with the second ACh exposure in one group of cells occurring 2 min after the addition of 20 μM glyburide (with glyburide infusion continuing throughout the second ACh infusion), and in a control group of cells the second ACh exposure occurred in the absence of glyburide. In cells superfused with ACh in the absence of glyburide (Fig. 4*A*), maximum ACh-induced changes in holding current averaged 858 ± 157 pA during the first ACh exposure ($n = 8$), and 553 ± 125 pA in the second exposure. The ratio of the ACh-induced change in holding current during the second exposure compared with that during the first exposure

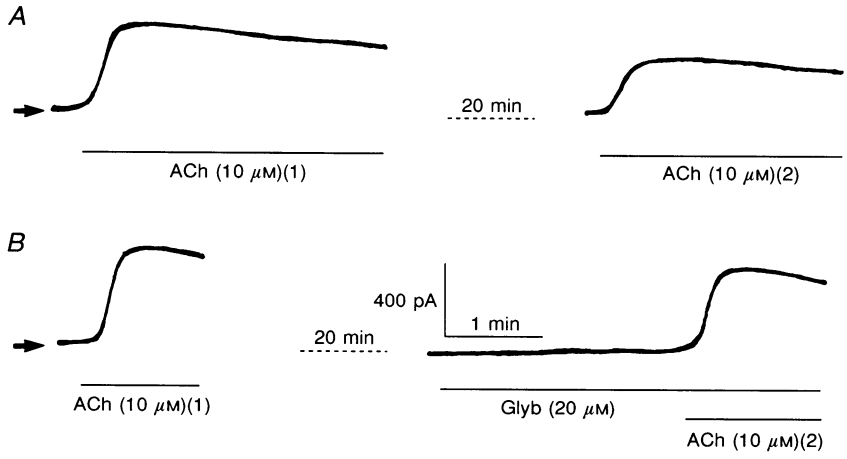

Figure 4. Glyburide does not prevent the holding current changes induced by ACh

Changes in the holding current at −40 mV induced by two periods of exposure to ACh separated by 20 min. General format as in Fig. 3. *A*, a second application, labelled (2), of ACh induced a smaller outward shift in the holding current than the first exposure, (1). *B*, after a first exposure to ACh, treatment with glyburide begun 2·5 min prior to a second exposure to ACh did not result in a smaller relative current shift than that seen in the absence of glyburide (*A*). Results shown in *A* and *B* are from different cells. Similar results were obtained for eight cells studied as in *A*, and six cells as in *B*.

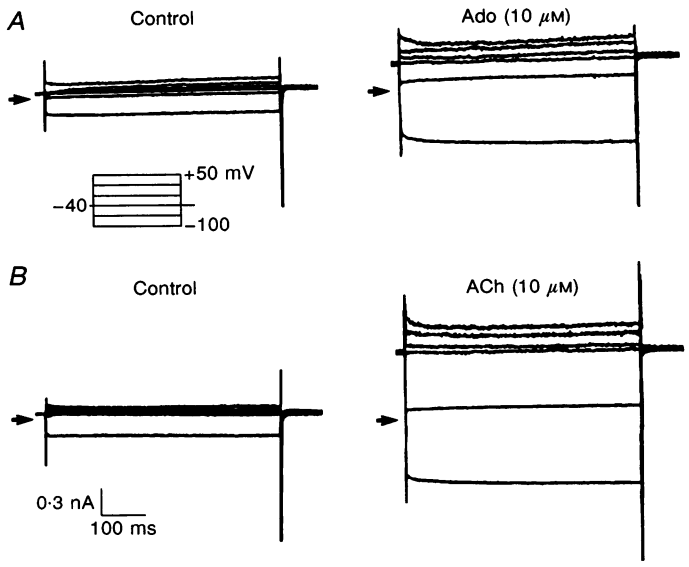

Figure 5. Effects of adenosine and ACh on currents activated by voltage steps

A, adenosine shifted the holding current at −40 mV in the outward direction, and increased the currents during voltage steps. *B*, ACh also shifted the holding current in the outward direction and increased the currents elicited by voltage steps. *A* and *B* are from different cells.

was 0·72 ± 0·15. When the second ACh exposure occurred in the presence of glyburide (Fig. 4*B*), the ACh-induced change in holding current averaged 784 ± 211 pA ($n = 7$) during the first exposure, and 530 ± 120 pA during the second. The ratio of the second to the first ACh-induced current was 0·74 ± 0·10 (*P* not significant *vs.* control group). Therefore, in contrast to adenosine, the effects of ACh on holding current were not affected by co-administration with glyburide.

Glyburide effects on voltage-dependent currents induced by adenosine and ACh

Voltage-dependent properties of the currents induced by adenosine and ACh were examined using 500 ms voltage steps from a holding potential of −40 mV to between −100 and +70 mV, in increments of 10 mV. Representative recordings are shown in Fig. 5. Adenosine and ACh significantly shifted the holding current in the outward direction, and increased the inwardly rectifying current

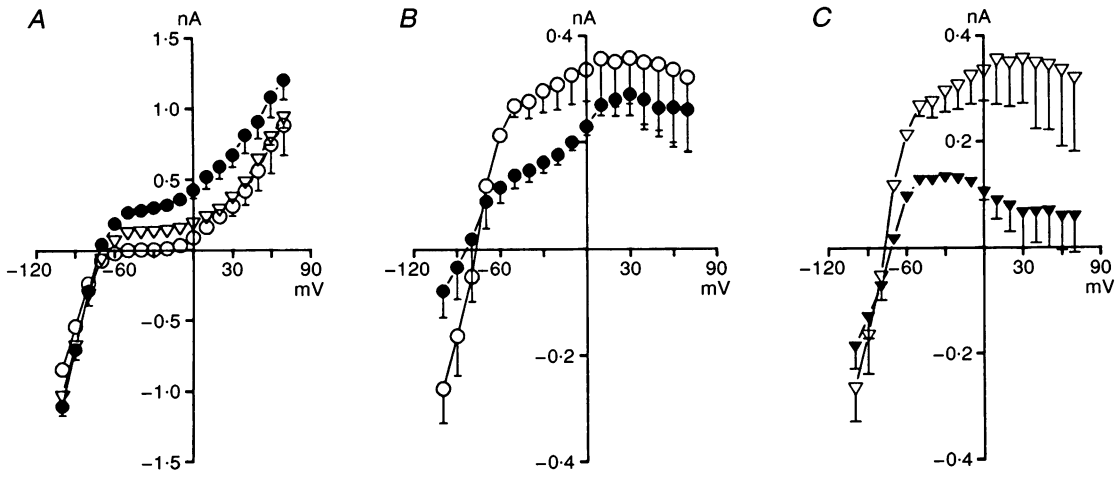

Figure 6. Glyburide inhibits adenosine-induced increases in membrane conductance

Effects of glyburide on current–voltage relations of adenosine-induced currents, as determined with pipettes containing 10 mM ATP. *A*, 10 μM adenosine (●) increased the current (measured at the end of the pulse) elicited by 500 ms voltage steps from a holding potential of −40 mV ($n = 6$ cells), and the addition of glyburide (20 μM; ▽) reduced the effects of adenosine. ○, control. *B*, total adenosine-induced current (○), and glyburide-sensitive component (●). *C*, total adenosine-induced current (▽), and glyburide-insensitive component (▼).

during voltage steps. The current–voltage (I–V) relations of adenosine- and ACh-induced currents (as measured at the end of voltage steps) are shown in Figs 6 and 7.

Figure 6 shows the I–V relations of adenosine-induced currents and the effects of glyburide, measured with 10 mM ATP in the pipette. Adenosine (10 μM) significantly increased the current flowing during voltage steps (Fig. 6*A*, $n = 6$). The addition of glyburide reduced the actions of adenosine (Fig. 6*A*, open triangles). The glyburide-sensitive current is shown in Fig. 6*B*. Both the adenosine-induced current and the glyburide-sensitive component reverse at −80 to −90 mV, consistent with a K^+ current mechanism. The glyburide-sensitive component showed inward rectification at voltages more positive than 0 mV. Figure 6*C* shows the glyburide-resistant portion of the adenosine-induced current (filled triangles). The glyburide-insensitive portion rectified at more negative voltages, in the range of −40 mV. Glyburide itself did not alter the I–V relation (data not shown).

Figure 7 shows I–V relations of ACh-induced current during sequential exposure periods in the same cells. Like adenosine, ACh significantly increased the current elicited by voltage steps. Under control conditions in seven cells, a second exposure to ACh 20 min after the first resulted in smaller currents (Fig. 7*A*). In another set of six cells, a second exposure to ACh in the presence of glyburide (started 2 min prior to ACh superfusion) produced similar current shifts (Fig. 7*B*). The current induced by ACh showed inward rectification at voltages more positive than −40 mV, like the glyburide-resistant component of adenosine-induced current shown in Fig. 6*C*.

Effects of adenosine in the presence of maximally effective concentrations of ACh

If a portion of the action of adenosine on membrane conductance is mediated by the activation of K^+_{ATP} channels, the addition of adenosine would be expected to increase the outward current elicited by depolarizing voltage steps in the presence of maximally effective concentrations of ACh. Figure 8 shows the results of experiments designed to test this concept. In an initial series of experiments, concentration–response relations were established for the effects of adenosine and ACh on whole-cell currents elicited by 300 ms voltage steps from −40 to +30 mV. Each cell was studied under control conditions and then in the presence of increasing concentrations of adenosine ($n = 6$) or ACh ($n = 5$), with 5 min of exposure to each concentration of each agent. As shown in Fig. 8*A*, maximum effects were achieved with concentrations of 10 μM for each agent. In the next series of studies, cells were exposed to 10 μM adenosine in the presence of 10 μM ACh. Figure 8*B* shows the results of one experiment. Prior to drug exposure, depolarization from −40 to +30 mV elicited an outward current of about 150 pA. In the presence of ACh, the current elicited by the same voltage step increased rapidly to over 1000 pA, and then decreased slightly over the subsequent 5 min. The addition of adenosine in the presence of ACh caused a further increase in outward current.

In many experiments, desensitization to the effect of ACh was more marked than in the experiment illustrated in Fig. 8*B*, and required 10 min to approach steady state. The addition of adenosine while current was rapidly

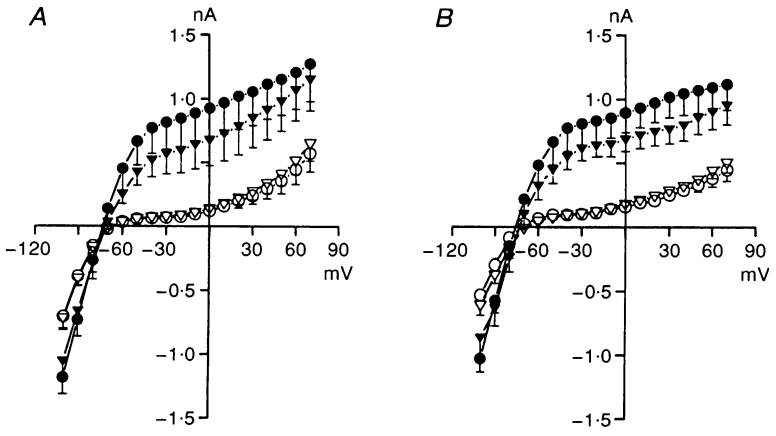

Figure 7. Glyburide does not alter the effects of ACh on membrane conductance

Current–voltage relations of ACh-induced currents in the absence and presence of glyburide. Currents were elicited by 500 ms pulses from −40 mV to the voltage indicated, and measured at the end of each pulse. *A*, in the absence of glyburide ($n = 6$ cells), the first exposure to 10 μM ACh (●) increased current amplitudes, an effect completely reversed by ACh washout (▽). A second 10 μM ACh treatment (▼) induced a smaller current increase. ○, control. *B*, after a first ACh exposure (●), currents returned to control values (○) upon washout of ACh, and were not altered by 20 μM glyburide in seven cells (▽). When a second exposure to 10 μM ACh was performed in the continued presence of glyburide (▼, same seven cells), the response to ACh was similar to that in the absence of glyburide as shown in *A*.

decreasing, because of desensitization to ACh, often resulted in a blunted response. We therefore studied the action of adenosine in the presence of steady-state ACh effects, and compared this response with the effect of adenosine in the absence of ACh.

Cells were first exposed to 10 μM adenosine for 5 min, and changes in current elicited by depolarization to +30 mV were measured. Adenosine was then washed out until control conditions were re-established, and ACh (10 μM) was superfused for 10 min. Adenosine (10 μM) was then added in the presence of ACh, and outward current prior to adenosine was compared with the current after 5 min of adenosine superfusion. Results from five cells are shown in Fig. 8*C*. Adenosine alone increased outward current during the voltage step by about 250 pA ($P < 0{\cdot}01$). The subsequent addition of ACh increased outward current to a greater extent, and the addition of adenosine in the presence of ACh caused a significant ($P < 0{\cdot}01$) additional increment in current. Note that the increment in current caused by adenosine (Ado-induced current) was significantly greater ($P < 0{\cdot}05$) in the absence of ACh compared with results in the presence of ACh, compatible with the possibility that a component of the action of adenosine was mediated by $I_{K,ACh}$.

Single K^+ channel currents induced by adenosine and ACh

The results described above indicate that the effects of adenosine on whole-cell K^+ currents are partially antagonized by glyburide, while those of ACh are not. Despite the presence of 5–10 mM ATP in the internal solution, it is possible that cellular dialysis alters the metabolic state of the cell, or changes the concentration of

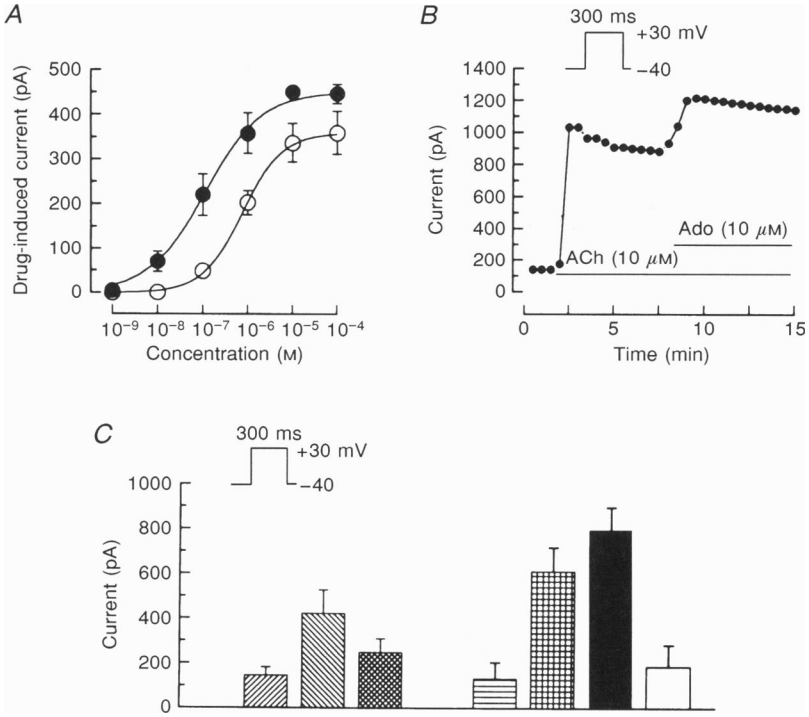

Figure 8. Adenosine increases membrane conductance in the presence of maximally effective concentrations of ACh

A, concentration–response curves were obtained by depolarizing the cell for 300 ms from −40 to +30 mV under control conditions, and then in the presence of increasing concentrations of adenosine (○, 6 cells) or ACh (●, 5 cells). The current elicited by depolarization under control conditions was subtracted from the current observed upon depolarization after 5 min at each drug concentration, in order to obtain drug-induced current. Maximal effects with both agents were achieved at a concentration of 10 μM. *B*, currents induced by depolarization prior to and after the addition of 10 μM ACh to the superfusate, and the subsequent inclusion of 10 μM adenosine along with ACh. *C*, outward current, elicited by the voltage protocol shown, under control conditions (▨), and 5 min after the addition of 10 μM adenosine (▧), showing a significant increase ($P < 0{\cdot}01$). Adenosine was subsequently washed out, and current returned to control values (▤). The addition of 10 μM ACh (▦) for 10 min significantly increased ($P < 0{\cdot}01$) the current elicited by the voltage step, and the subsequent introduction of adenosine (10 μM for 5 min, ■) in the presence of ACh further increased the depolarization-induced current ($P < 0{\cdot}01$ *vs.* ACh alone). The adenosine-induced current under control conditions (10 μM adenosine − control, ▩) was significantly greater ($P < 0{\cdot}05$) than that in the presence of ACh ((ACh + Ado) − ACh, □).

some key intracellular constituent, favouring the activation of $I_{K,ATP}$. In order to address this issue, and to obtain precise information about the channels activated by adenosine and ACh, we studied single-channel activity in cell-attached patches. Bath and pipette solutions contained 140 mM K^+, in order to set the resting membrane potential close to 0 mV. In the absence of pipette adenosine or ACh and in the presence of 0·5 mM Ba^{2+} in the bath and pipette ($n = 8$), single-channel currents were rarely observed (1 of 8 patches). When adenosine (10 μM, $n = 5$) or ACh (10 μM, $n = 3$) were added to the superfusate, no new single-channel activity appeared. However, clear channel activity was observed when adenosine (12 of 14 patches, $P < 0{\cdot}01$ *vs.* control) or ACh (5 of 6 patches, $P < 0{\cdot}01$ *vs.* control) were included in the pipette solutions at the same (10 μM) concentrations. Figure 9 illustrates the results of typical experiments in which single-channel activity was seen in the presence of adenosine or ACh in the pipette. ACh induced a single population of channels, with a mean conductance of 41 ± 5 pS ($n = 5$). ACh-induced channels showed strong inward rectification, with no clear outward currents detectable (Fig. 9*B*). Adenosine induced channels with at least two distinct conductances (Fig. 9*A*). The large-conductance channel (level O_2 in Fig. 9*A*) had a conductance of 72 ± 5 pS ($n = 6$) in the negative voltage range (−80 to −10 mV). Adenosine also induced a channel with a lower conductance, and single-channel current amplitudes (level O_1) similar to those induced by ACh. Unlike ACh, adenosine-induced channels were able to conduct current in the outward direction. Mean *I–V* relations of large-conductance adenosine-stimulated ($n = 6$) and ACh-induced ($n = 5$) single K^+ channel currents are shown in Fig. 9*C*.

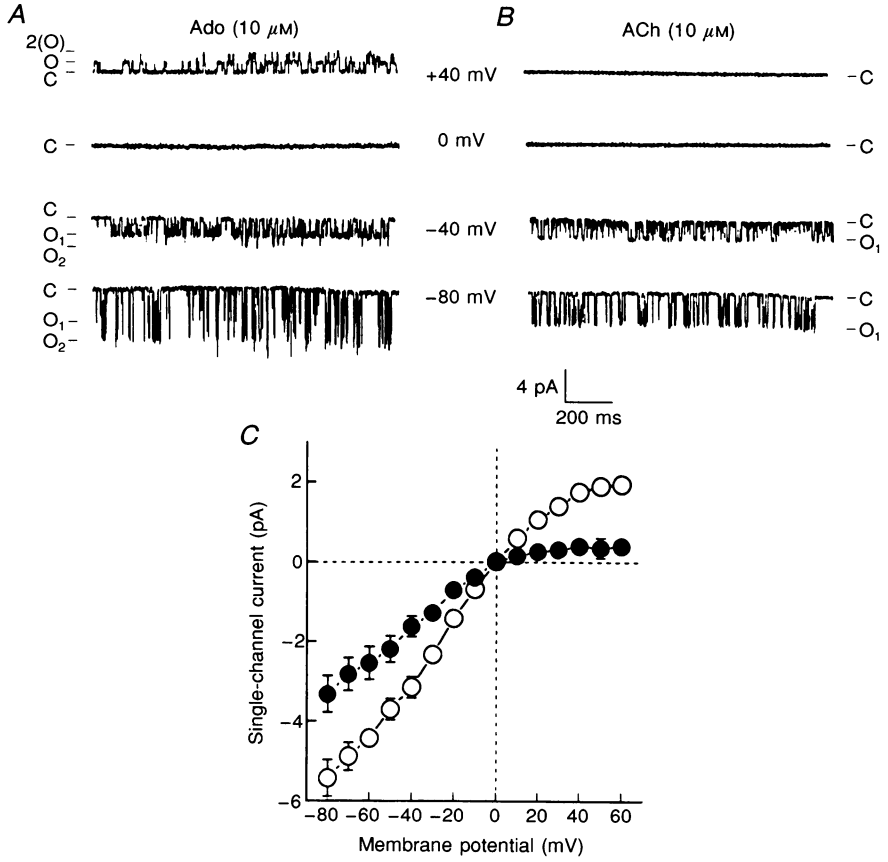

Figure 9. Single-channel currents induced by adenosine and ACh

Single-channel currents recorded in cell-attached mode from one cell with 10 μM adenosine (*A*) in the pipette and another cell with 10 μM ACh (*B*) in the pipette. In the presence of adenosine, two open-channel levels (O_1 and O_2) were observed during hyperpolarization, one of which (O_1) corresponded to the level of currents carried by a single type of channel seen in the presence of ACh, C indicates the closed channel level. During depolarization, no channel openings were seen in the presence of ACh, but clear openings occurred in the presence of adenosine. The higher-conductance channel in the presence of adenosine had a conductance similar to that recorded for $I_{K,ATP}$. Ba^{2+} (0·5 mM), present in the pipette, is known to produce voltage-dependent open-state block of K^+_{ATP} channels upon hyperpolarization, possibly explaining the greater open duration of K^+_{ATP} channels upon depolarization and the observation of currents carried by two such channels at level 2(O). *C*, current–voltage relations for high-conductance channels activated by adenosine (○, $n = 6$), and channels activated by ACh (●, $n = 5$).

Figure 10 illustrates the effects of glyburide on adenosine-stimulated single K^+ channel currents. In the presence of adenosine, single-channel activity showed inward rectification but nonetheless clearly carried outward current at positive potentials (Fig. 10*A*). In addition to a large-conductance channel with a conductance in the range of 70 pS in the negative voltage range (level O_2), there are lower current levels, suggesting the presence of a second, lower-conductance channel (level O_1). In addition, there are openings (e.g. see arrow in Fig. 10*A*) suggesting that both the low- and high-conductance channels could be open simultaneously. Figure 10*B* shows currents recorded from the same patch after the addition of glyburide (20 μM) to the superfusate. The amplitude and frequency of single-channel activity is reduced, and no outward current is recorded. Single-channel current amplitudes after glyburide (O_1) correspond to the lower-amplitude current levels in the presence of adenosine alone (Fig. 10*A*). Figure 10*C* and *D* shows all-points histograms for data recorded at −80 mV from the cell the single-channel currents of which are shown in Fig. 10*A* and *B*. In the presence of adenosine alone (Fig. 10*C*),

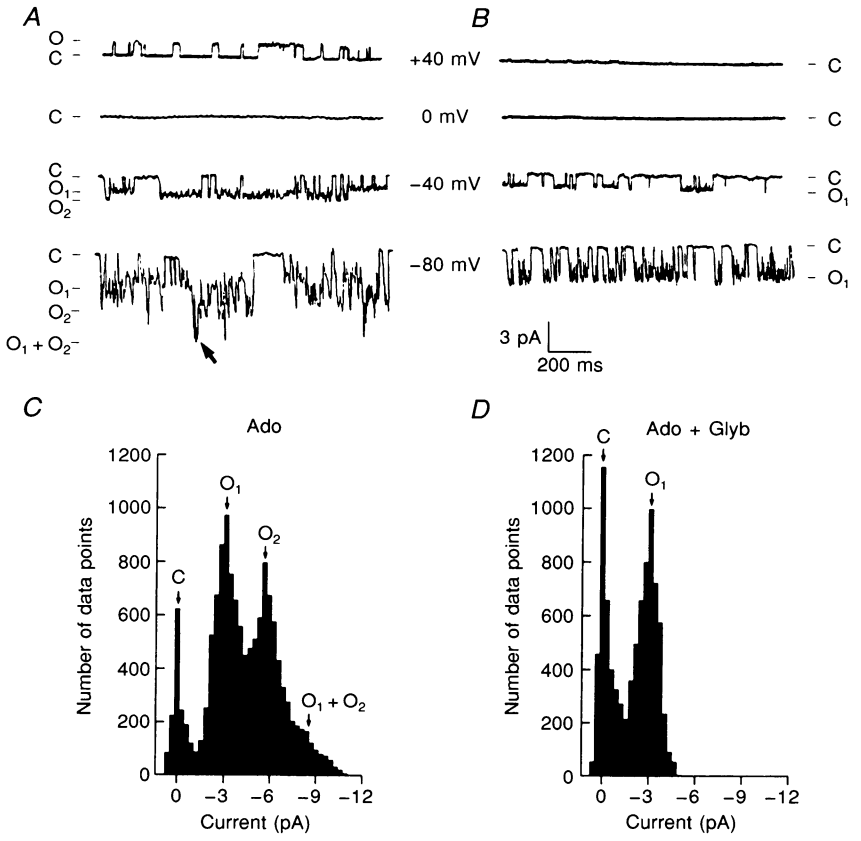

Figure 10. Effect of glyburide on adenosine-regulated channel currents in a cell-attached patch

A, with the presence of 10 μM adenosine in the pipette, single-channel currents were recorded at various membrane potentials. During hyperpolarization, two discrete open-channel levels (O_1 and O_2) were seen, as well as occasional openings to a third level (indicated by arrow) which was equal to the sum of $O_1 + O_2$. *B*, the addition of 20 μM glyburide to the superfusate of the cell depicted in *A* suppressed the larger-amplitude openings (to levels O_2 and $O_1 + O_2$), leaving apparently unaltered the openings to O_1. Outward current was not carried upon depolarization by the channel remaining in the presence of glyburide, suggesting that the outward current seen in its absence (level O_1, *A*) was carried by the channel opening to O_2 upon hyperpolarization. Similar results were obtained in four cells, with single-channel slope conductance between −80 and −10 mV averaging 68 ± 2 pS for the high-conductance channel observed in the presence of adenosine alone, and 36 ± 5 pS for the channel remaining when glyburide was added to the superfusate. *C*, all-points histogram obtained from the cell illustrated in *A* and *B* with adenosine in the pipette, upon hyperpolarization to −80 mV. Levels corresponding to the C, O_1, O_2, and $O_1 + O_2$ channels shown in *A* are indicated. *D*, after the addition of glyburide, hyperpolarization to −80 mV results in a bimodal distribution with peaks corresponding to C and O_1, but the higher amplitude openings corresponding to O_2 and $O_1 + O_2$ in *A* and *C* are suppressed.

peaks are present at the closed level (C) and at the two open levels (O_1 and O_2) shown in Fig. 10*A*. There is also a shoulder which corresponds to current amplitudes equal to the sum of levels O_1 and O_2, as indicated in the Fig. 10*C*. When glyburide is added, only two peaks are seen, corresponding to levels C and O_1 in the other panels of Fig. 10, and the activity at levels O_2 and $O_1 + O_2$, seen in Fig. 10*C*, is absent. We interpret these results as suggesting that adenosine activates two different types of channels, and that both channels can be open simultaneously. The addition of glyburide abolishes activity of the high-conductance channel and combined openings, leaving only activity corresponding to the low-conductance channel activated by adenosine. Similar results were obtained in five other cells. The conductance of the high-amplitude adenosine-stimulated channel at negative voltages averaged 68 ± 2 pS. The mean conductance after the addition of glyburide was 36 ± 5 pS. Only low-conductance channels were observed when both adenosine (10 μM) and glyburide (20 μM) were included in the pipette solution in three other cells. These data suggest that adenosine activates at least two channels: (1) a higher-conductance channel, and (2) a lower-conductance channel, with a conductance similar to that of channels activated by ACh. Glyburide selectively blocks the high-conductance channel.

DISCUSSION

The present study demonstrates that the effects of adenosine on action potential duration and ionic currents in guinea-pig atrium are inhibited by glyburide. Single-channel recording shows that adenosine activates two populations of channels, one with conductance and rectification properties similar to those of ACh-activated channels, and the other distinguished by a higher unitary conductance and less inward rectification. The single-channel conductance of the higher-conductance channel, in the range of 70 pS at negative voltages, along with its ability to carry outward current, matches very closely with properties typical of K^+_{ATP} channels under similar conditions (Trube & Hescheler, 1984; Nichols & Lederer, 1991). Glyburide selectively inhibits the high-conductance channel activated by adenosine. These results are compatible with the hypothesis that $I_{K,ATP}$ mediates a significant portion of the electrophysiological actions of adenosine on the guinea-pig atrium.

Relation to other studies of the ionic mechanisms of adenosine

Adenosine is generally believed to produce cardiac electrophysiological effects by activating K^+_{ACh} channels (Belardinelli & Isenberg, 1983*a*; Kurachi *et al.* 1986; Pelzer & Trautwein, 1987; Belardinelli *et al.* 1990; Visen, Wu & Belardinelli, 1990). There are many similarities in the actions of adenosine and ACh – both increase cardiac K^+ conductance (Belardinelli & Isenberg, 1983*b*), decrease transmembrane Ca^{2+} currents (Iijima, Irisawa & Kameyama, 1985; Visen *et al.* 1990), antagonize isoprenaline effects (Schrader *et al.* 1977; Dobbson, 1978; Bailey, Watanabe, Besch & Lathrop, 1979; Belardinelli & Isenberg, 1983*a*), and activate single K^+ channel currents (Kurachi *et al.* 1986) in guinea-pig atrial cells. Recent work suggests that the actions of adenosine in vascular smooth muscle can be mediated via $I_{K,ATP}$ (Daut *et al.* 1990; Liepert *et al.* 1992; Dart & Standen, 1993). In swine coronary arteries, adenosine activates $I_{K,ATP}$ via an A_1 receptor (Dart & Standen, 1993), the same receptor subtype believed to mediate the cardiac electrophysiological actions of adenosine (Belardinelli *et al.* 1989, 1990). Kirsch *et al.* (1990) showed that adenosine can activate K^+_{ATP} channels in excised inside-out patches from rat ventricular myocytes in the presence of low bath ATP concentrations, an effect that could be mimicked by the addition of free α-subunits of the inhibitory G-protein (α_{i1}*, α_{i2}* or α_{i3}*). They also demonstrated that adenosine can activate a tolbutamide-sensitive outward current in cells dialysed with 0·1 mM ATP. They interpreted their results as indicating a role for adenosine in explaining the activation of $I_{K,ATP}$ during myocardial ischaemia. We now report evidence to suggest that adenosine activates $I_{K,ATP}$, even in the absence of reduced intracellular ATP concentrations.

Kirsch *et al.* (1990) did not observe the activation of K^+_{ATP} channels in cell-attached patches in the presence or absence of adenosine. This discrepancy with our findings could be due to differences in species (guinea-pig *vs.* rat) or tissue (atrial *vs.* ventricular). Alternatively, the difference could be due to the manner of adenosine administration. We found that adenosine superfusion did not activate either K^+_{ACh} or K^+_{ATP} channels in cell-attached patch experiments. On the other hand, when adenosine was included in the pipette, channels with the characteristics of both $I_{K,ATP}$ and $I_{K,ACh}$ were seen. Kurachi *et al.* (1986) similarly observed that adenosine activated K^+ channels only when it was included in the pipette solution. This result suggests that adenosine activates channels that are in close physical proximity to the adenosine receptor, rather than acting via a diffusible second messenger. Kirsch *et al.* (1990) do not state whether they exposed cell-attached patches to adenosine via superfusion or in the pipette – if only superfusion was used, this might explain why they did not observe $I_{K,ATP}$ channels in cell-attached patches upon exposure to adenosine.

We observed, as did Kurachi *et al.* (1986), that adenosine activates channels with properties of $I_{K,ACh}$. On the other hand, the actions of adenosine clearly differed from those of ACh in our experiments, in that adenosine also activated a higher-conductance, glyburide-sensitive channel capable of carrying outward currents under symmetrical K^+ conditions. These results suggest that the actions of ACh and adenosine to increase K^+ currents in

guinea-pig atrium are not completely due to increases in the cytoplasmic concentration of a common diffusible second messenger. The ability of adenosine to result in K^+_{ATP} channel opening could be explained by preferential coupling, almost certainly involving G_i* (Kurachi *et al.* 1986; Kirsch *et al.* 1990), of the adenosine receptor to local K^+_{ATP} channels in a membrane-delimited fashion (Kirsch *et al.* 1990; Nichols & Lederer, 1991). The lack of similar effects of ACh in our experiments could be explained by a preferential localization of adenosine receptors in physical proximity to K^+_{ATP} channels. Alternatively, differences in coupling by G-protein subunits to K^+_{ATP} and K^+_{ACh} channels may be involved in explaining the activation of K^+_{ATP} that we observed with adenosine but not ACh. The possibility also exists that glyburide alters the coupling of adenosine to membrane channels. The fact that adenosine and ACh only activate channels when they are included in the pipette, and not by superfusion (as shown both in the present experiments and previously by Kurachi *et al.* 1986) suggests that channel activation via adenosine and ACh receptors is a local phenomenon, and does not result from a diffusible second messenger.

Ito *et al.* (1992) showed that in inside-out patches from guinea-pig atrial and ventricular myocytes, K^+_{ATP} channel activity could be detected in the presence of 1 μM ACh. This observation may be related to the specific experimental conditions they used (inside-out patch, 100 μM ATP in the bath). The results of experiments at multiple levels of complexity are consistent in showing that the effects of adenosine can be inhibited by glyburide (at all three levels), and differ partially from those of ACh (at the whole-cell voltage clamp and single-channel level). The results of the single-channel experiments are the strongest single piece of evidence implicating K^+_{ATP} channels in mediating adenosine action, while results at the other two levels point to such a mechanism.

Limitations

The glyburide concentrations that we used (20 μM in voltage clamp studies, IC_{50} of 31 μM in standard microelectrode experiments) are relatively high, particularly when compared with the concentrations that are effective in blocking pancreatic $I_{K,ATP}$ (Zunkler, Lenzen, Manner, Panten & Trube, 1988; Misler, Gillis & Tabacharani, 1989). They are, nevertheless, in the same range as the K_d (dissociation constant) of 5–20 μM reported in previous studies of cardiac and vascular smooth muscle (Nichols & Lederer, 1991). The need for adenosine in the pipette to activate channels in cell-attached patches precludes the possibility of studying directly the effect of the addition of adenosine on single-channel activity observed in the absence of the drug. Similar difficulties were noted by Kurachi *et al.* (1986). The consistent absence of single-channel activity under control conditions, its presence when adenosine was included in the pipette, the suppression of the high-conductance channel by the addition of glyburide, and the absence of high-conductance channel activity when glyburide was included with adenosine in the pipette, all point to the activation of $I_{K,ATP}$ by adenosine in cell-attached patch experiments.

The EC_{50} for the effects of adenosine on atrial APD was substantially larger than the concentration that produced important changes in outward currents at the whole-cell level. This discrepancy may be due to dialysis of cellular contents during whole-cell voltage clamp, or to the activity of adenosine uptake and degradation processes which very effectively extract adenosine from the interstitial fluid of multicellular preparations (Belardinelli *et al.* 1982; Froldi & Belardinelli, 1990).

We did not study directly the ionic selectivity of channels activated by adenosine and ACh. However, the reversal potentials of whole-cell and single-channel currents induced by adenosine and ACh are compatible with channels that are highly selective for K^+ ions. Extracellular Cl^- replacement did not alter the actions of adenosine (Fig. 2), suggesting that changes in Cl^- conductance are unlikely to play an important role in mediating the actions of adenosine on repolarization under the conditions that we used.

We used 0·5 mM Ba^{2+} in the bath and pipette solutions to avoid contamination by I_{K1} in the cell-attached patch studies. Barium produces a voltage-dependent block of K^+_{ATP} channels, reducing mean open time without altering single-channel current amplitude or conductance (Quayle, Standen & Stanfield, 1988). Consequently, K^+_{ATP} channel kinetics are substantially changed in the presence of external Ba^{2+}, and we did not analyse single-channel kinetics. On the other hand, K^+_{ATP} conductance properties are not altered by Ba^{2+}, and we observed typical K^+_{ATP} unitary current amplitudes in the presence of adenosine.

Conclusions

Since the discovery of K^+_{ATP} channels by Noma (1983), their importance has been increasingly recognized. Their inhibition by ATP in micromolar concentrations led to a debate about the importance of K^+_{ATP} channels in acute myocardial ischaemia (Nichols & Lederer, 1991), until studies of the modulating action of ischaemic products such as protons (Fan & Makielski, 1993) and adenosine (Kirsch *et al.* 1990) indicated the possibility of $I_{K,ATP}$ activation at concentrations of intracellular ATP achieved during the early phases of acute myocardial ischaemia. The present study, along with earlier investigations in vascular smooth muscle (Daut *et al.* 1990; Liepert *et al.* 1992; Dart & Standen, 1993), indicate a potential new role for $I_{K,ATP}$, that of a target for pharmacological modulation of membrane conductance in the absence of ischaemic conditions. Thus, the electrophysiological

actions of adenosine (slowed atrioventricular (AV) nodal conduction, abbreviated atrial action potential duration and refractoriness), as well as its effects on arrhythmias (termination of AV nodal re-entry, occasional induction of atrial fibrillation), may also be mediated by $I_{K,ATP}$, and not solely by $I_{K,ACh}$, as previously believed.

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