

## Identification and properties of an ATP-sensitive K<sup>+</sup> current in rabbit sino-atrial node pacemaker cells

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1. Single myocytes were isolated from rabbit sino-atrial (SA) node by enzymatic dissociation. Spontaneous pacemaker activity, whole-cell and single-channel currents were recorded under conditions known to modulate ATP-sensitive K<sup>+</sup> (K<sub>ATP</sub>) channels.
2. The K<sub>ATP</sub> channel openers, cromakalim and pinacidil, slowed or abolished the pacemaker activity, and caused hyperpolarization of the maximum diastolic potential (MDP). Glibenclamide, a K<sub>ATP</sub> channel blocker, reversed these effects. Cromakalim- and pinacidil-activated currents reversed near the potassium equilibrium potential,  $E_K$ . Glibenclamide had no effect on the L-type calcium current,  $I_{Ca(L)}$ , the hyperpolarization-activated inward current,  $I_f$ , or the delayed rectifier potassium current,  $I_K$ .
3. Sodium cyanide, which inhibits mitochondrial ATP production, induced a macroscopic current that reversed near  $E_K$  and was blocked by glibenclamide.
4. In excised, inside-out patches from SA node cells, single K<sub>ATP</sub> channels showed a slope conductance of  $52 \pm 8$  pS (mean  $\pm$  s.d.) when measurements were made at negative voltages in symmetric, 140 mM K<sup>+</sup>. Channels from ventricular myocytes showed a somewhat larger slope conductance ( $70 \pm 5$  pS).
5. Raising the intracellular ATP concentration caused a concentration-dependent reduction in the open probability of the K<sub>ATP</sub> channels ( $IC_{50}$ , 16  $\mu$ M; Hill coefficient,  $\sim 1$ ; at both pH 7.4 and 6.8).
6. In excised inside-out patches, cromakalim or pinacidil induced significant increases in K<sub>ATP</sub> channel activity in the presence of 50  $\mu$ M or 1 mM intracellular ATP. This channel activity was blocked by glibenclamide.
7. Our results suggest that sino-atrial node cells express a distinct isoform of K<sub>ATP</sub> channel which may play an important role in pharmacological and pathophysiological modulation of pacemaker activity.

ATP-sensitive K<sup>+</sup> (K<sub>ATP</sub>) channels have been identified in a number of different tissues, including mammalian heart (Noma, 1983) where they have been studied extensively (for reviews see Nichols & Lederer, 1991; Wilde & Janse, 1994). Considerable attention has been focused on the role of K<sub>ATP</sub> channels in the genesis of cardiac arrhythmias following ischaemia/reperfusion in mammalian ventricle. The available evidence suggests that during ischaemia, activation of K<sub>ATP</sub> channels may be responsible for the shortening of the action potential and increased K<sup>+</sup> efflux (Gasser & Vaughan-Jones, 1990; Nichols, Ripoll & Lederer, 1990). K<sub>ATP</sub> channels can also regulate the contractility and excitability of cardiac muscle during metabolic inhibition (Lederer, Nichols & Smith, 1989; Weiss, Venkatesh & Lamp, 1992).

Most studies of K<sub>ATP</sub> current in mammalian heart have used myocytes from atrium and ventricle. At present no detailed electrophysiological information is available regarding the properties of K<sub>ATP</sub> channels in the primary pacemaker cells from the sino-atrial (SA) node (Rosen *et al.* 1991; Irisawa, Brown & Giles, 1993). The literature on the effects of K<sub>ATP</sub> channel openers on heart rate ranges from descriptions of no effect (Grover, McCullough, Henry, Conder & Sleph, 1989; Gautier, Bertrand & Guiraudou, 1991) to reports of a moderate decrease in sinus pacemaker rate (Sato & Hashimoto, 1984; Taira, 1987). However, there is also a report of cardiac standstill induced by the K<sub>ATP</sub> channel openers (Gotanda, Sato & Taira, 1988).

If these K<sup>+</sup> channels are present in SA node they may be involved in the regulation of heart rate in either normal or

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pathophysiological conditions (Wilde & Janse, 1994). For example, they may contribute to the pathogenesis of the sick sinus syndrome seen in some ischaemic heart diseases (Shaw, Linker, Heaver & Evans, 1987; Alboni *et al.* 1991). Moreover, clinical applications of  $K_{ATP}$  channel openers and blockers are increasing (i.e. antihypertensive, anti-arrhythmic), and therapeutic decisions should include consideration of their direct effects on pacemaker rhythm.

In the present experiments, the following questions were addressed. (1) Are  $K_{ATP}$  channels present in SA node and, if so, what are their single-channel properties? (2) Can physiological, pharmacological and pathological conditions which are known to activate  $K_{ATP}$  channels alter SA node pacemaker activity?

Isolated single cells from the rabbit heart SA node were studied by measuring spontaneous action potentials and transmembrane ionic currents at both the whole-cell and single-channel levels under conditions known to modulate the activity of  $K_{ATP}$  channels.

## METHODS

### Cell isolation

The method for isolating single cells from the SA node of rabbit hearts has been described previously (Han, Habuchi & Giles, 1994a; Han, Shimoni & Giles, 1994c). Ventricular myocytes were isolated using the procedure described by Giles & Imaizumi (1988) and were used to allow comparisons of the properties of single  $K_{ATP}$  channels from two different regions of the heart. Rabbits were anaesthetized by injection of pentobarbitone (65 mg ml<sup>-1</sup>, 1 ml (kg body weight)<sup>-1</sup>), heparin (1000 IU ml<sup>-1</sup>) and 2% benzyl alcohol into the marginal ear vein, and then killed by cervical dislocation and exsanguination.

### Solutions

**Whole-cell recordings.** The bicarbonate-buffered Tyrode solution used for cell isolation contained (mM): NaCl, 121; KCl, 5; sodium acetate, 2.8; MgCl<sub>2</sub>, 1.0; Na<sub>2</sub>HPO<sub>4</sub>, 1.0; NaHCO<sub>3</sub>, 24; glucose, 5.5; and CaCl<sub>2</sub>, 0.5. This solution was equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (pH 7.4). The KB solution (Isenberg & Klockner, 1982) used for storing the cells contained (mM): potassium glutamate, 90; potassium oxalate, 10; KCl, 25; KH<sub>2</sub>PO<sub>4</sub>, 10; NaOH, 6; MgSO<sub>4</sub>, 1.0; taurine, 20; Hepes, 5; and glucose, 10. pH was adjusted to 7.2 with KOH. Hepes-buffered Tyrode solution, in which all experiments were carried out, contained (mM): NaCl, 145; KCl, 5.4; MgCl<sub>2</sub>, 1.0; Na<sub>2</sub>HPO<sub>4</sub>, 1.0; Hepes, 5.0; CaCl<sub>2</sub>, 1.8; and glucose, 10. pH was adjusted to 7.4 with NaOH.

**Single-channel recordings.** The pipette contained the following solution (mM): NaCl, 140; KCl, 5; Hepes, 10; CaCl<sub>2</sub>, 1; MgCl<sub>2</sub>, 1; and glucose, 10; buffered to pH 7.4 with NaOH. The standard bath solution contained (mM): KCl, 140; Hepes, 10; EGTA, 5; MgCl<sub>2</sub>, 1.4; and glucose, 10. EGTA was included in the bath solution to prevent the activation of large-conductance Ca<sup>2+</sup>-activated potassium channels and Ca<sup>2+</sup>-induced run-down of  $K_{ATP}$  channels. The pH was adjusted to either 7.4 or 6.8 with KOH. ATP (as K<sub>2</sub>ATP) was added as required from a 10 mM stock solution made immediately before use. Current-voltage relationships were recorded in symmetric conditions: the standard bath solution was used as pipette solution, with the exception that

Mg<sup>2+</sup> was omitted. Test solutions were applied to the patch either by a common outlet at a flow rate of 2 ml min<sup>-1</sup> or by a multi-input perfusion device at a flow rate of 100 μl min<sup>-1</sup>.

### Whole-cell recordings and data acquisition

The nystatin (0.3 mg ml<sup>-1</sup>)-perforated patch technique (Horn & Marty, 1988) was used to current and voltage clamp the cells in the whole-cell configuration (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The pipette solution contained (mM): KCl, 140; NaCl, 6; MgCl<sub>2</sub>, 1; Hepes, 5. pH was adjusted to 7.2 with KOH and DC resistance was 2–3 MΩ. Nystatin was first dissolved in dimethyl sulphoxide (DMSO; Sigma) and then added to the pipette solution. These recordings were made at 32.5 ± 1 °C. Membrane currents were digitized at 2.5 kHz. The recording chamber volume was 0.5 ml and a constant flow rate of 1.5 ml min<sup>-1</sup> was used to superfuse the cells.

### Single-channel recordings and data acquisition

Standard patch clamp techniques (Hamill *et al.* 1981) were used to record single-channel currents using the inside-out patch configuration. Pipettes were pulled from borosilicate glass (PG52151-4; World Precision Instruments). The tips were then coated in silicon resin (Sylgard 184; Dow Corning) and fire-polished. Typical pipette resistance was 3–7 MΩ. After the establishment of a gigohm seal, the pipette and attached cell were lifted slightly from the base of the chamber and an inside-out membrane patch was excised by means of a short rapid burst of solution, which tore the cell away from the pipette.

Single-channel currents were normally recorded at a holding potential of 0 mV. These were filtered at 5 kHz and amplified (Axopatch 200; Axon Instruments), digitized (Neuro-corder DR-384; Neuro Data Instruments Corp., New York), and then stored on video tape. Data were replayed through a 4-pole Bessel filter (LPF-100; Warner Instruments Corp., Hamden, CT, USA) and computer interface (Axolab 1100; Axon Instruments) connected to a Compaq PC (386) for analysis. Data were analysed using pCLAMP version 5.5 and version 6.0 software (Axon Instruments). Mean current amplitudes were calculated from amplitude histograms constructed from data segments 30–60 s in duration, recorded under ATP-free conditions at a filter frequency (–3 dB) and sampling rate of 200 and 500 Hz, respectively, unless otherwise stated. Events lists were constructed from data segments 30 s in duration, from which the open probabilities were derived (filter frequency, 200 Hz; sampling rate, 500 Hz). All other filter frequencies and sampling rates are given in figure legends.

$K_{ATP}$  channel open probability was expressed as the product  $NP_o$ , where  $N$  is the number of channels in the patch and  $P_o$  is the mean open probability.  $NP_o$  was calculated by dividing the mean patch current (over a 20–30 s test period) by the mean unitary current amplitude. Unitary current amplitudes were calculated from the difference between peaks in a multiple Gaussian fit to all-points current amplitude histograms, which were constructed from data segments 20–30 s in duration.  $NP_o$  data were usually expressed in normalized form for each patch, i.e.  $NP_o(\text{test})/NP_o(\text{initial [ATP]})$ , where initial [ATP] = 0 unless otherwise stated. All single-channel recordings were made at room temperature (20–22 °C).

The activity of  $K_{ATP}$  channels in most tissues slowly decreases with time after patches are excised into the ATP-free solution. This phenomenon is known as 'run-down' (Kakei & Noma, 1984). In our studies of the sensitivity of the  $K_{ATP}$  channels to intracellular ATP, data were discarded if a significant run-down was observed when returning to an ATP-free solution after exposure to different intracellular concentrations of ATP ([ATP]<sub>i</sub>).

## Drugs

Glibenclamide (Sigma) was stored as a 10 mM stock solution in DMSO. Cromakalim and pinacidil were dissolved in DMSO at a stock concentration of 100 mM. Cromakalim (BRL 38227/Levcromakalim) was obtained from SmithKline Beecham Pharmaceuticals, Betchworth, Surrey, UK and pinacidil was obtained from Lilly Research Laboratories (Indianapolis, IN, USA). All other agents were purchased from Sigma.

## Statistics

Statistical significance was evaluated by Student's paired or unpaired *t* tests, where appropriate. Differences with values of  $P < 0.05$  were considered to be significant. Values expressed in text are means  $\pm$  s.d. unless otherwise stated.

## RESULTS

### Effects of cromakalim, pinacidil and glibenclamide on spontaneous pacemaker activity

The electrophysiological characteristics of the single SA node cells used in this study have been described previously (Irisawa *et al.* 1993; Han *et al.* 1994*a,b*). Stable spontaneous action potentials could be recorded routinely when the nystatin-perforated patch technique (Horn & Marty, 1988; Denyer & Brown, 1990; Han *et al.* 1994*b*) was used. Figure 1 illustrates the effects of two  $K_{ATP}$  channel openers, cromakalim (50  $\mu$ M) and pinacidil (50  $\mu$ M), and the  $K_{ATP}$  channel blocker, glibenclamide (10  $\mu$ M), on the spontaneous pacemaker activity of single SA node cells. In Fig. 1*A*, action potentials (from top to bottom) were recorded under control conditions, after 2 min exposure to cromakalim, and following wash-out of cromakalim (4 min). Next the cell was re-exposed to cromakalim (2 min), and then glibenclamide was added in the presence of cromakalim. In Fig. 1*B*, action potentials (from top to bottom) were recorded under control conditions, after 2 min exposure to pinacidil, after wash-out of pinacidil (4 min), after a second 2 min exposure to pinacidil, and finally after addition of glibenclamide in the presence of pinacidil. Note that both  $K_{ATP}$  channel openers, cromakalim and pinacidil, hyperpolarized the SA node cell and slowed its spontaneous pacemaker rate. In the ten cells exposed to cromakalim, the maximum diastolic potential hyperpolarized from  $-56.3 \pm 3.7$  to  $-71 \pm 5.3$  mV; pinacidil had a similar effect, hyperpolarizing cells from  $57.4 \pm 2.3$  to  $-68.8 \pm 4.4$  mV ( $n = 5$  for each drug). The spontaneous pacemaker rate was decreased by cromakalim from  $183 \pm 22$  to  $32 \pm 10$  spikes  $\text{min}^{-1}$  and by pinacidil from  $177 \pm 29$  to  $40 \pm 14$  spikes  $\text{min}^{-1}$  ( $n = 4$  for each drug). In one cell (not included in the averages) from each drug-treated group, pacemaker activity was completely abolished and the cell remained strongly hyperpolarized. These effects of cromakalim and pinacidil were reversible upon their removal, and they were quickly and almost completely blocked by the  $K_{ATP}$  channel blocker, glibenclamide. These pharmacological data suggest that modulation of  $K_{ATP}$  channels is responsible for the observed

changes in the spontaneous pacemaker rate and maximum diastolic potential during superfusion with these agents.

### Lack of effect of glibenclamide on pacemaker activity and underlying ionic currents

Interpretation of the above results would be complicated, and might be impossible, if glibenclamide altered other time- and voltage-dependent ionic currents in SA node cells. The results shown in Fig. 2 demonstrate that glibenclamide (10  $\mu$ M) does not have any significant non-specific effects in SA node cells. In Fig. 2*A*, the traces of spontaneous action potentials (from top to bottom) were recorded under control conditions, after exposure to glibenclamide for 2 and 4 min, and finally following 7 min wash-out of glibenclamide. No significant changes in the spontaneous pacemaker rate or the maximum diastolic potential were observed. The spontaneous pacemaker rate and maximum diastolic potential were  $171 \pm 31$  spikes  $\text{min}^{-1}$  and  $57.5 \pm 4.5$  mV, respectively, under control conditions; they remained at  $174 \pm 26$  spikes  $\text{min}^{-1}$  and  $54.4 \pm 5.7$  mV, respectively, after 4 min exposure to glibenclamide ( $n = 9$ ). The superimposed current traces in Fig. 2*B* also show that there are no significant effects of glibenclamide (10–15  $\mu$ M) on three time- and voltage-dependent ion currents in SA node. These are (from top to bottom): L-type  $\text{Ca}^{2+}$  current,  $I_{\text{Ca(L)}}$ , delayed rectifier  $\text{K}^+$  current,  $I_{\text{K}}$ , and hyperpolarization-activated inward current,  $I_{\text{f}}$ . In each panel 'a' denotes the control trace and 'b' the data recorded in the presence of glibenclamide (10  $\mu$ M for 4 min). The holding potential was  $-40$  mV.  $I_{\text{Ca(L)}}$  and  $I_{\text{K}}$  were activated by depolarizing to 0 and  $+30$  mV, respectively, and  $I_{\text{f}}$  was activated by hyperpolarizing to  $-100$  mV. Very similar results were obtained in each of the six SA node cells that were studied, leading us to conclude that the action of glibenclamide in the rabbit SA node results from an inhibition of  $K_{ATP}$  channels.

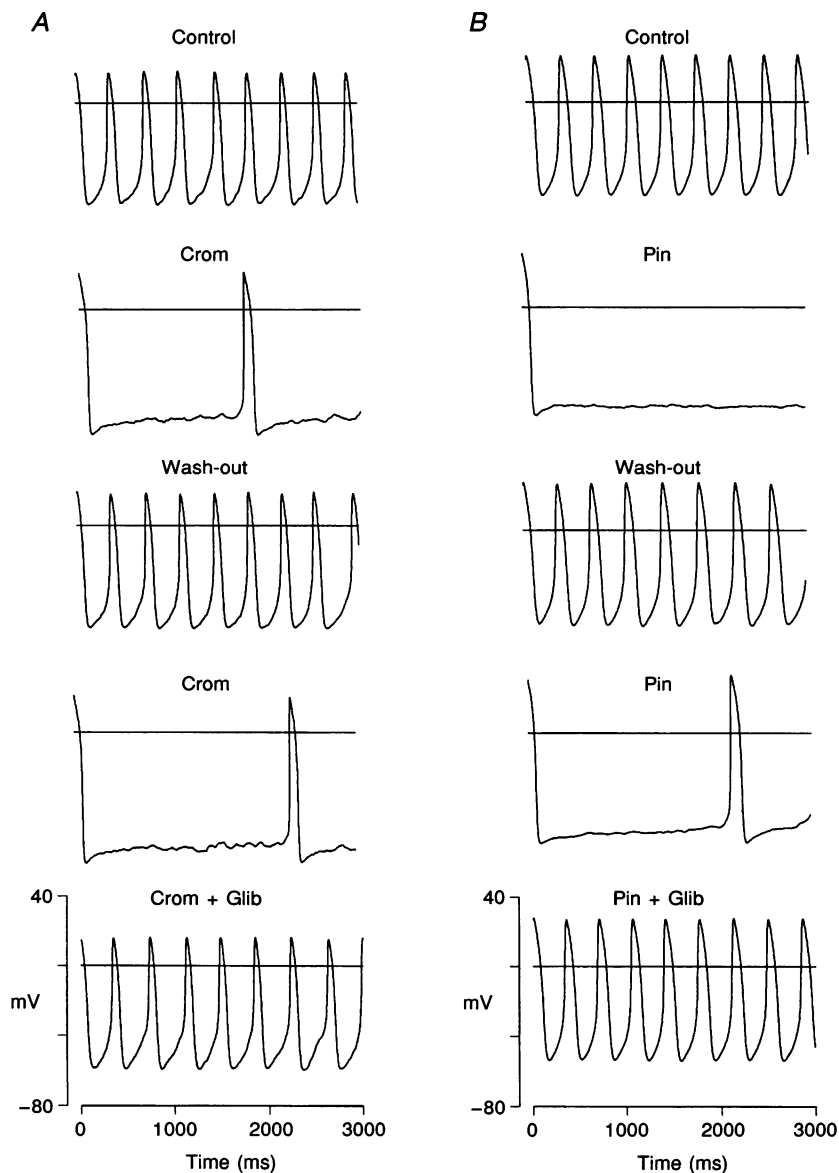
### Activation of $I_{\text{K(ATP)}}$ in SA node cells

#### Effects of $K_{ATP}$ channel openers and blockers

The hyperpolarization of the maximum diastolic potential in SA node cells suggests that cromakalim and pinacidil activate an outward current, and measurements under whole-cell voltage clamp confirmed this (Fig. 3). Figure 3*A* and *B* shows activation of  $I_{\text{K(ATP)}}$  by cromakalim (50  $\mu$ M) and pinacidil (50  $\mu$ M), respectively, as well as the blocking effect of glibenclamide (10  $\mu$ M). In both panels, the 200 ms hyperpolarizing voltage step to  $-75$  mV from a holding potential of  $-40$  mV in control conditions activated only a small current,  $I_{\text{f}}$ . Exposure to cromakalim or pinacidil for 5 min resulted in a dramatic increase in amplitude of the holding current in the outward direction. Subsequent application of glibenclamide quickly (30 s to 1 min) reduced the outward current to near control levels. Note that no significant current changes were observed at  $-75$  mV, which corresponds to the reversal potential for the induced current (Fig. 3*C*) and is very near the equilibrium potential for  $\text{K}^+$  in single SA node cells (Habuchi, Han & Giles, 1995).

The amplitude of the changes in holding current, which at  $-40$  mV was mostly  $I_{K(ATP)}$ , varied in the SA node cells that we studied. Eight cells were exposed to cromakalim; of these, one cell did not show any change in the holding current, three cells showed moderate change (ranging from  $0.03$  to  $0.1$  nA in magnitude) and the remaining four cells

developed a relatively large ( $> 0.3$  nA) outward current. In a total of four cells in which the effect of pinacidil was tested, the drug-induced activation of  $I_{K(ATP)}$  was  $< 0.1$  nA in two cells and  $> 0.2$  nA in the remaining two cells. Glibenclamide ( $10 \mu\text{M}$ ) completely blocked each cromakalim- and pinacidil-induced outward current. The current–



**Figure 1. Effects of cromakalim, pinacidil and glibenclamide on spontaneous action potentials in single cells from rabbit SA node**

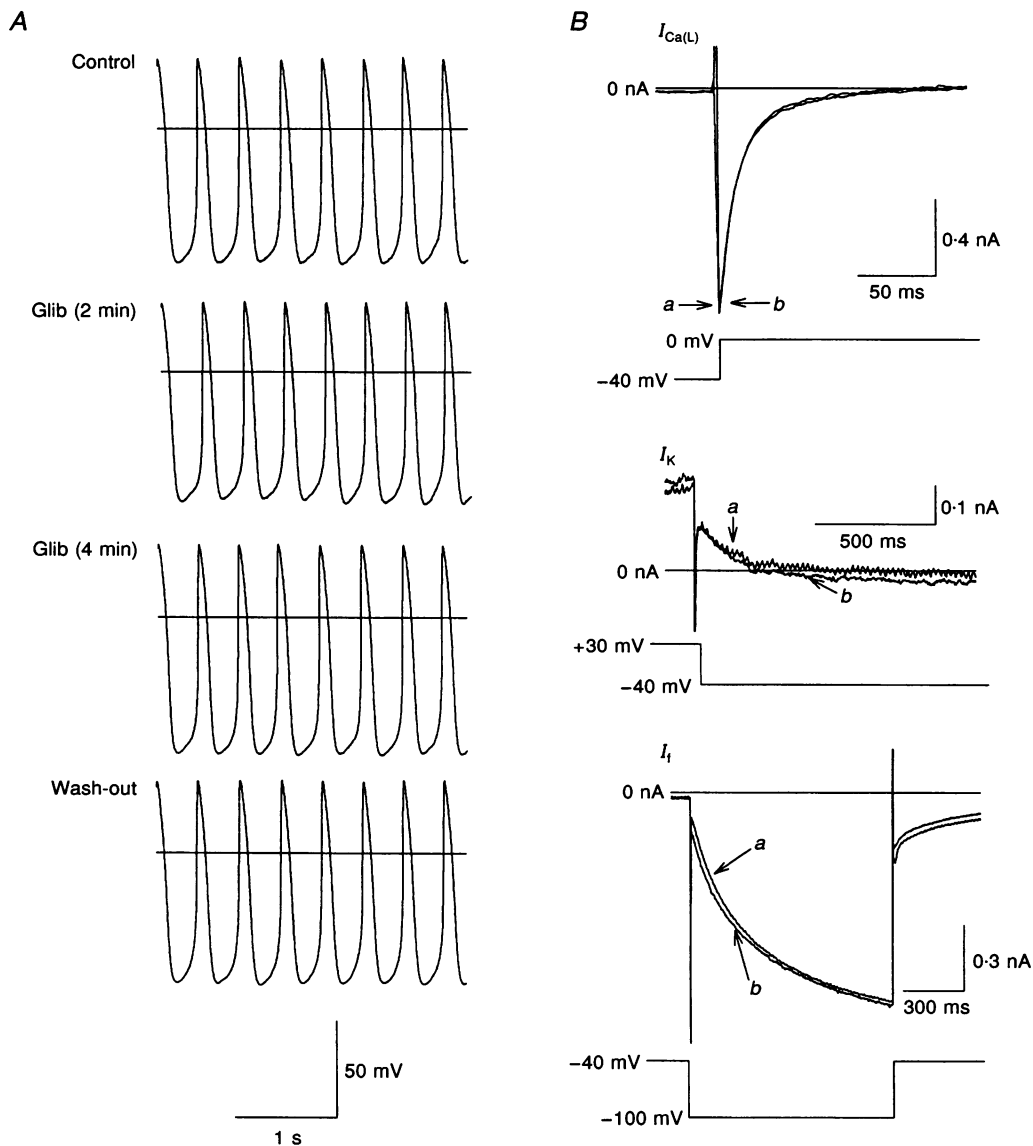
Drugs were applied at the following concentrations: cromakalim (Crom),  $50 \mu\text{M}$ ; pinacidil (Pin),  $50 \mu\text{M}$ ; and glibenclamide (Glib),  $10 \mu\text{M}$ . *A*, action potentials (from top to bottom) were recorded under control conditions, and then after 2 min exposure to cromakalim, 4 min wash-out of cromakalim, 2 min re-exposure to cromakalim, and finally 1 min after exposure to glibenclamide in the presence of cromakalim. *B*, action potentials (from top to bottom) were recorded under control conditions and then after 2 min exposure to pinacidil, 4 min wash-out of pinacidil, 2 min re-exposure to pinacidil, and 1 min after application of glibenclamide in the presence of pinacidil. The horizontal lines indicate the  $0$  mV level. Note that the  $K_{ATP}$  channel openers, cromakalim and pinacidil, markedly hyperpolarized the maximum diastolic potential (by approximately  $15$  and  $10$  mV, respectively) and slowed the pacemaker rate. These effects were almost completely reversible upon wash-out, and they were blocked by the  $K_{ATP}$  channel antagonist glibenclamide.

voltage relationship plotted in Fig. 3C was taken from a cell which showed relatively large activation of  $I_{K(ATP)}$  by cromakalim.

### Effects of metabolic inhibition

Metabolic compromise is known to activate  $I_{K(ATP)}$  in the atrial and ventricular myocardium. This effect can also be demonstrated in the SA node. In Fig. 4A the superimposed current traces were recorded under control conditions in

response to the voltage protocols illustrated. Amongst the essential features of the ionic currents in primary pacemaker cells are the absence of both inward rectifier  $K^+$  current ( $I_{K1}$ ) and transient outward  $K^+$  current ( $I_{to}$ ), and the presence of prominent  $I_{Ca(L)}$ ,  $I_f$  and  $I_K$  (Irisawa *et al.* 1993). Exposure to 3 mM sodium cyanide (NaCN), which blocks the mitochondrial respiratory chain and thus inhibits the production of ATP from this source, resulted in a large glibenclamide-sensitive outward shift in the holding



**Figure 2. Lack of a direct effect of glibenclamide on SA node cells**

A, action potentials (from top to bottom) were recorded under control conditions, following exposure to glibenclamide (Glib;  $10 \mu\text{M}$ ) for 2 and 4 min, and finally 7 min after wash-out of glibenclamide. The horizontal lines indicate the 0 mV levels. B, superimposed current traces showing the lack of direct effects of glibenclamide ( $10 \mu\text{M}$ ) on the three major currents in rabbit SA node cells (from top to bottom),  $I_{Ca(L)}$ ,  $I_K$  and  $I_f$ . Current traces denoted by 'a' were recorded under control conditions; those denoted by 'b' were recorded during superfusion with glibenclamide. The holding potential was  $-40$  mV.  $I_{Ca(L)}$  and  $I_K$  were activated by depolarizing to 0 and  $+30$  mV, respectively, and  $I_f$  was activated by hyperpolarizing to  $-100$  mV.  $I_K$  (middle) is shown as the decaying tail current seen on repolarization to  $-40$  mV. Note that glibenclamide had no significant effects on pacing rate and the maximum diastolic potential, nor did it change  $I_{Ca(L)}$ ,  $I_K$  or  $I_f$  at a concentration ( $10 \mu\text{M}$ ) which strongly blocked  $I_{K(ATP)}$ .

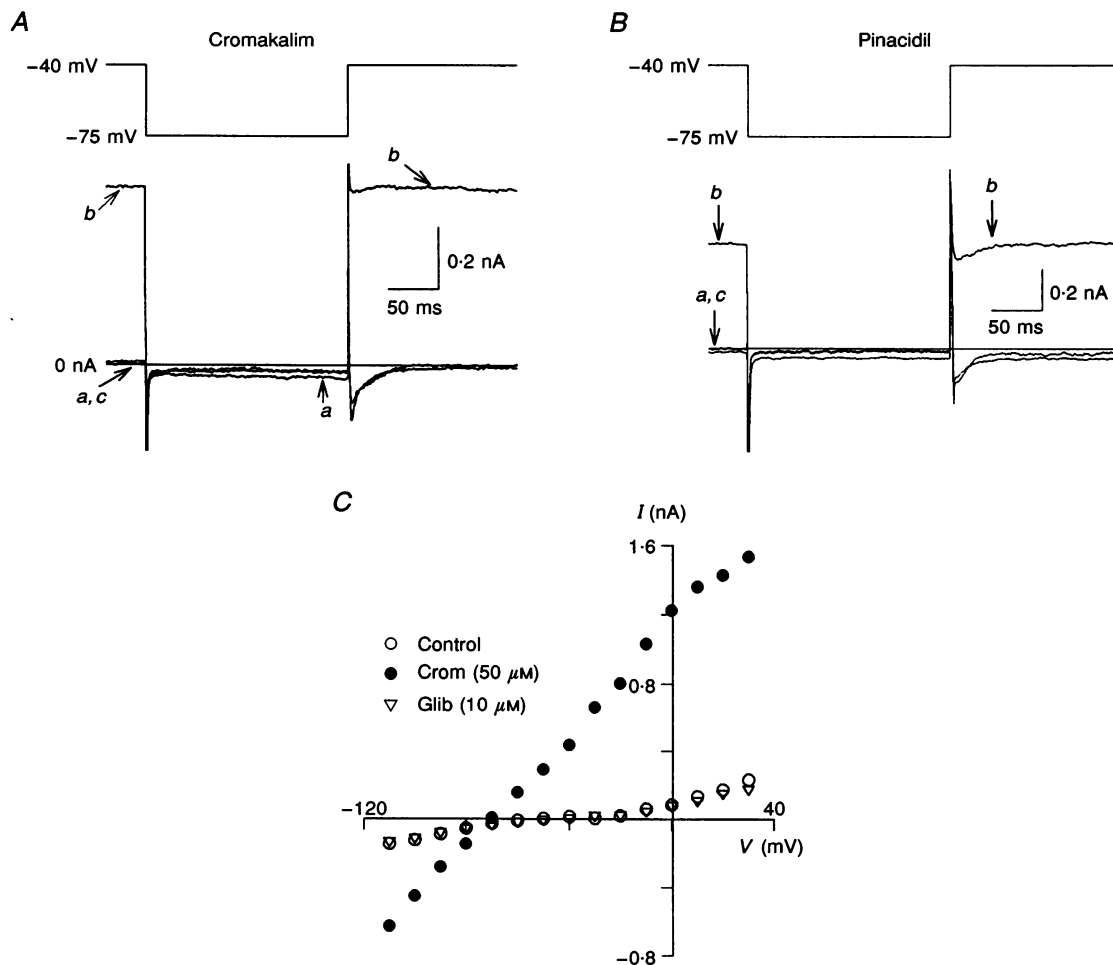
current and the appearance of a large instantaneous current seen immediately following a voltage step (Fig. 4). This NaCN-induced current reversed near  $E_K$  (approximately  $-75$  mV).

### Properties of single $K_{ATP}$ channels in SA node cells

Recordings in the inside-out patch configuration were obtained from SA node cells to identify the single-channel events underlying  $I_{K(ATP)}$  and to determine some of their biophysical properties. Upon excision of a patch into the ATP-free high- $K^+$  solution, spontaneous single-channel events could be observed consistently. This activity was abolished in the presence of  $0.5$ – $1$  mM ATP. At a holding potential of  $0$  mV, the mean unitary current amplitudes

were  $1.07 \pm 0.10$  pA (pH  $7.4$ ,  $n = 8$ ) and  $1.02 \pm 0.08$  pA (pH  $6.8$ ,  $n = 8$ ). As shown in Fig. 5C, at negative voltages, the slope conductance of the  $I$ - $V$  data recorded under symmetric conditions ( $140$  mM KCl) was  $52 \pm 8$  pS ( $n = 4$ ). Under identical conditions, patches excised from ventricular myocytes exhibited channel activity with a unitary conductance of  $70 \pm 5$  pS ( $n = 4$ ; see Fig. 5C). The open probability of the  $K_{ATP}$  channels in SA node was independent of the holding potential. Figure 5A shows a typical pattern of response of this single  $K_{ATP}$  channel activity at different values of  $[ATP]_i$  in a patch which contained several  $K_{ATP}$  channels.

As expected,  $K_{ATP}$  channel activity was inhibited in a concentration-dependent manner with increasing  $[ATP]_i$



**Figure 3.**  $I_{K(ATP)}$  activation by cromakalim and pinacidil and block by glibenclamide in SA node cells

In *A* and *B*, the voltage-clamp steps are shown on top; and the superimposed current traces are shown below. Measurements were made in control conditions (*a*), in the presence of cromakalim or pinacidil (*b*), and in the presence of cromakalim or pinacidil plus glibenclamide (*c*). Application of cromakalim ( $50 \mu\text{M}$ ; *A*), or pinacidil ( $50 \mu\text{M}$ ; *B*) resulted in a large outward shift in the holding current, which subsequently was quickly ( $30$  s to  $1$  min) and almost completely blocked by glibenclamide ( $10 \mu\text{M}$ ). *C*, the current-voltage relationships are plotted from a cell in which cromakalim produced a relatively large  $I_{K(ATP)}$ . Voltages are nominal values; a junction potential correction of  $+3$  mV should be added to approximate better the absolute transmembrane potential difference.

(Noma, 1983; for review see Nichols & Lederer, 1991). However, reducing the pH from 7.4 to 6.8 had no significant effect on the sensitivity of channels to  $[ATP]_i$ . The apparent  $IC_{50}$  for ATP at both pH 7.4 and 6.8 ( $n = 9$  and 8 patches, respectively) was between 15 and 17  $\mu M$  in the presence of internal 1.4 mM  $Mg^{2+}$  (see Fig. 5B). The ATP dose-response curves in Fig. 5B were fitted using the least-squares method (see eqn (1) below) to yield values for the Hill coefficient ( $n_H$ ) of ATP binding of 1.2 (pH 7.4) and 1.1 (pH 6.8).

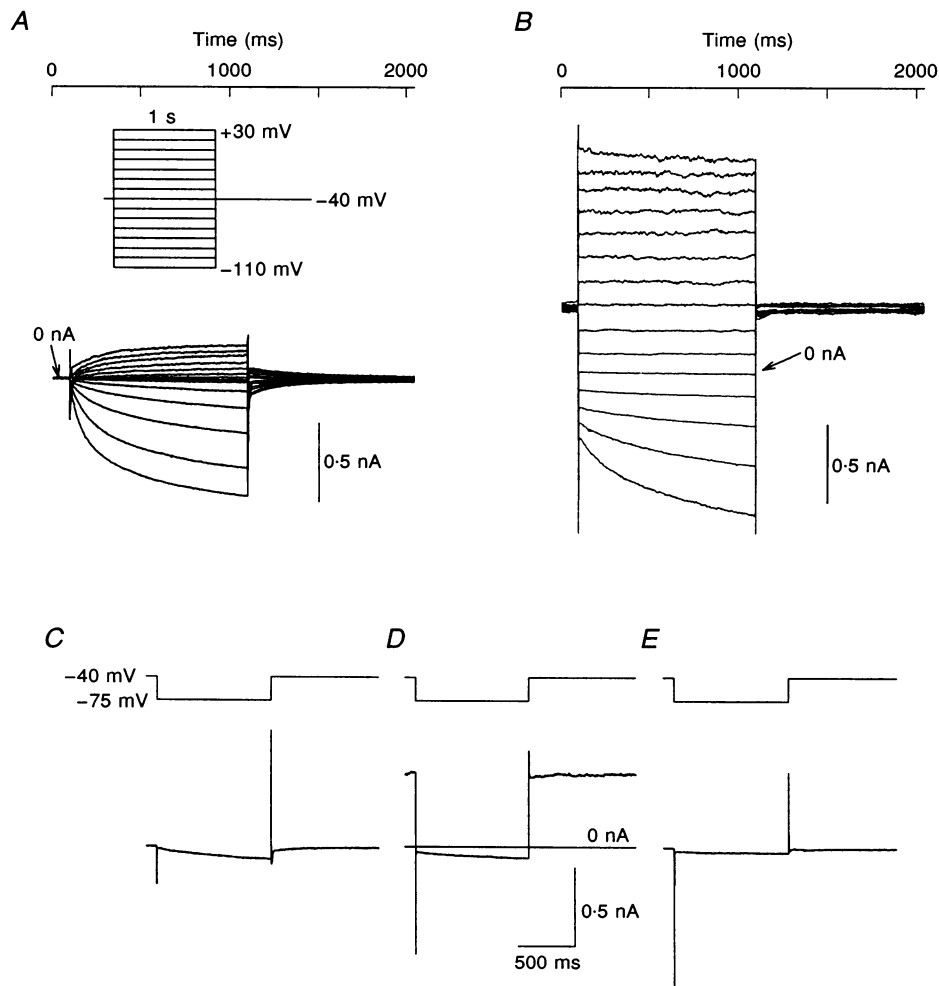
$$\begin{aligned} \text{Normalized } NP_o &= NP_o / NP_{o(\max)} \\ &= 1 / \{1 + ([ATP] / IC_{50})^{n_H}\}, \quad (1) \end{aligned}$$

where  $NP_o$  is the product of  $P_o$  (open probability) and  $N$  (the number of channels in a patch), estimated as described in the Methods.  $NP_{o(\max)}$  denotes  $NP_o$  in the absence of ATP,  $[ATP]$  is the ATP test concentration,  $IC_{50}$  signifies

the ATP concentration required for half-maximal inhibition and  $n_H$  is the Hill coefficient. The normalized  $NP_o$  is used as a measure of relative open probability in Figs 5B and 7.

### Pharmacological modulation of single $K_{ATP}$ channels from rabbit SA node

To compare our action potential and whole-cell current data with the underlying single-channel results, the pharmacological modulation of single  $K_{ATP}$  channels was studied in the presence of the agonists, cromakalim and pinacidil, and the antagonist, glibenclamide. Figure 6A shows recordings of  $K_{ATP}$  channel activity made in the excised inside-out patch configuration from a patch containing at least five channels. Elevating  $[ATP]_i$  from 0 to 50  $\mu M$  resulted in a noticeable reduction in channel activity. In the presence of 50  $\mu M$  intracellular ATP, application of cromakalim (50  $\mu M$ ) resulted in activation of channel activity to levels higher than under ATP-free



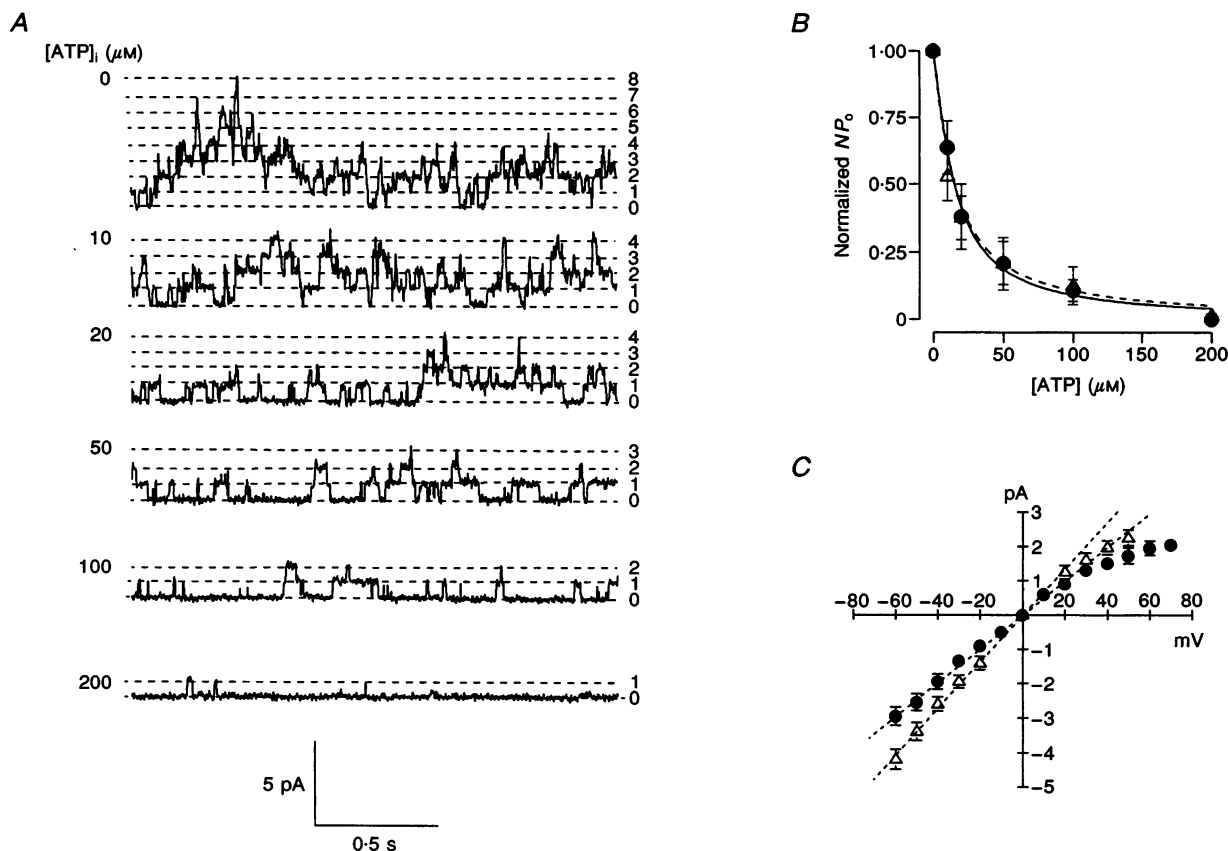
**Figure 4.** Metabolic inhibition activates  $I_{K(ATP)}$  in SA node cells

*A*, whole-cell currents recorded under control conditions. *B*, whole-cell currents recorded after NaCN (3 mM) treatment for 60 min. The three lower traces show transmembrane ionic currents before application of NaCN (*C*), in the presence of NaCN (*D*) and after addition of glibenclamide (10  $\mu M$ ), which blocks the current induced by metabolic inhibition (*E*).  $I_{Ca(L)}$  was blocked by 50  $\mu M$   $Cd^{2+}$  in these experiments.

conditions. Note that the cromakalim-induced activity was reduced markedly by 10  $\mu\text{M}$  glibenclamide (Fig. 6A and E). Data from several patches are summarized quantitatively in Fig. 7A. Very similar results were obtained using a different  $\text{K}_{\text{ATP}}$  channel opener, pinacidil (see Fig. 7B).

The efficacy of the  $\text{K}_{\text{ATP}}$  channel openers, cromakalim and pinacidil, in activating  $\text{K}_{\text{ATP}}$  channels is reflected in the changes in normalized  $NP_o$  in Fig. 7, which includes pooled data from excised inside-out patches. In Fig. 7A, 50  $\mu\text{M}$  ATP significantly reduced  $NP_o$  to  $0.21 \pm 0.14$  ( $n = 8$ ) of maximal activity recorded in 0 mM ATP; application of 50  $\mu\text{M}$  cromakalim in the presence of 50  $\mu\text{M}$  ATP increased  $NP_o$  to  $0.81 \pm 0.24$ . In the presence of both intracellular ATP and cromakalim, 10  $\mu\text{M}$  glibenclamide suppressed the  $NP_o$  to  $0.16 \pm 0.15$ . In Fig. 7B, 50  $\mu\text{M}$  ATP caused a reduction of  $NP_o$  to  $0.32 \pm 0.17$  ( $n = 5$ ) of maximal activity and 100  $\mu\text{M}$  pinacidil increased the  $NP_o$  to

$0.72 \pm 0.18$ . In the presence of both intracellular ATP and pinacidil (50 and 100  $\mu\text{M}$ , respectively), application of 10  $\mu\text{M}$  glibenclamide reduced the  $NP_o$  to  $0.12 \pm 0.14$ , a level which is even lower than that observed with 50  $\mu\text{M}$  ATP alone. Upon removal of cromakalim and pinacidil,  $\text{K}_{\text{ATP}}$  channel activity quickly returned to levels observed before drug exposure. In contrast, attempts to wash out glibenclamide resulted in only a slow and partial restoration of channel activity in each of the patches studied. It should be noted that, at the single-channel level, the extent to which cromakalim and pinacidil activated  $\text{K}_{\text{ATP}}$  channels was variable and in several patches in the presence of 50  $\mu\text{M}$  ATP (see Fig. 6A), cromakalim increased the maximum number of channels observed simultaneously open above that seen in the absence of ATP. The error bars (Fig. 7A) in the presence of cromakalim reflect this variability.



**Figure 5.** Properties of single  $\text{K}_{\text{ATP}}$  channels in SA node

A, single-channel recordings obtained from an inside-out patch containing at least 8 channels. Data segments show channel activity at selected intracellular ATP concentrations (pH 7.4; holding potential, 0 mV). The horizontal dashed lines denote the current which was observed at each unitary level. B, effect of ATP on the open probability, represented by normalized  $NP_o$  (see text), of  $\text{K}_{\text{ATP}}$  channels in the inside-out patch configuration at pH 7.4 ( $\bullet$ ) and 6.8 ( $\Delta$ ). The continuous line is the fit of eqn (1) to the data at pH 7.4, the dashed line is the fit to data at pH 6.8. Points were calculated from data segments 30 s in duration. C, current-voltage relationship for single SA node  $\text{K}_{\text{ATP}}$  channels ( $\bullet$ ) and single  $\text{K}_{\text{ATP}}$  channels from rabbit ventricular myocytes ( $\Delta$ ) recorded under symmetrical  $\text{K}^+$  (140 mM) conditions (see text for solutions). The unitary slope conductance at negative voltages was  $52 \pm 8$  and  $70 \pm 5$  pS for SA node and ventricular myocyte  $\text{K}_{\text{ATP}}$  channels, respectively. Sampling rate and filter frequency were 500 and 200 Hz, respectively. Bars in B and C represent  $\pm$  s.d.

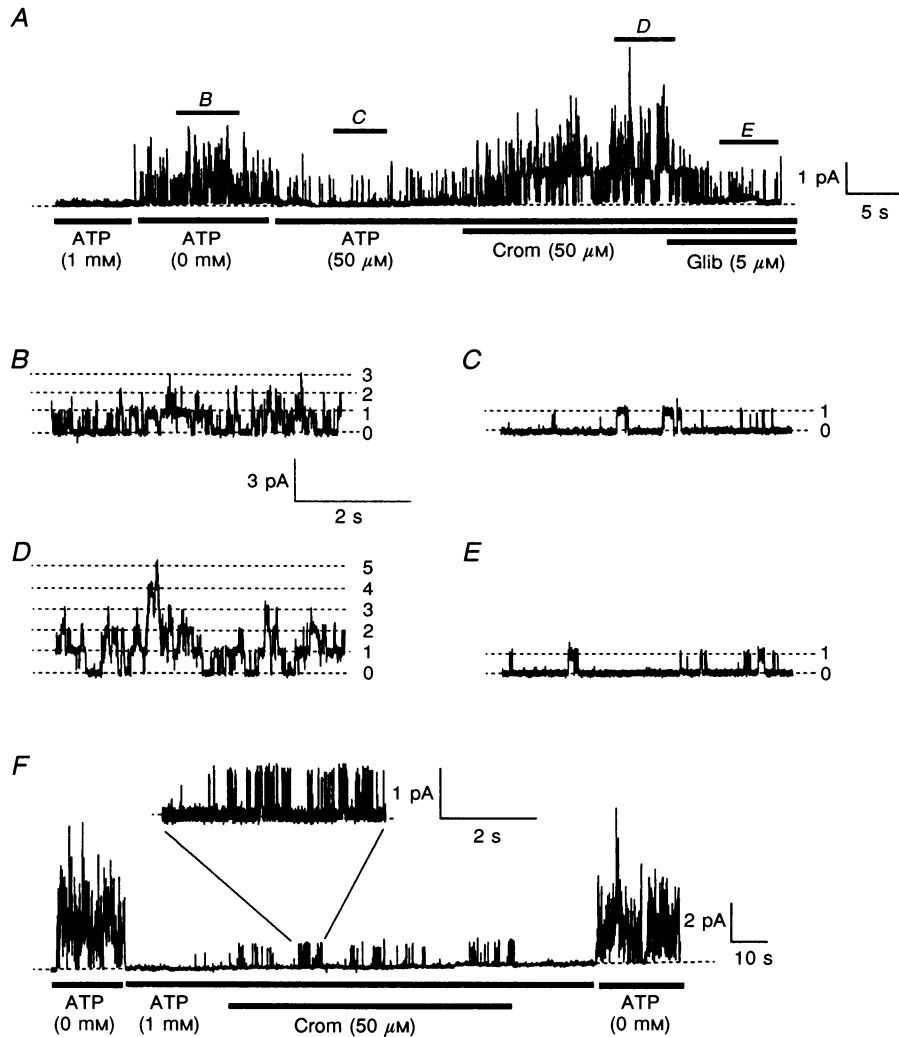


In the above experiments both cromakalim and pinacidil consistently activated  $K_{ATP}$  channels in the presence of  $50 \mu\text{M}$  ATP. However, this concentration of ATP is much lower than the physiological ATP level in intact mammalian heart, which is in the range of 1–10 mM (Nichols & Lederer, 1991; Traut, 1994). Accordingly, four experiments were done in which cromakalim was applied in the presence of 1 mM ATP. As expected,  $K_{ATP}$  channel activity was almost completely abolished in the presence of 1 mM ATP (normalized  $NP_o < 0.001$ , see Fig. 6*F*). Note,

however, that even in the presence of 1 mM ATP,  $K_{ATP}$  channel activity was substantially increased by application of  $50 \mu\text{M}$  cromakalim (normalized  $NP_o = 0.003\text{--}0.01$ ,  $n = 4$ , see Fig. 6*F*) and removal of cromakalim resulted in channel activity being abolished.

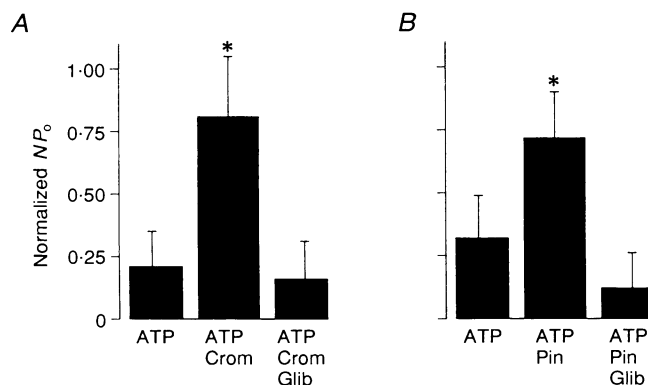
### Open and closed times for a single $K_{ATP}$ channel from SA node

The high density of  $K_{ATP}$  channels in SA node cells made it impossible to excise patches containing only one channel,



**Figure 6.** The effects of cromakalim and glibenclamide on SA node  $K_{ATP}$  channel activity

*A*, single-channel currents recorded from an inside-out patch at a holding potential of 0 mV. This patch contained at least 5 channels. Sampling rate and filter frequency were 250 and 100 Hz, respectively. *B–E*, expanded data segments of 5 s duration corresponding to the filled bars labelled in *A*. Sampling rate and filter corner frequency (lowpass) were 1 kHz and 500 Hz, respectively. Dotted lines denote unitary single-channel current levels. *F*, activation of  $K_{ATP}$  channels in rabbit SA node by  $50 \mu\text{M}$  cromakalim in the presence of 1 mM ATP. Single-channel currents recorded from a single excised inside-out patch containing at least 6 channels. Sampling rate and filter frequency were 250 and 100 Hz, respectively. *F* (inset), expanded data segment corresponding to the section of data indicated. Sampling rate and filter frequency were 1 kHz and 500 Hz, respectively. Dotted line denotes closed level. The pipette contained the following solution (mM): NaCl, 140; KCl, 5; Hepes, 10;  $\text{CaCl}_2$ , 1;  $\text{MgCl}_2$ , 1; and glucose, 10; pH 7.4. The bath/internal solution contained (mM): KCl, 140; Hepes, 10; EGTA, 5;  $\text{MgCl}_2$ , 1.4; and glucose, 10; pH 7.4. No significant run-down was observed during the course of these experiments.

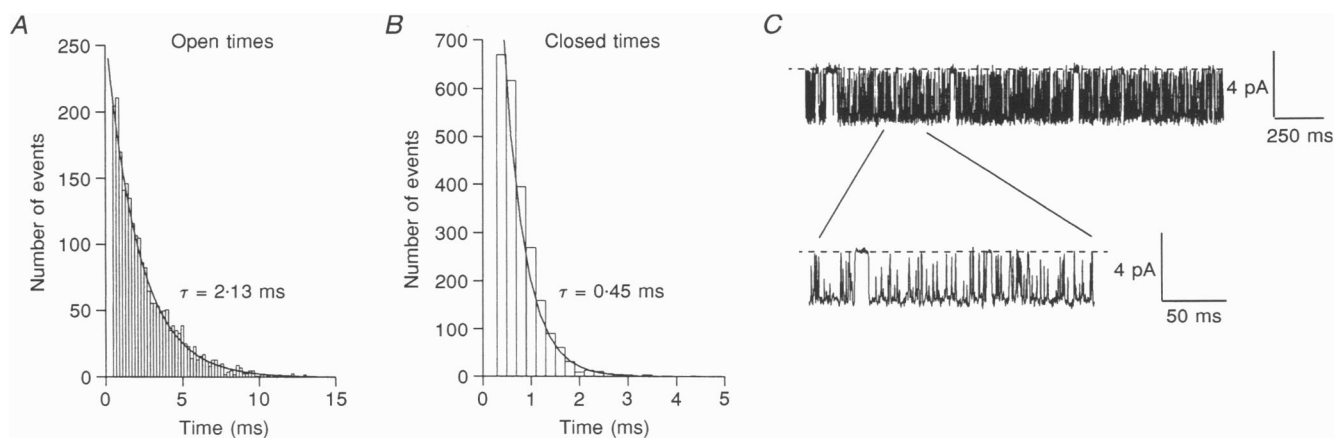


**Figure 7. Effects of cromakalim and pinacidil on the open probability of K<sub>ATP</sub> channels recorded from inside-out patches**

Changes in open probability are reflected by changes in the normalized NP<sub>o</sub>, as defined in the text. *A*, effect of 50 μM cromakalim and 10 μM glibenclamide in the presence of 50 μM intracellular ATP. *B*, effect of 100 μM pinacidil and 10 μM glibenclamide in the presence of 50 μM intracellular ATP. Bars represent the s.d. (8 patches in *A*, 5 patches in *B*). Data were taken from record segments 30 s in duration at a sampling rate and filter frequency of 500 and 200 Hz, respectively. The asterisks denote results ( $P < 0.05$ ) significantly different from data in the presence of 50 μM ATP alone, or in the presence of both the potassium channel opener and glibenclamide.

even when very small high-resistance (10–15 MΩ) pipettes were used. However, in the example shown in Fig. 8, only a single channel showed significant activity. A second channel was present in the patch, but opened rarely. If these two channels exhibited identical and independent behaviour, they would have been open simultaneously 68% of the time, based on the measured open and closed times. This is clearly not the case. It was therefore possible to measure the open and closed times of a single channel. Only a single

exponential component was observed for either open or closed times within a burst of activity. In this respect, and based on the measured values for mean open time (2.1 ms) and closed time (0.45 ms), the kinetics for this channel were very similar to those measured for K<sub>ATP</sub> channels from rat ventricular myocytes under similar, but not identical, conditions (Zilberter, Burnashev, Papin, Portnov & Khodorov, 1988).



**Figure 8. Open and closed time distributions of a single K<sub>ATP</sub> channel from SA node**

Open (*A*) and closed time histograms (*B*) were constructed from a 15 s segment of data recorded immediately after exposing the patch to ATP-free conditions from an initial ATP concentration of 1 mM. Potassium concentration was 140 mM on both sides of the membrane; holding potential was  $-60$  mV. The numbers of events used to construct the histograms in *A* and *B* were 2334 and 1715, respectively. Both histograms were fitted with a single exponential using a least-squares method, and time constants ( $\tau$ ) are shown above each curve. *C*, single-channel trace of 2 s duration taken during the 15 s data segment used to construct the histograms. *C* (inset), expanded section of data (200 ms) from the section indicated in *C*. The data were filtered at 2 kHz and sampled at 5 kHz. Dashed line denotes zero current level.

## DISCUSSION

### $K_{ATP}$ channels from SA node: a distinct isoform?

Overall, our data show that SA node tissue expresses  $K_{ATP}$  channels that share the major pharmacological properties with  $K_{ATP}$  channels from other heart tissues. However, their unitary conductance and their reduced sensitivity to intracellular pH suggest that the channels from SA node represent a distinct subtype of this  $K^+$  channel family.

### Pharmacological properties

Glibenclamide selectively blocks  $K_{ATP}$  channels (Escande, 1989; Hamada *et al.* 1990) when applied at concentrations up to 100  $\mu\text{M}$ . In the present study, glibenclamide at concentrations of 10–15  $\mu\text{M}$  did not affect the pacemaker rate or the maximum diastolic potential of single SA node cells in the absence of the  $K_{ATP}$  channel openers (Fig. 2A). Moreover, there was no significant effect on any of the three time- and voltage-dependent ionic currents,  $I_{Ca(L)}$ ,  $I_K$  and  $I_f$ , which underlie SA node pacemaker activity (Fig. 2B). Thus, inhibition of the cromakalim-, pinacidil- and NaCN-induced whole-cell and/or single-channel currents by glibenclamide can be attributed to specific block of  $K_{ATP}$  channels.

Either cromakalim or pinacidil increased single-channel activity in excised patches from SA node, and glibenclamide inhibited this channel activity. These observations are consistent with our whole-cell data, and they confirm that  $K_{ATP}$  channels are expressed in SA node, and that pacemaker rhythm is decreased by selective activation of  $K_{ATP}$  channels.

### Single-channel properties

We have identified unitary potassium current fluctuations that are sensitive to ATP. This result, together with the insensitivity to voltage, inward rectification at positive potentials and inhibition by glibenclamide, confirms that these events are due to the activity of  $K_{ATP}$  channels. The single-channel conductance measured in our study was 52 pS at room temperature (20–22 °C). This is smaller than the value we measured in ventricular myocytes under identical conditions (70 pS, Fig. 5C; but see Zilberter *et al.* 1988). It is also smaller than has generally been observed in other cardiac cell types, or in smooth muscle (for review see Nichols & Lederer, 1991), but considerably larger than a value measured in follicular cells (19 pS, Honoré & Lazdunski, 1993). These findings show that the conductance of  $K_{ATP}$  channels varies widely among different species and cell types, and may provide a useful criterion for identifying different isoforms of the channel.

Intraburst open and closed times for the SA node  $K_{ATP}$  channel were similar to the fast components seen in studies of ventricular myocytes (Zilberter *et al.* 1988; McLarnon, Hamman & Tibbits, 1993). No evidence for a second, longer closed time was obtained; and previous reports describing

the existence and duration of this component have been variable (Kakei & Noma, 1984; Zilberter *et al.* 1988; McLarnon *et al.* 1993). The kinetic properties of the  $K_{ATP}$  channels in SA node, therefore, do not distinguish them from  $K_{ATP}$  channels in other cardiac cell types.

### Dependence of channel activity on ATP and pH

Fits to our data yielded a Hill coefficient for ATP binding in the range of 1.1–1.2. Values similar to this have been published from measurements on rabbit ventricular myocytes (Han, So, Kim & Earm, 1993), but values of  $\sim 2$  have been reported in other studies (for example, see Nichols *et al.* 1990; Light, Allen, Walsh & French, 1995; for review see Nichols & Lederer, 1991). The sensitivity to intracellular ATP ( $IC_{50} = 16 \mu\text{M}$ ) of SA node  $K_{ATP}$  channels is similar to that observed in atrial and ventricular myocytes (Findlay, 1988; Lederer & Nichols, 1989; Light *et al.* 1995); however,  $IC_{50}$  values as high as 114  $\mu\text{M}$  have also been reported (Nichols *et al.* 1990).

It is noteworthy that lowering intracellular pH from 7.4 to 6.8 did not alter the intracellular ATP dependence of the  $K_{ATP}$  channels in SA node. In ventricular myocytes (Koyano, Kakei, Nakashima, Yoshinaga, Matsuoka & Tanaka, 1993) and skeletal muscle (Standen, 1992), lowering of intracellular pH during metabolic insults significantly increases the open probability of these channels, even at physiological levels of intracellular ATP. The lack of sensitivity of SA node  $K_{ATP}$  channels to a decrease in intracellular pH may help to stabilize heart rate by limiting the degree of activation of  $I_{K(ATP)}$  during ischaemia.

### Metabolic inhibition and $K_{ATP}$ channel activation

Metabolic compromise which inhibits ATP generation has been shown to activate the  $K_{ATP}$  channels in ventricular myocardium (Weiss *et al.* 1992; Wilde & Janse, 1994). This effect was also demonstrated in the present study in SA node (Fig. 4). However, additional more complex changes in the transmembrane action potentials and ionic currents are also observed when SA node cells are exposed to metabolic inhibition (Han, Habuchi & Giles, 1994*b*).

### Physiological and pathophysiological implications

#### $K_{ATP}$ channel activity during normal metabolism

Most reports suggest that  $K_{ATP}$  channel blockers do not affect the cardiac action potential configuration (e.g. Fig. 2) and this has been taken as evidence that  $K_{ATP}$  channels are not activated to a significant degree under normal conditions (e.g. Wilde & Janse, 1994). Below we suggest that, in rabbit SA node,  $K_{ATP}$  channel activity may be so close to levels that would substantially influence action potentials that normal regulatory mechanisms, as distinct from a metabolic emergency, could shift  $K_{ATP}$  channel activity into and out of a range that would exert an important influence on the electrical activity of the heart.

In SA node pacemaker cells, cromakalim activates  $K_{ATP}$  channels (Fig. 6F) even in the presence of 1 mM ATP. This demonstrates that  $K_{ATP}$  channel openers can modulate  $I_{K(ATP)}$  under conditions approaching those in intact cells, consistent with our whole-cell recordings. To examine this issue in more detail, we calculated the open probability of SA node  $K_{ATP}$  channels using parameters from our whole-cell data. Addition of  $K_{ATP}$  channel openers causes a 10–15 mV hyperpolarization, from approximately –60 to –75 mV. Assuming ohmic behaviour (single channel conductance  $\sim 50$  pS) over this limited range, and knowing the input resistance for a typical cell ( $\sim 10^9 \Omega$ ) we calculated the current ( $\sim 10$  pA) required to produce this shift in maximum diastolic potential. The result implies that about thirty channels per cell are open simultaneously. The surface area of an SA node cell was estimated from its capacitance ( $\sim 35$  pF). The  $K_{ATP}$  channel density in SA node cells was estimated to be  $\sim 3 \mu\text{m}^{-2}$  from the average pipette diameter and the average number of channels observed per patch. Using this density, we calculated that a  $K_{ATP}$  channel open probability of  $\sim 0.003$  would be required to produce the observed hyperpolarization. This prediction is close to the open probability of 0.003–0.01, which we measured using inside-out patches when cromakalim was applied in the presence of a nearly physiological (1 mM) ATP concentration. Thus, even minimal activation of the  $K_{ATP}$  channels could result in significant changes in the maximum diastolic potential and action potential configuration (cf. Nichols & Lederer, 1991). Consequently, SA node must be kept in mind as a potential site of action when  $K_{ATP}$  channel agonists are administered, or when interactions with  $K_{ATP}$  channels of transmitters, such as adenosine or acetylcholine (Li, Feng, Shrier & Nattel, 1995), are considered.

Based on our dose–response data (Fig. 5B), an ATP concentration of about 2 mM would give  $P_o = 0.003$  in the absence of any extrinsic  $K_{ATP}$  channel modulators. This [ATP] is within the normal range of 1–10 mM (Nichols & Lederer, 1991; Traut, 1994). Furthermore, our recent work on  $K_{ATP}$  channels from rabbit ventricular myocytes (Light *et al.* 1995) indicates that, in excised patches, phosphorylation by purified protein kinase C reduces  $K_{ATP}$  channel open probability. In contrast, at 1 mM ATP, phosphorylation by protein kinase C upregulates activity (P. E. Light, A. A. Sabir, B. G. Allen, M. P. Walsh & R. J. French, unpublished observations). These mechanisms could easily change, by 10-fold, the ATP concentration at which  $K_{ATP}$  channels attain an open probability of  $\sim 0.003$  and thus begin to exert a significant influence on the action potential. These predictions depend on the assumption that the Hill equation (eqn (1)) accurately describes  $P_o$  down to very low levels. Nonetheless, they make the important point that a realistic, small modulation of  $K_{ATP}$  channels could raise their activity to a level that would significantly affect action potential waveform, pacemaker depolarization

and spontaneous rhythm in the SA node, as well as in ventricle.

### Modulation of pacemaker activity

In our study, both cromakalim and pinacidil significantly hyperpolarized the maximum diastolic potential and slowed the pacemaker rate in SA node cells. These effects were observed with the nystatin-perforated patch technique (which should maintain  $[\text{ATP}]_i$  at nearly physiological levels) and were blocked by glibenclamide. The results demonstrate that pharmacological modulation of SA node  $K_{ATP}$  channels is capable of affecting heart rate, and they provide an alternative rationale for the negative chronotropic effects of some  $K_{ATP}$  channel openers, which have been observed in whole heart preparations (Satoh & Hashimoto, 1984; Taira, 1987; Gotanda *et al.* 1988). Thus this tissue must be considered as a potential target whenever  $K_{ATP}$  channel modulators are administered. The fact that metabolic depletion can activate  $I_{K(ATP)}$  in SA node cells suggests that this conductance may be important in sinus node artery disease (Alboni *et al.* 1991) and in the pathogenesis of sick sinus syndrome, which is a common correlate of cardiac ischaemia (Shaw *et al.* 1987).

The pharmacological action of  $K_{ATP}$  channel modulators is complex, and this is compounded by our demonstration that  $K_{ATP}$  channels are present in the primary pacemaker tissue. Although counter-examples have been reported under certain specific conditions,  $K_{ATP}$  channel openers generally protect myocardial tissue against ischaemic damage but also tend to be pro-arrhythmic. On the other hand,  $K_{ATP}$  channel blockers can specifically block hypoxia/ischaemia-induced antiarrhythmias but also lead to increased tissue damage (e.g. Hiraoka, Sawanobori, Adaniya & Yukisada, 1994). Extensive discussion of these issues is provided in the ‘Viewpoint’ and ‘Feedback’ sections of the January (1994) issue of *Cardiovascular Research* (volume 28, no. 1). Our own work and other recent studies suggest the feasibility of developing new drugs that are more precisely targeted, and hence less likely to cause damaging side-effects. First, our data indicate that the SA node  $K_{ATP}$  channels differ in subtle, but perhaps functionally important, ways from those in ventricular myocytes. Second, although their complete molecular identity is not established,  $K_{ATP}$  channels appear to belong to the family of inwardly rectifying  $K^+$  channels which, like the voltage-dependent  $K^+$  channel family, probably shows extensive heterogeneity of functional properties based on a heteromeric assembly of subunits (Ashford, Bond, Blair & Adelman, 1994; Krapivinsky, Gordon, Wickham, Velimirovic, Krapivinsky & Clapham, 1995). Third, a  $K_{ATP}$  channel blocker that specifically inhibits ischaemia-induced effects has already been reported (McCullough, Normandin, Conder, Sleph, Dzwonczyk & Grover, 1991). Together, these points suggest that systematic attempts to identify  $K_{ATP}$  channel isoforms specific to

different cell types and tissues, and to document their properties and develop isoform-specific pharmacological modulators should be a fruitful avenue for future work. If, for example, cardiac rhythm could be modulated solely through action on K<sub>ATP</sub> channels in the SA node, pro-arrhythmic effects resulting from dispersion of refractory period in the ventricle would be avoided, as would side-effects in vascular smooth muscle, pancreas and other tissues.

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### Acknowledgements

We thank the Medical Research Council of Canada (MRC), the Heart and Stroke Foundation of Alberta, and the Alberta Heritage Foundation for Medical Research (AHFMR) for financial assistance. X.H. held a postdoctoral fellowship from the MRC; W.R.G. is an AHFMR Medical Scientist and R.J.F. is an AHFMR Senior Scholar. Ms K. Burrell provided skilled secretarial assistance.

Received 24 January 1995; accepted 20 July 1995.