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Rapid Report: An ATP-sensitive K+ conductance in dissociated neurones from adult rat intracardiac ganglia

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Abstract

1. An ATP-sensitive K+ (KATP) conductance has been identified using the perforated patch recording configuration in a population (52%) of dissociated neurones from adult rat intracardiac ganglia. The presence of the sulphonylurea receptor in approximately half of the intracardiac neurones was confirmed by labelling with fluorescent glibenclamide-BODIPY.

2. Under current clamp conditions in physiological solutions, levcromakalim (10 µM) evoked a hyperpolarization, which was inhibited by the sulphonylurea drugs glibenclamide and tolbutamide.

3. Under voltage clamp conditions in symmetrical (140 mM) K+ solutions, bath application of levcromakalim evoked an inward current with a density of ~8 pA pF_1 at _50 mV and a slope conductance of ~9 nS, which reversed close to the potassium equilibrium potential (*E*K). Cell dialysis with an ATP-free intracellular solution also evoked an inward current, which was inhibited by tolbutamide.

4. Bath application of either glibenclamide (10 µM) or tolbutamide (100 µM) depolarized adult intracardiac neurones by 3–5mV, suggesting that a KATP conductance is activated under resting conditions and contributes to the resting membrane potential.

5. Activation of a membrane current by levcromakalim was concentration dependent with an EC50 of 1.6 µM. Inhibition of the levcromakalim-activated current by glibenclamide was also concentration dependent with an IC50 of 55 nM.

6. Metabolic inhibition with 2,4-dinitrophenol and iodoacetic acid or superfusion with hypoxic solution (*P*O2 ~16 mmHg) also activated a membrane current. These currents exhibited similar *I*–*V* characteristics to the levcromakalim-induced current and were inhibited by glibenclamide.

7. Activation of KATP channels in mammalian intracardiac neurones may contribute to changes in neural regulation of the mature heart and cardiac function during ischaemia–reperfusion.

Introduction

The location of the mammalian intracardiac ganglia in the atrial epicardium makes them susceptible to the effects of myocardial ischaemia and reperfusion associated with coronary heart disease (Horackova & Armour, 1995; Armour, 1999). Each ganglionated plexus in the human heart is perfused by two or more arterial branches that arise from different major coronary arteries and the firing activity of intrinsic cardiac neurones is modified by transient coronary occlusion (Huang et al. 1993). Coronary occlusion results in a lack of oxygen and metabolic substrates to the ganglia as well as a build up of the local products of ischaemia such as oxygen-derived free radicals. Hypoxia has been shown to activate ATP-sensitive K+ (KATP) channels in numerous cell types (pancreatic β cells, cardiac, skeletal and smooth muscle cells, endothelial cells, central neurones), which serve to transduce changes in cell

metabolism into changes in membrane potential. KATP channels are closed in the presence of micromolar concentrations of cytoplasmic ATP and are open when the ATP concentration decreases below a threshold. KATP channels can be identified pharmacologically by their sensitivity to the K+ channel openers levcromakalim, pinacidil and diazoxide, and to the sulphonylurea drugs glibenclamide and tolbutamide (see Edwards & Weston, 1993).

KATP channels have been suggested to be present in the canine intracardiac ganglia and to be functionally important during oxidative challenge. Recordings from canine intracardiac ganglia in situ using extracellular microelectrodes demonstrate that administration of cromakalim in the local blood supply causes a decrease in neuronal activity (Thompson et al. 1998). Recently, sensory neurones in the guinea-pig enteric nervous system have been shown to sense changes in extracellular glucose levels via KATP channel activity and are sensitive to tolbutamide (Liu et al. 1999). However, to our knowledge, there has been no report of KATP channels in mammalian autonomic neurones. KATP channel activation may contribute to changes in neuronal excitability in response to ischaemia (hypoxia and/or metabolic inhibition) by inducing membrane hyperpolarization. Our results provide evidence for an ATP-sensitive K+ conductance in adult rat intracardiac neurones. Preliminary reports of some of these results have been presented to The Physiological Society (Hogg & Adams, 2000).

Methods

The isolation of parasympathetic neurones from neonatal and adult rat intracardiac ganglia has been described previously (Hogg *et al.* 2001). Briefly, neonatal (2-8 days) and young adult Wistar rats (> 6 weeks, 160-180 g) were killed by stunning and cervical dislocation in accordance with the guidelines of the University of Queensland Animal Experimentation Ethics Committee. The hearts were excised, the atria isolated and the intracardiac ganglia dissected from the fat pads of the dorsal surfaces of the atria. The ganglia were incubated in a saline solution containing either 0.1 mg ml^{-1} trypsin (Sigma) and 0.8-1 mg ml^{-1} collagenase (Worthington-Biochemical Type 2), or collagenase only, at 37 °C for 60 min. The ganglia were dispersed by trituration and dissociated neurones were plated on laminin-coated glass coverslips and incubated at 37 °C under a 95 % air:5 % CO_2 atmosphere for 24-72 h.

Recordings were made using the perforated patch whole-cell configuration with a final concentration of 240 µg ml^{-1} amphotericin B in 0.4 % DMSO in the pipette solution. Patch electrodes were pulled from thin-walled borosilicate glass (GC150TF; Harvard Apparatus Ltd, Edenbridge, Kent, UK) and after fire polishing had resistances of ≈1 MΩ. Access resistances using the perforated patch configuration were 4-8 MΩ before series resistance compensation, which was routinely 80 %. Membrane current and voltage were recorded using an Axopatch 200A patch clamp amplifier (Axon Instruments Inc., Union City, CA, USA), filtered at 1 kHz and digitized at 5 kHz (Digidata 1200A interface, Axon Instruments Inc.) to be stored on the hard disk of a PC for viewing and analysis. Voltage ramps were applied using pCLAMP software (version 6.1.3, Axon Instruments Inc.); five ramps (65 mV s^{-1}) applied at 300 ms intervals were averaged. Slope conductances were determined from the net current activated by levcromakalim or under metabolic inhibition or hypoxic conditions. The Hill equation was fitted to the data using the least squares non-linear curve fitting routine in Microcal Origin 6.0 (Microcal Software Inc., Northampton, MA, USA). No corrections were made for liquid junction potentials. Data are presented as means ±s.e.m.

Labelling with glibenclamide-BODIPY

The sulphonylurea receptor (SUR) was detected in adult rat intracardiac neurones by fluorescent glibenclamide-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) (Molecular Probes Inc., Eugene, OR, USA). Dissociated neurones were incubated with 40 nm glibenclamide-BODIPY at 4 °C for 30 min. Under these conditons, labelling should be specific, as previously described (Löhrke *et al.* 1997). Neurones were viewed on a BioRad M600 UV laser confocal microscope system, with standard settings.

Solutions

The control external solution was physiological saline solution (PSS) containing (mm): 140 NaCl, 3 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 7.7 glucose and 10 Hepes-NaOH, pH 7.2. The pipette solution for perforated patch whole-cell recordings contained (mm): 75 K_2SO_4, 55 KCl, 5 $MgSO_4$ and 10 Hepes-*N*-methyl-d-glucamine, pH 7.2. For dialysed whole-cell experiments, the pipette solution contained (mm): 140 KCl, 1.2 $MgCl_2$, 2 K_4BAPTA, 0.5 KADP and 10 Hepes-KOH, pH 7.2. The extracellular K^+ concentration was changed by equimolar substitution of KCl for NaCl. Symmetrical 140 mm K^+ solutions contained 5 mm TEACl extracellularly. The osmolality of all solutions was

monitored by a vapour pressure osmometer (Wescor 5500, Logan, UT, USA) and was in the range 285-295 mmol kg^{-1}. Hypoxic solutions were bubbled with 100 % nitrogen for at least 2 h prior to the experiment. O_2 partial pressure (P_{O2}) was measured via a probe in the recording chamber attached to an Oxygen meter (Strathkelvin Instruments, Glasgow, UK) and was ≈16 mmHg compared with ≈190 mmHg for normoxic solutions. Pharmacological agents were bath applied at the concentrations indicated. All chemicals used were of analytical grade. The following drugs were used: amphotericin B, 2,4-dinitrophenol, glibenclamide, iodoacetic acid, tolbutamide, tetraethylammonium chloride (Sigma Chemical Co., St Louis, MO, USA) and levcromakalim (BRL 38227; SmithKline Beecham Pharmaceuticals, UK).

Results

Glibenclamide-BODIPY labels a population of adult intracardiac neurones

To detect the presence of the SUR in neurones from adult rat intracardiac ganglia, dissociated neurones were incubated with fluorescent glibenclamide-BODIPY. Neurones were identified visually using Nomarski-interference optics and approximately half of the neurones identified exhibited intense fluorescence indicating SUR-positive neurones. In Fig. 1A, 12 of 30 neurones identified in the field of view exhibited significant glibenclamide-BODIPY binding, indicating that the SUR is present in the plasma membrane of a subpopulation of intracardiac neurones.

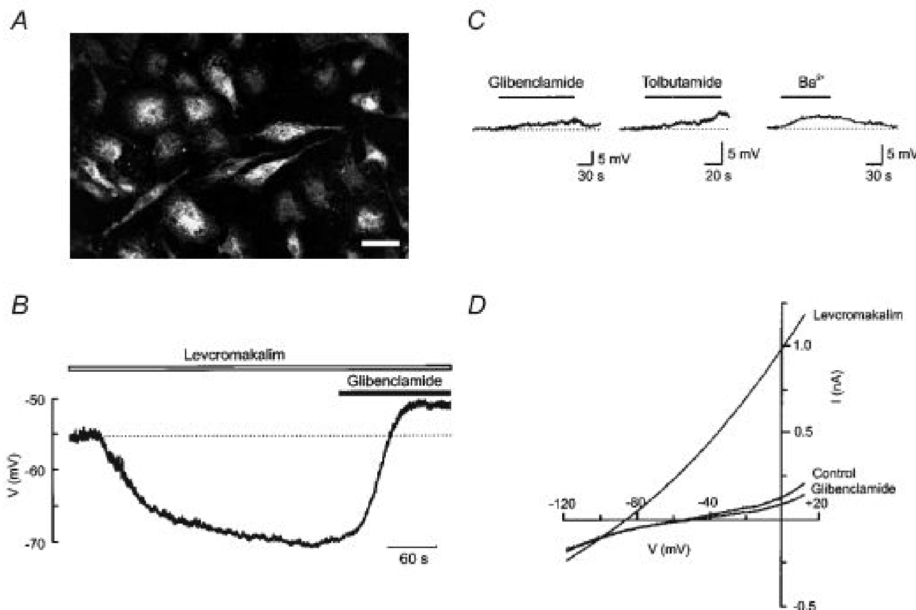

Figure 1. Binding of glibenclamide-BODIPY and levcromakalim-activated currents in a population of adult rat intracardiac ganglion neurones

A, confocal image of fluorescent glibenclamide-BODIPY binding to dissociated intracardiac neurones. Scale bar represents 50 μm. *B*, under current clamp conditions, 3 μM levcromakalim evoked a 16 mV hyperpolarization, which was inhibited by 10 μM glibenclamide. *C*, effect of bath application of 10 μM glibenclamide, 100 μM tolbutamide and 10 μM Ba^{2+} on the resting membrane potential. *D*, under voltage clamp conditions in normal PSS, 3 μM levcromakalim shifted the reversal potential of the membrane current obtained in response to voltage ramps (−120 to +10 mV) from −53 mV to −87 mV. Glibenclamide (10 μM) reversed the effect of levcromakalim.

Levcromakalim activates a conductance increase in adult rat intracardiac neurones

The K_{ATP} channel opener levcromakalim (3-10 μm) caused an increase in membrane conductance in 52 % (54/102) of adult rat intracardiac neurones studied. Under current clamp conditions in physiological ionic conditions, bath application of levcromakalim (3 μm) hyperpolarized intracardiac neurones by 15.5 ± 2.9 mV (n = 4) from the

resting membrane potential (RMP) (Fig. 1*B*). The RMPs of adult (-52.4 ± 1.3 mV, *n* = 10) and neonatal (-52.5 ± 1.8 mV, *n* = 11) intracardiac neurones were not significantly different (*P* > 0.05). The levcromakalim-induced hyperpolarization was not affected by the Ca^{2+}-activated K^+ channel inhibitor charybdotoxin (100 nm), but was reversed completely by bath application of 10 µm glibenclamide, and the RMP became slightly depolarized (Fig. 1*B*). In 6 of 11 adult neurones, glibenclamide (10 µm) alone caused a mean depolarization of 4.8 ± 1.1 mV (*n* = 6), which was not readily reversible. Similarly, bath application of 100 µm tolbutamide depolarized neurones by 3.3 ± 1.7 mV (*n* = 3) and the inwardly rectifying K^+ channel inhibitor Ba^{2+} (10 µm) caused a mean depolarization of 4.9 ± 1.1 mV (*n* = 10) (Fig. 1*C*). In contrast, neither levcromakalim (10 µm) nor glibenclamide (10 µm) caused a conductance change in *neonatal* rat intracardiac neurones (*n* = 12, data not shown).

Under voltage clamp conditions in physiological solutions, membrane currents in response to voltage ramps reversed between -50 and -55 mV. Bath application of levcromakalim (3 µm) shifted the reversal potential towards more negative membrane potentials, close to the predicted K^+ equilibrium potential (E_K) (Fig. 1*D*). The mean shift in reversal potential observed in the presence of levcromakalim was -28 ± 3 mV (*n* = 3). In symmetrical 140 mm K^+ solutions, activation of K_{ATP} channels by levcromakalim (3 µm) evoked an inward current at a holding potential of -50 mV, which had a current density of 8.1 ± 2.6 pA pF^{-1}(*n* = 6) at -50 mV and was inhibited by glibenclamide (10 µm) (Fig. 2*A*). The mean capacitance of isolated neurones from adult rat intracardiac ganglia was 54.3 ± 8.5 pF (*n* = 8). The *I-V* relationship of the levcromakalim-sensitive current (levcromakalim - control), obtained from voltage ramps, was linear between -100 and -20 mV and had a slope conductance of 9.0 ± 3.1 nS, corresponding to a conductance density of 165 ± 41 pS pF^{-1}(*n* = 6). The extrapolated reversal potential was close to 0 mV (Fig. 2*B*), as predicted for a K^+-selective conductance in symmetrical K^+ solutions.

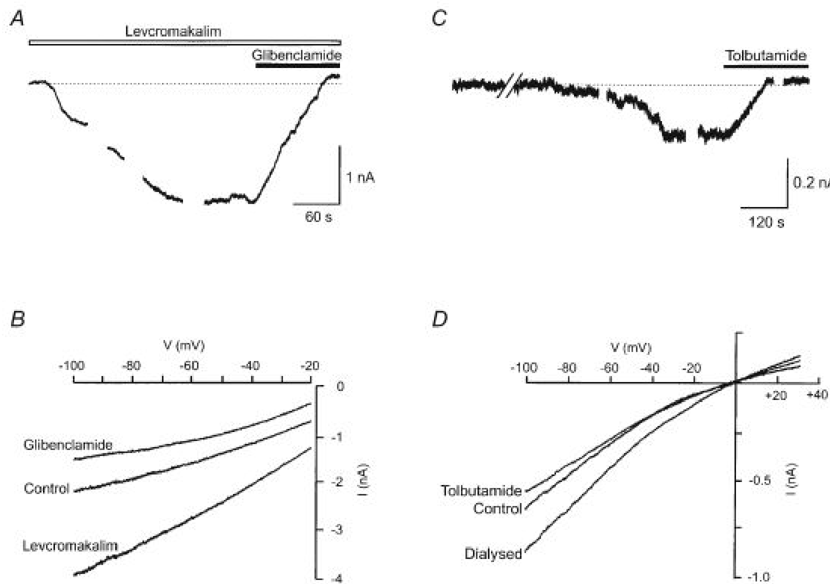

Figure 2. Levcromakalim-induced current in an adult intracardiac neurone obtained in symmetrical K^+ conditions

A, levcromakalim (3 µM) activated an inward current at a holding potential of −50 mV in symmetrical (140 mM) K^+, which was inhibited by 10 µM glibenclamide. Voltage ramps (−100 to −20 mV) were applied at the gaps in the current trace. *B*, *I–V* relationship obtained from voltage ramps in control conditions and in the presence of 3 µM levcromakalim and levcromakalim plus 10 µM glibenclamide, from the same neurone as in *A*. *C*, cell dialysis with an ATP-free (0.5 mM ADP) intracellular solution evoked an inward current at −50 mV in symmetrical K^+ solutions. The inward current was inhibited by bath application of 200 µM tolbutamide. Gap in the current trace is 7 min. *D*, *I–V* relationship obtained in response to voltage ramps applied after obtaining the dialysed whole-cell recording configuration, following 17 min dialysis and following inhibition of the current by 200 µM tolbutamide.

Activation of a KATP conductance by cell dialysis with an ATP-free intracellular solution

Using the conventional whole-cell recording configuration, cell dialysis of voltage-clamped adult intracardiac neurones with an ATP-free intracellular solution evoked an inward current at a holding potential of -50 mV in symmetrical K^+ solutions. Cell dialysis with an ATP-free intracellular solution containing 0.5 mm ADP evoked an inward current in 4 of 7 neurones, which was inhibited by 200 µm tolbutamide (Fig. 2*C*). The mean slope

conductance was 5.2 ± 1.1 nS *(n* = 4) and the reversal potential of the ***I-V*** relationship for the current induced by the dialysed (ATP-free) intracellular solution was close to 0 mV (Fig. 2*D)*

Activation of a KATP conductance by K+ channel openers

Activation of the K_{ATP} current by levcromakalim was concentration dependent. Cumulative bath application of levcromakalim caused a progressive increase in the amplitude of the ATP-sensitive K^+ current (Fig. 3*A*i). Maximal activation was obtained with 10 µm levcromakalim and further increasing the concentration to 30 µm attenuated the current activation. The dose-response relationship for activation of the K_{ATP} current is shown in Fig. 3*A*ii and the fitted curve gave an EC_{50} value of 1.6 µm.

Sulphonylurea inhibition of the K_{ATP} conductance

Inhibition of the levcromakalim-activated current by glibenclamide was also concentration dependent (Fig. 3***B***i). Cumulative addition of glibenclamide to the levcromakalim-induced current caused progressive inhibition. The dose-response relationship for inhibition of the levcromakalim-activated current by glibenclamide is shown in Fig. 3***B***ii. The IC_{50} determined from the fitted curve was 55 nm and inhibition was maximal with 10 µm glibenclamide. Tolbutamide was less potent than glibenclamide; 100 µm tolbutamide inhibited the levcromakalim-induced hyperpolarization by 33 ± 17 %*(n* = 3).

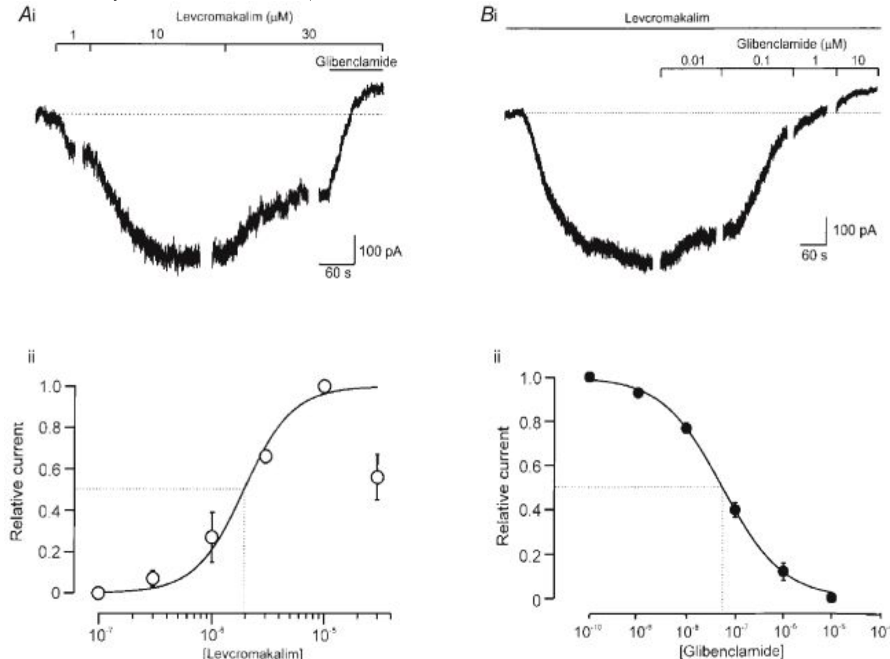

Figure 3. Current activation by levcromakalim and inhibition by glibenclamide are concentration dependent

*A*i, cumulative addition of levcromakalim causes a concentration-dependent increase in the amplitude of the ATP-sensitive K^+ current. Maximal current activation was obtained with 10 µM levcromakalim. *A*ii, concentration dependence of levcromakalim-induced current. Currents were normalized to the maximal current amplitude in the presence of 10 µM levcromakalim. The fit of the data from 0.1 to 10 µM levcromakalim gave an EC_{50} of 1.6 µM (dotted line) and a Hill coefficient of 0.6 (data points, $n = 3–6$). *B*i, cumulative addition of glibenclamide inhibits the levcromakalim (3 µM)-activated current. *B*ii, concentration dependence of the inhibition of the levcromakalim-activated current by glibenclamide. Data were normalized to the maximum current amplitude, which was taken as the difference between the maximum levcromakalim-activated current and the maximum inhibition obtained with a saturating concentration of glibenclamide. The curve fit gave an IC_{50} of 55 nM (dotted line) and a Hill coefficient of 0.7 (data points, $n = 4$).

Metabolic inhibition and hypoxia activate a K_{ATP} conductance

Inhibition of intracellular ATP production by bath application of 50 µm 2,4-dinitrophenol and 1 mm iodoacetic acid (Katnik & Adams, 1995) activated a membrane current in isolated intracardiac neurones 1-5 min following application. Under current clamp conditions, metabolic inhibition resulted in a hyperpolarization of the RMP, which was antagonized by 10 µm glibenclamide (Fig. 4*A*i). Under voltage clamp conditions in symmetrical K^+ solutions, metabolic inhibition evoked an inward current at a holding potential of -50 mV, which was inhibited by glibenclamide (Fig. 4*A*ii). The *I-V* relationship in response to voltage ramps obtained in the presence of metabolic inhibition was linear and reversed close to 0 mV (Fig. 4*A*iii). The slope conductance of the inward current induced by metabolic inhibition was 11.6 ± 4.1 nS (n = 3).

Under current clamp conditions, changing to a hypoxic perfusing solution (P_{O2}≈16 mmHg) hyperpolarized neurones by 10 ± 3.3 mV (n = 7) as shown in Fig. 4*B*i. The hypoxia-induced hyperpolarization was reversed by 10 µm glibenclamide. Under voltage clamp conditions in symmetrical K^+, hypoxia activated an inward current at a holding potential of -50 mV, which was also inhibited by 10 µm glibenclamide (Fig. 4*B*ii). The *I-V* relationship of the hypoxia-induced current (hypoxia - control) was linear with a slope conductance of 14.7 ± 5.5 nS (n = 5) and reversed close to 0 mV (Fig. 4*B*iii).

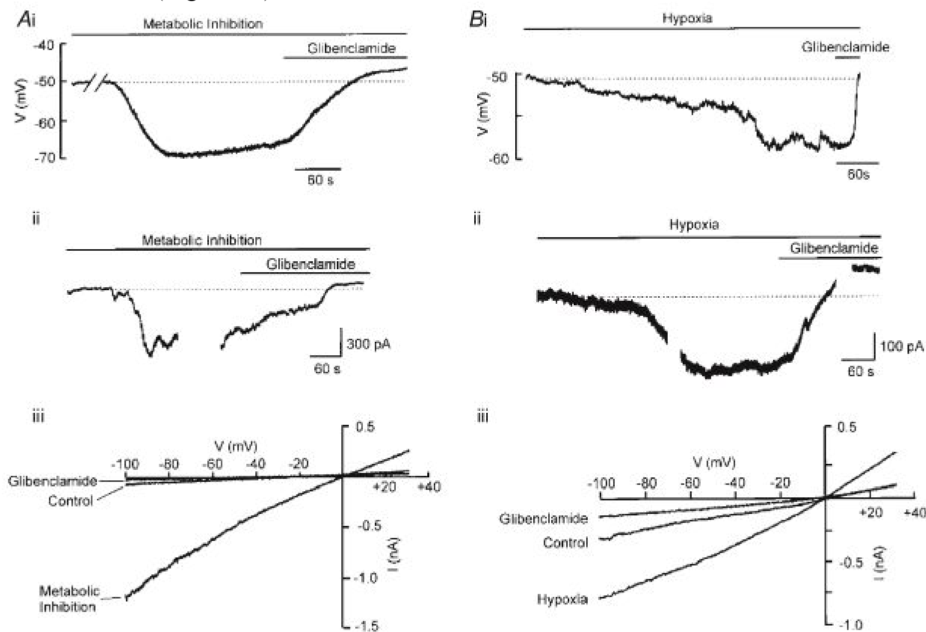

Figure 4. Activation of membrane currents by metabolic inhibition and hypoxia

*A*i, under current clamp conditions, metabolic inhibition (2,4-dinitrophenol + iodoacetic acid) caused membrane hyperpolarization, which was inhibited by 10 μM glibenclamide. *A*ii, under voltage clamp conditions, metabolic inhibition evoked an inward current at −50 mV in symmetrical K^+, which was inhibited by 10 μM glibenclamide. *A*iii, the corresponding *I–V* relationships obtained in response to voltage ramps (−100 to +30 mV). *B*i, under current clamp conditions in physiological solutions, hypoxia (P_{O_2} ~16 mmHg) hyperpolarized the neurone. *B*ii, under voltage clamp conditions, hypoxia activated an inward current at −50 mV in symmetrical K^+, which was inhibited by 10 μM glibenclamide. *B*iii, *I–V* relationships obtained in response to voltage ramps in the same cell, in control and hypoxic conditions and following the application of 10 μM glibenclamide in hypoxic conditions.

Discussion

The present findings demonstrate that an ATP-sensitive K^+ current is present in a population of intracardiac ganglion neurones from adult rats. The K_{ATP} current was activated by the K^+ channel opener levcromakalim, metabolic inhibition or hypoxia causing membrane hyperpolarization. In neurones that exhibited a K_{ATP} conductance, bath application of glibenclamide inhibited the activated K_{ATP} current and depolarized the neurone beyond the RMP. Glibenclamide, tolbutamide and Ba^{2+} each depolarized intracardiac neurones suggesting the K_{ATP} current was activated under resting conditions and contributed to the RMP in adult neurones. Cell dialysis with an ATP-free

intracellular solution also evoked an inward current, which was inhibited by tolbutamide. In normal physiological ionic conditions, the levcromakalim-activated current reversed close to E_K and in isotonic K^+ solutions the current reversed at 0 mV, suggesting that K_{ATP} channels are K^+ selective. The EC_{50} for half-maximal activation of the K_{ATP} current by levcromakalim was 1.6 μm, similar to that observed in smooth muscle cells from rabbit mesenteric artery (1.9 μm, Quayle *et al.* 1995) and endothelial cells from rabbit arteries (6.5 μm, Katnik & Adams, 1995). The IC_{50} value of 55 nm for inhibition of the levcromakalim-activated current by glibenclamide is comparable to that obtained in rabbit mesenteric artery myocytes (101 nm, Quayle *et al.* 1995) and rabbit endothelial cells (43 nm, Katnik & Adams, 1995).

The activation of K_{ATP} channels by metabolic inhibition or hypoxia suggests that K_{ATP} channels play a role in intracardiac neurones by hyperpolarizing the cell and decreasing neuronal excitability in response to metabolic stress or hypoxia. Furthermore, metabolic inhibition may raise the cytoplasmic Ca^{2+} concentration and activate Ca^{2+}-dependent K^+ channels, which have been shown to contribute to the action potential afterhyperpolarization in rat intracardiac neurones (Franciolini *et al.* 2001). *In situ* recordings of firing activity from intracardiac ganglion neurones in anaesthetized dogs show that transient coronary artery occlusion affected spontaneous firing activity (Huang *et al.* 1993). In addition, changes in neuronal firing, observed upon application of hydrogen peroxide, were not observed in the presence of cromakalim, suggesting K_{ATP} channels may be a target for reactive oxygen species and are involved in changes in neuronal activity associated with reperfusion (Thompson *et al.* 1998). It is well established that hypoxia activates K_{ATP} channels and pretreatment of rat neocortical brain slices with K_{ATP} channel agonists has been shown to be neuroprotective, counteracting hypoxia-induced cell injury (Garcia de Arriba *et al.* 1999).

K_{ATP} channels consist of the pore-forming Kir6.2 or 6.1 subunits, which associate with different regulatory SUR isoforms. The presence of the SUR in approximately half of the intracardiac neurones was confirmed by labelling with fluorescent glibenclamide-BODIPY. The sulfonylurea receptor isoforms (SUR1 in pancreatic β cells, SUR2A in heart and SUR2B in smooth muscle) confer different sensitivities on the K_{ATP} channel to metabolic inhibition and drugs (see Ashcroft & Gribble, 2000). Kir6.2-SUR1 channels expressed in *Xenopus* oocytes are insensitive to cromakalim (Gribble *et al.* 1997), suggesting that a SUR2 subunit may be involved in the regulation of K_{ATP} channels in rat intracardiac neurones. However, recent RT-PCR studies have demonstrated K_{ATP} channel formation by SUR1 with Kir6.2 and Kir6.1 subunits in rat dorsal vagal neurones (Karschin *et al.* 1998) and rat ventromedial hypothalamic neurones (Lee *et al.* 1999), respectively. Furthermore, a subset of guinea-pig enteric neurones that are glucoresponsive display Kir6.2 and SUR1 immunoreactivity (Liu *et al.* 1999).

At least three distinct populations of postganglionic intracardiac neurones have been identified on the basis of their morphological and electrical properties (Allen & Burnstock, 1987; Xi *et al.* 1994; Edwards *et al.* 1995). We have previously reported that a subpopulation (≈1/3) of adult intracardiac neurones express an inwardly rectifying K^+ current, sensitive to block by 10 μm Ba^{2+}, which is not present in neonatal neurones (Hogg *et al.* 2001). The greater sample size of the present study suggests that a higher proportion of adult rat intracardiac neurones express inward rectifier K^+ channels and that the complement of K^+ channels changes in autonomic neurones during postnatal development. The physiological and pathophysiological modulation of K_{ATP} channels in intrinsic cardiac ganglia may be important in neuroeffector transmission in the heart and regulation of heart rate. The presence of K_{ATP} channels in adult intracardiac neurones only, may contribute to the differential effects of hypoxia on cardiovascular responses (i.e. heart rate) in neonates *versus* adults (see Gootman & Gootman, 2000).

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