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Direct interaction of Na-azide with the K_{ATP} channel

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1 The effects of the metabolic inhibitor sodium azide were tested on excised macropatches from *Xenopus* oocytes expressing cloned ATP-sensitive potassium (K_{ATP}) channels of the Kir6.2/SUR1 type.

2 In inside-out patches from oocytes expressing Kir6.2ΔC36 (a truncated form of Kir6.2 that expresses in the absence of SUR), intracellular Na-azide inhibited macroscopic currents with an IC_{50} of 11 mM. The inhibitory effect of Na-azide was mimicked by the same concentration of NaCl, but not by sucrose.

3 Na-azide and NaCl blocked Kir6.2/SUR1 currents with IC_{50} of 36 mM and 19 mM, respectively. Inhibition was abolished in the absence of intracellular Mg^{2+}. In contrast, Kir6.2ΔC36 currents were inhibited by Na-azide both in the presence or absence of intracellular Mg^{2+}.

4 Kir6.2/SUR1 currents were less sensitive to 3 mM Na-azide in the presence of MgATP. This apparent reduction in sensitivity is caused by a small activatory effect of Na-azide conferred by SUR.

5 We conclude that, in addition to its well-established inhibitory effect on cellular metabolism, which leads to activation of K_{ATP} channels in intact cells, intracellular Na-azide has direct effects on the K_{ATP} channel. Inhibition is intrinsic to Kir6.2, is mediated by Na^+, and is modulated by SUR. There is also a small, ATP-dependent, stimulatory effect of Na-azide mediated by the SUR subunit. The direct effects of 3 mM Na-azide on K_{ATP} channels are negligible in comparison to the metabolic activation produced by the same Na-azide concentration.
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Keywords: ATP-dependent potassium channel; potassium channel; ATP; azide; sodium block; SUR; Kir6.2; inward rectifier

Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; K_{ATP} channel, ATP-sensitive potassium channel; P_o, single-channel open probability; SUR, sulphonylurea receptor

Introduction

ATP-sensitive K-channels (K_{ATP} channels) couple electrical activity to the metabolic state of the cell in a variety of tissues, including muscle, nerve and endocrine cells (Ashcroft & Ashcroft, 1990; Nichols & Lederer, 1991; Quayle *et al*., 1997; Trapp & Ashcroft, 1997). These channels are inhibited by ATP and activated by MgADP, and it is currently thought that metabolic regulation of the K_{ATP} channel is achieved through changes in the cytosolic levels of these nucleotides. K_{ATP} channels are also blocked by sulphonylurea drugs, which are used to treat type-2 diabetes mellitus, and activated by a group of chemically unrelated drugs collectively known as K-channel openers (Ashcroft & Ashcroft, 1992; Edwards & Weston, 1993; Ashcroft & Gribble,1998).

ecent studies have demonstrated that the β-cell K_{ATP} channel is an octameric 4:4 complex of two structurally distinct proteins: an inwardly-rectifying K-channel subunit, Kir6.2, and a sulphonylurea receptor, SUR1 (Inagaki *et al*., 1995; 1997; Sakura *et al*., 1995; Clement *et al*., 1997; Shyng & Nichols, 1997). Both subunits are required for functional channel expression. Kir6.2 acts as an ATP-sensitive pore while SUR1 is a regulatory subunit which endows Kir6.2 with sensitivity to sulphonylureas, K-channel openers and the potentiatory effects of Mg-nucleotides, such as MgADP (Nichols *et al*., 1996; Gribble *et al*., 1997b; Shyng *et al*., 1997; Trapp *et al*., 1997b; Tucker *et al*., 1997). Two different sulphonylurea receptor genes have been cloned, SUR1 and SUR2, which encode proteins with different pharmacological sensitivity and which show different tissue expression (Chutcow *et al*., 1996; Inagaki *et al*., 1996; Isomoto *et al*., 1996). K_{ATP} channels in pancreatic β-cells comprise Kir6.2 and SUR1 subunits while those of cardiac muscle appear to be composed of Kir6.2 and a splice variant of SUR2, SUR2A (Aguilar-Bryan & Bryan, 1999).

RAlthough wild-type K_{ATP} channels require both types of subunit (Kir6.2 and SUR) for functional activity, a mutant form of Kir6.2 with a C-terminal truncation of 26 or 36 amino acids (Kir6.2ΔC) is capable of independent expression (Tucker *et al*., 1997). Kir6.2ΔC therefore provides a useful tool for studying the effects of agents on the pore-forming subunit of the K_{ATP} channel.

Sodium azide is widely used both *in vivo* and *in vitro* as a metabolic inhibitor. It acts by inhibiting cytochrome C oxidase, the last enzyme in the mitochondrial electron transport chain, and thereby produces a fall in the intracellular ATP concentration. Application of Na-azide both to pancreatic β-cells and to *Xenopus* oocytes expressing the cloned β-cell K_{ATP} channel Kir6.2/SUR1, produces a marked increase in the K_{ATP} current (Misler *et al*., 1986; Gribble *et al*., 1997a). This effect is believed to result from the drop of intracellular ATP (and concomitant increase of MgADP) produced by the metabolic inhibition. However, azide has also been reported to interact with various ATP-binding proteins including the F1/F0 ATP-ase and the nucleotide binding domains of CFTR (Vasilyeva *et al*., 1982; Ko & Pedersen, 1995). This raises the possibility that the activation of K_{ATP} channels induced by azide, may reflect not only inhibition of the mitochondrial metabolism, but also a direct effect on the channel itself. Indeed, it has

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recently been reported that azide directly activates K_{ATP} channels in the CRI-G1 insulinoma cell line (Harvey *et al*., 1999).

In contrast, there is also evidence that Na-azide may have an inhibitory effect on the β-cell K_{ATP} channel. First, Na-azide blocks whole cell K_{ATP} currents in isolated pancreatic β-cells (Trapp *et al*., 1997a). Secondly, although Na-azide has a primary stimulatory effect on whole-cell Kir6.2/SUR1 currents expressed in *Xenopus* oocytes, there is often a further increase in current when the inhibitor is washed off, suggesting that Na-azide may also have an additional inhibitory effect (Gribble *et al*., 1997a).

In order to investigate the direct effects of Na-azide on the K_{ATP} channel in detail, we examined the effect of the inhibitor on cloned K_{ATP} channels expressed in *Xenopus* oocytes. We observed that direct application of Na-azide to the intracellular solution causes inhibition of Kir6.2/SUR1 currents, and that this effect is mediated by interaction of Na^+ ions with the Kir6.2 subunit of the channel.

Methods

Molecular biology

Mouse Kir6.2 (Genbank D50581, Inagaki *et al*., 1995; Sakura *et al*., 1995), and rat SUR1 (Genbank L40624, Aguilar-Bryan *et al*., 1995; kindly provided by Dr G. Bell, University of Chicago) were used in this study. Kir6.2 with a C-terminal truncation of 36 amino acids (Kir6.2ΔC36) was also used (Tucker *et al*., 1997). Site-directed mutagenesis was carried out by subcloning the appropriate fragments into the pALTER vector (Promega, Madison, WI, U.S.A.). Mutations are indicated by the single amino acid letter code. Synthesis of capped mRNA was carried out using the mMessage mMachine large scale *in vitro* transcription kit (Ambion, Austin, TX, U.S.A.).

Electrophysiology

Female *Xenopus laevis* were anaesthetized with MS222 (2 g l^{-1} added to the water). One ovary was removed *via* a mini-laparotomy, the incision sutured and the animal allowed to recover. Once the wound had completely healed, the second ovary was removed in a similar operation and the animal was then killed by decapitation whilst under anaesthesia. Immature stage V-VI *Xenopus* oocytes were incubated for 60 min with 1.0 mg ml^{-1} collagenase (Sigma, type V) and manually defolliculated. Oocytes were injected with $\sim$2 ng of mRNA encoding Kir6.2ΔC36 or were coinjected with $\sim$0.1 ng full-length Kir6.2 and $\sim$2 ng of SUR1 or SUR2A (giving a 1 : 20 ratio). Control oocytes were injected with water. The final injection volume was $\sim$50 nl per oocyte. Isolated oocytes were maintained in tissue culture and studied 1–4 days after injection (Gribble *et al*., 1997a).

Macroscopic currents were recorded from giant excised inside-out patches at a holding potential of 0 mV and at 20–24°C (Gribble *et al*., 1997a). Currents were evoked by repetitive 3 s voltage ramps from -110 mV to $+100$ mV and recorded using an EPC7 patch-clamp amplifier (HEKA Electronik, Lambrecht, Germany). They were filtered at 0.2 kHz, digitized at 0.5 kHz using a Digidata 1200 Interface and analysed using pClamp software (Axon Instruments, Burlingame, U.S.A.). Single-channel currents were recorded at -60 mV, filtered at 1 kHz and sampled at 3 kHz.

The pipette solution contained (mM): KCl 140, $MgCl_2$ 1.2, $CaCl_2$ 2.6, HEPES 10 (pH 7.4 with KOH) and the internal (bath) solution contained (mM): KCl 110, $MgCl_2$ 2, $CaCl_2$ 1, KOH 30, EGTA 10, HEPES 10 (pH 7.2 with KOH) and nucleotides as indicated. Mg^{2+} free solution contained no added $MgCl_2$ and 10 EDTA instead of EGTA. Na-azide and NaCl were added to the solution as indicated. Solutions containing nucleotides were made up fresh each day. The pH of all solutions was checked and readjusted, if required, after drug and nucleotide addition. Rapid exchange of solutions was achieved by positioning the patch in the mouth of one of a series of adjacent inflow pipes placed in the bath.

Data analysis

The slope conductance was measured by fitting a straight line to the current-voltage relation between -20 mV and -100 mV: the average of five consecutive ramps was calculated in each solution. Concentration-response relationships were measured by alternating the control solution with a test concentration of Na-azide or NaCl. The extent of inhibition was then expressed as a fraction of the mean of the value obtained in the control solution before and after application of the test agent. Concentration-response curves were fitted to the Hill equation (equation 1).

$$G/G_C = 1/(1 + [X]/IC_{50})^h)$$

where [X] is the NaCl or Na-azide concentration, IC_{50} is the concentration at which inhibition is half maximal and h is the slope factor (Hill coefficient). Single-channel currents were measured at -60 mV and analysed using pClamp software. The single-channel current amplitude was determined by fitting gaussian distributions to amplitude histograms.

Results

Our aim was to determine whether Na-azide has a direct effect on the β-cell type of K_{ATP} channel, independent of its ability to block metabolism. We therefore first examined the effect of Na-azide on the pore-forming subunit Kir6.2, by expressing Kir6.2ΔC36 in the absence of SUR. We subsequently studied the interaction of Na-azide with Kir6.2/SUR1.

Effects of Na-azide on Kir6.2ΔC36 currents

Inside-out patches excised from *Xenopus* oocytes expressing Kir6.2ΔC36 exhibited large inwardly rectifying ATP-sensitive K^+ currents, that were absent in water-injected oocytes. As shown in Figure 1A, these currents were inhibited by application of 10 mM Na-azide to the intracellular membrane surface. Na-azide reduced the mean slope conductance by $47 \pm 2\%$ ($n = 8$). The relationship between channel inhibition and the Na-azide concentration is shown in Figure 1B. Assuming a complete block of the K_{ATP} current at very high concentrations of Na-azide, the data can be fitted by the Hill equation with an IC_{50} of 11 ± 1 mM ($n = 7$) and a Hill coefficient (slope factor) of 0.74 ± 0.04. Thus, at concentrations commonly used as a metabolic poison (3 mM), the inhibitory effect of Na-azide was relatively small (20–25%). The block was also immediate and rapidly reversible, in contrast to what is observed for activation of K_{ATP} currents by azide in intact oocytes (Gribble *et al*., 1997a).

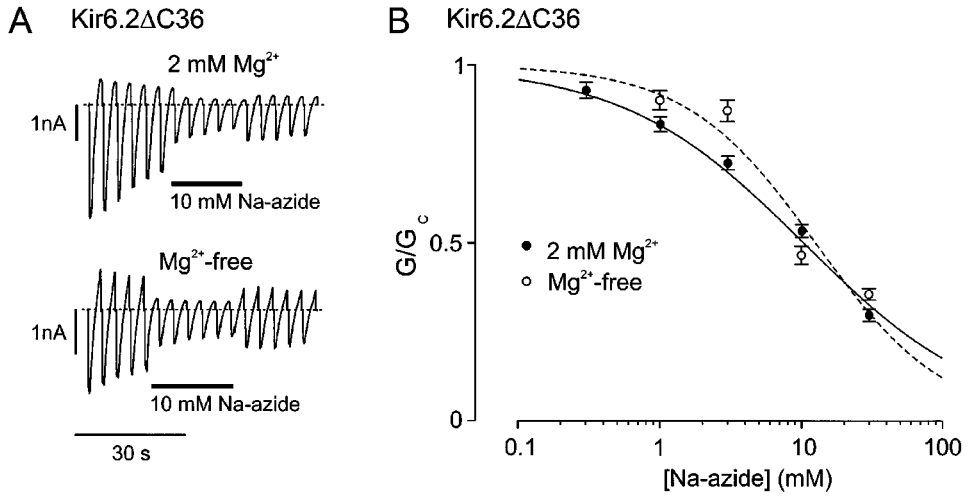

Figure 1 Effects of Na-azide on Kir6.2ΔC36 currents. (A) Macroscopic Kir6.2ΔC36 currents recorded in the presence or absence of internal Mg^{2+} from an inside-out patch in response to a series of voltage ramps from −110 mV to +100 mV. Na-azide (10 mM) was added to the internal solution as indicated by the bars. The dashed lines indicate the zero current level. (B) Mean Na-azide concentration-response relationships for Kir6.2ΔC36 currents in the presence ($n=6$) or absence ($n=4$) of 2 mM intracellular $MgCl_2$ (1.2 mM free Mg^{2+}). Test solutions were alternated with control solutions and the slope conductance (G) is expressed as a fraction of the mean of that obtained in control solution before and after exposure to Na-azide (G_c). The lines are the best fit of the Hill equation to the data using the mean values for IC_{50} and h given in the text.

It has previously been reported that interaction of Na-azide with native K_{ATP} channels is influenced by Mg^{2+} ions (Harvey *et al*., 1999). We therefore examined the effect of removing intracellular Mg^{2+} on Na-azide inhibition. In the absence of Mg^{2+}, 10 mM Na-azide blocked Kir6.2ΔC36 currents by 53±3% ($n=4$). The Na-azide concentration-response curve was best fitted by the Hill equation with an IC_{50} of 13±3 mM and a Hill coefficient of 0.95±0.26 ($n=4$). These values are not significantly different from those obtained in the presence of 2 mM $MgCl_2$, and imply that Mg^{2+} does not influence the block of Kir6.2ΔC36 by Na-azide.

Effects of NaCl on Kir6.2ΔC36 currents

The activity of inwardly rectifying K^+ channels of the Kir3.× type is modulated by Na^+ ions (Sui *et al*., 1996; Ho & Murrell-Lagnado, 1999). We therefore explored whether the inhibitory effect of Na-azide is due to Na^+ ions, by examining the effect of intracellular NaCl on Kir6.2ΔC36. Figure 2A shows that 10 mM NaCl blocked Kir6.2ΔC36 currents by 48±4% ($n=8$). This effect was not mimicked by 30 mM sucrose, suggesting that it is not due to the change in the osmolarity of the solution (Figure 2B). Concentration-response curves for inhibition of Kir6.2ΔC36 currents by NaCl revealed a very similar inhibitory potency to that found for Na-azide (compare Figure 2C with 1B). Assuming a complete block of the current at high NaCl concentrations, an IC_{50} value of 16±1 mM ($n=5$) was obtained. This value is only slightly greater than that found for Na-azide (11 mM), indicating that inhibition by Na-azide is principally the result of the blocking action of Na^+ ions. The Hill coefficient was 1.02±0.10 ($n=5$).

Effects of Na-azide on Kir6.2/SUR1 currents

We next explored whether the presence of the sulphonylurea receptor subunit SUR1 influences the ability of Na-azide to inhibit the K_{ATP} channel. Figure 3A shows that 10 mM Na-azide inhibited Kir6.2/SUR1 currents by 32±5% ($n=6$) in the presence of 2 mM Mg^+. This is significantly less block than that observed for Kir6.2ΔC36 currents (47%, $P<0.05$). The inhibition was concentration dependent and, assuming complete block at high concentrations, the best fit of the Hill equation to the data gave values of 36±11 mM for the IC_{50} and of 0.56±0.10 ($n=6$) for the Hill coefficient (Figure 3B). For comparison, the IC_{50} for Na-azide block of Kir6.2ΔC36 was 11 mM. Thus SUR1 is able to decrease the apparent efficacy of Na-azide inhibition. Furthermore, the fact that the Hill coefficient is less than unity suggests that Na-azide interacts with the channel at more than one site, and that these sites show negative cooperativity.

An even greater discrepancy between the behaviour of Kir6.2ΔC36 and Kir6.2/SUR1 channels was observed in the absence of intracellular Mg^{2+}. In Mg^{2+}-free solution, the inhibitory effect of Na-azide on the Kir6.2/SUR1 inward current was abolished and even as much as 30 mM Na-azide was without significant effect (Figure 3A,B). In contrast, Na-azide inhibited Kir6.2ΔC36 currents equally effectively in the presence or absence of Mg^{2+}. The Mg^{2+}-sensitivity of the NaCl block must therefore be conferred by SUR1.

These results demonstrate that the sulphonylurea receptor SUR1 reduces the apparent efficacy of Na-azide inhibition, and that this effect is greater in the absence of Mg^{2+}.

Effects of NaCl on Kir6.2/SUR1 currents

In contrast to the inhibition produced by Na-azide, the block by NaCl was not significantly different between Kir6.2ΔC36 channels and Kir6.2/SUR1 channels ($P>0.05$). Figure 4 shows that 10 mM NaCl inhibited Kir6.2/SUR1 inward currents by 61±2% ($n=7$) and that the concentration-response relationship could be fitted with an IC_{50} of 19±3 mM ($n=5$) and a Hill coefficient of 0.76±0.10 ($n=5$). This compares with an IC_{50} of 16 mM for Kir6.2ΔC36. This result suggests that the ability of SUR1 to reduce the Na-azide inhibition of the K_{ATP} current involves the azide moiety. One possible explanation for this finding is that azide causes a stimulation of K_{ATP} channel activity, which partially offsets the inhibitory action of Na^+ ions; and that this effect is mediated by SUR1.

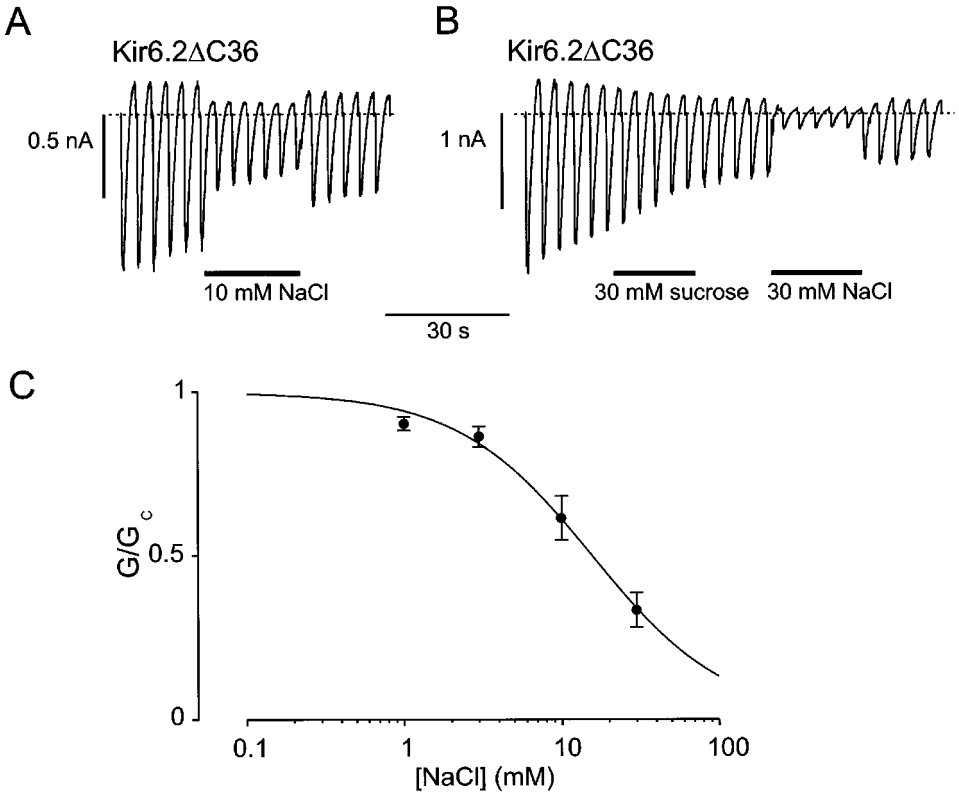

Figure 2 Effects of NaCl on Kir6.2ΔC36 currents. (A,B) Macroscopic currents recorded from inside-out patches excised from oocytes expressing Kir6.2ΔC36. Currents were recorded in response to voltage ramps from −110 mV to +100 mV. NaCl or sucrose were added to the internal solution as indicated by the bars. $MgCl_2$ (2 mM) was present in all solutions. The dashed lines indicate the zero current level. (C) Mean NaCl concentration-response relationship for Kir6.2ΔC36 currents ($n=6$). The line is the best fit of the Hill equation to the data using the mean values for IC_{50} and h given in the text.

As was the case for Na-azide, however, NaCl had a reduced inhibitory effect on inward Kir6.2/SUR1 currents in the absence of intracellular Mg^{2+}: 30 mM NaCl inhibited the current by $27\pm6\%$ ($n=4$) in Mg-free solution, compared with 60% in the presence of 2 mM Mg^{2+} (Figure 4B). The Mg^{2+}-sensitivity of NaCl block must be conferred by SUR1, as it is not observed for Kir6.2ΔC36.

Effects of NaCl and Na-azide on the single-channel current amplitude of Kir6.2/SUR1 channels

The macroscopic current is determined by the product of the single-channel current amplitude, the open probability (P_o) and the number of channels in the patch. Single Kir6.2/SUR1 channel recordings showed that the current amplitude at −60 mV was not noticeably affected by 10 mM NaCl, being 3.73 ± 0.02 pA ($n=3$) under control conditions and 3.67 ± 0.01 pA in the presence of 10 mM NaCl. Similar results were found for Na-azide: the single-channel current was 3.75 ± 0.02 pA and 3.92 ± 0.02 pA ($n=3$) in the absence and presence of 10 mM Na-azide, respectively. These drugs therefore mediate their effects on the macroscopic current by decreasing P_o or the number of functional channels.

Influence of nucleotides on the Na-azide block of Kir6.2/SUR1 currents

It has been reported that intracellular Na-azide has a stimulatory effect on native K_{ATP} channels recorded in inside-out patches excised from the CRI-G1 β-cell line in the presence of 100 μM MgATP (Harvey *et al*., 1999). This was not the case, however, when the cloned β-cell K_{ATP} channel was expressed in *Xenopus* oocytes (Figure 5A). Nevertheless, although Na-azide failed to activate Kir6.2/SUR1 currents in the presence of MgATP, the extent of inhibition produced by 3 mM Na-azide was slightly, but significantly ($P<0.01$), reduced: 3 mM Na-azide blocked the current by $24\pm2\%$ ($n=6$) in the absence of ATP, and by $6\pm4\%$ ($n=7$) in the presence of 100 μM ATP.

The marked inhibition produced by 100 μM MgATP makes it difficult to construct the Na-azide concentration-response curve at this nucleotide concentration. We therefore measured the concentration-response curve for Na-azide inhibition in the presence of 10 μM ATP (Figure 5B). Assuming a complete block of the K_{ATP} current at very high concentrations of Na-azide, the data can be fitted by the Hill equation with an IC_{50} of 41 ± 6 mM ($n=6$) and a Hill coefficient of 0.72 ± 0.07. Inhibition by 3 mM Na-azide in the presence of 10 μM ATP amounted to 12%, more than that seen in the presence of 100 μM MgATP (6%), but significantly ($P<0.05$) less than observed in the absence of nucleotide (24%).

As suggested above, Na-azide may have both stimulatory and inhibitory effects on Kir6.2/SUR1 currents. The reduced inhibition produced by Na-azide in MgATP solution might therefore result from either a decreased block or an enhanced activation of the channel. To determine which is the case we employed a mutant K_{ATP} channel (Kir6.2-R50G/SUR1) that shows greatly reduced inhibition by ATP (Gribble *et al*., 1998; Proks *et al*., 1999). MgATP has two effects on the K_{ATP} channel–it inhibits the channel by interaction with the Kir6.2 subunit (Tucker *et al*., 1997) and activates it *via* the SUR subunit (Gribble *et al*., 1998). In case of Kir6.2-R50G/SUR1, channel activation prevails and

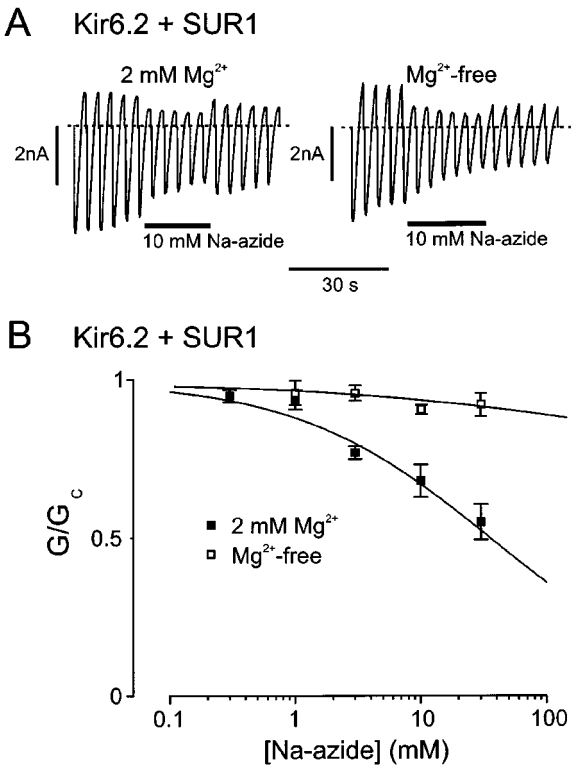

Figure 3 Effects of Na-azide on Kir6.2/SUR1 currents. (A) Macroscopic currents recorded in the presence or absence of internal Mg^{2+} from oocytes expressing Kir6.2 and SUR1 in response to a series of voltage ramps from −110 mV to +100 mV. Na-azide (10 mM) was added to the internal solution as indicated by the bars. The dashed line indicates the zero current level. (B) Mean Na-azide concentration-response relationships for Kir6.2/SUR1 currents in the presence ($n=6$) or absence ($n=4$) of 2 mM intracellular $MgCl_2$. The lines are the best fit of the Hill equation to the data using the mean values for IC_{50} and h given in the text.

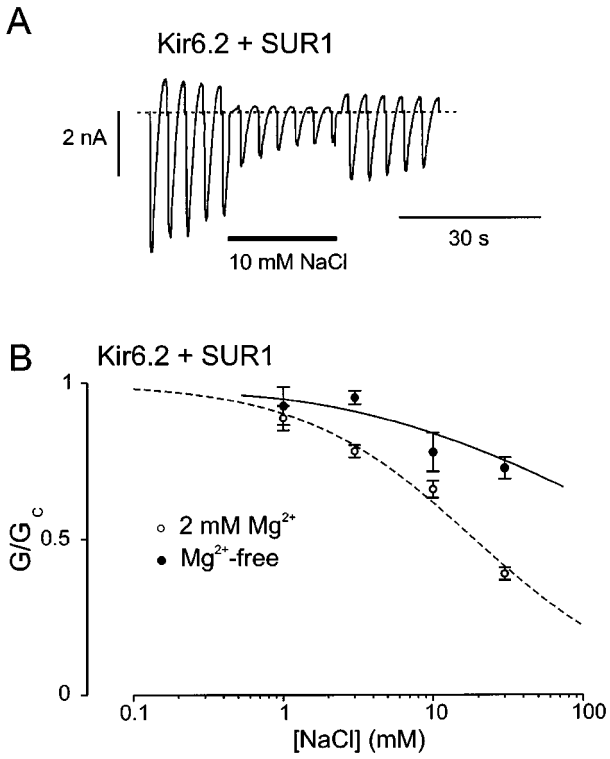

Figure 4 Effects of NaCl on Kir6.2/SUR1 currents. (A) Macroscopic Kir6.2/SUR1 currents recorded from an inside-out patch in response to voltage ramps from −110 mV to +100 mV. NaCl (10 mM) was added to the internal solution as indicated by the bar: 2 mM $MgCl_2$ was present throughout. (B) Mean concentration-response relationships for inhibition of Kir6.2/SUR1 currents by NaCl in the presence ($n=4$), or in the absence ($n=4$) of internal $MgCl_2$. The dashed line is the best fit of the Hill equation to the data using the mean values for IC_{50} and h given in the text. The solid line is drawn through the data points by eye.

1 mM ATP approximately doubles the macroscopic current (Figure 6A).

In the absence of ATP, 3 mM Na-azide inhibited Kir6.2-R50G/SUR1 and Kir6.2/SUR1 currents with similar potency. The fact that Na-azide blocks wild-type and ATP-insensitive K_{ATP} channels with similar efficacy indicates that inhibition by ATP and Na-azide are produced by separate mechanisms. In the presence of ATP, however, there was a small but significant activation of Kir6.2-R50G/SUR1 currents. This result demonstrates that Na-azide has a stimulatory effect on the K_{ATP} channel, and suggests that this potentiation is enhanced by MgATP.

The stimulatory effect of Mg-nucleotides on the K_{ATP} channel is mediated by the nucleotide binding domains (NBDs) of SUR and can be abolished by mutation of a conserved lysine residue in either NBD1 or NBD2 (Gribble *et al*., 1997b; 1998; Trapp *et al*., 1997b; Shyng *et al*., 1997). To determine if the NBDs are also required for the ability of MgATP to support Na-azide activation we used a mutant SUR in which the conserved lysine in NBD1 was changed to alanine (SUR1-K719A). Mutation of this residue is predicted to substantially decrease ATP binding and/or hydrolysis (Ueda *et al*., 1997). Inhibition of Kir6.2-R50G/SUR1-K719A currents by 3 mM Na-azide amounted to 23±1% ($n=3$) in the absence of ATP, which was not significantly different ($P>0.05$) from that observed for Kir6.2-R50G/SUR1 currents (Figure 6C). As previously reported (Gribble *et al*., 1998), 1 mM ATP did not activate Kir6.2-R50G/SUR1-K719A currents – instead the current was blocked by 44±4% ($n=6$). Application of 3 mM Na-azide in the presence of 1 mM ATP led to a further decrease in current of 26±3% (Figure 6B; $n=3$). This was not significantly different to the inhibition produced by Na-azide in the absence of ATP ($P>0.05$). This suggests that the MgATP-dependent stimulatory effect of Na-azide is mediated by the NBDs of SUR1.

Discussion

Our results demonstrate that intracellular Na^+ ions have a direct inhibitory effect on the β-cell type of K_{ATP} channel (Kir6.2/SUR1) that can fully account for the inhibitory action of Na-azide. Azide ions have a small stimulatory action on Kir6.2/SUR1 currents, but this is normally masked by the inhibitory effect of Na^+ ions. Indeed, we did not observe any net activation of K_{ATP} currents by Na-azide in inside-out membrane patches. Thus the stimulatory action of Na-azide on whole-cell cloned K_{ATP} channels in intact oocytes (Gribble *et al*., 1997a) is not due to a direct effect on the K_{ATP} channel and is probably mediated entirely by cytosolic changes that result from inhibition of mitochondrial metabolism.

Our results argue that the principal site at which both NaCl and Na-azide inhibit the K_{ATP} channel lies on Kir6.2, because inhibition is observed when Kir6.2ΔC36 is expressed in the absence of a sulphonylurea receptor. Because both agents produce a similar amount of block, it appears that this

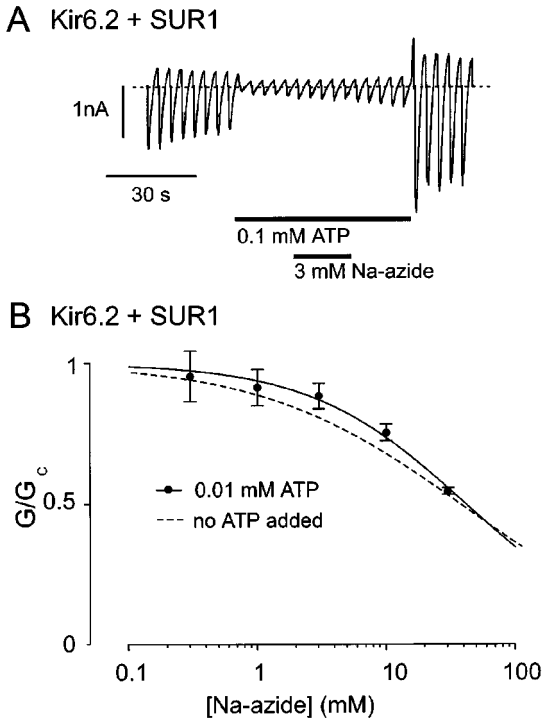

Figure 5 Effects of Na-azide on Kir6.2/SUR1 currents in the presence of ATP. (A) Macroscopic Kir6.2/SUR1 currents recorded from an inside-out patch in response to voltage ramps from −110 mV to +100 mV. The internal solution contained 2 mM $MgCl_2$, and Na-azide (3 mM) and ATP (0.1 mM) were added to the internal solution as indicated by the bars. (B) Mean Na-azide concentration-response relationship for Kir6.2/SUR1 currents in the presence of 2 mM $MgCl_2$ and 10 μM ATP ($n=4$). The solid line is the best fit of the Hill equation to the data using the mean values for IC_{50} and h given in the text. The dashed line is the same as that fitted to the concentration-response relationship in the absence of ATP in Figure 3.

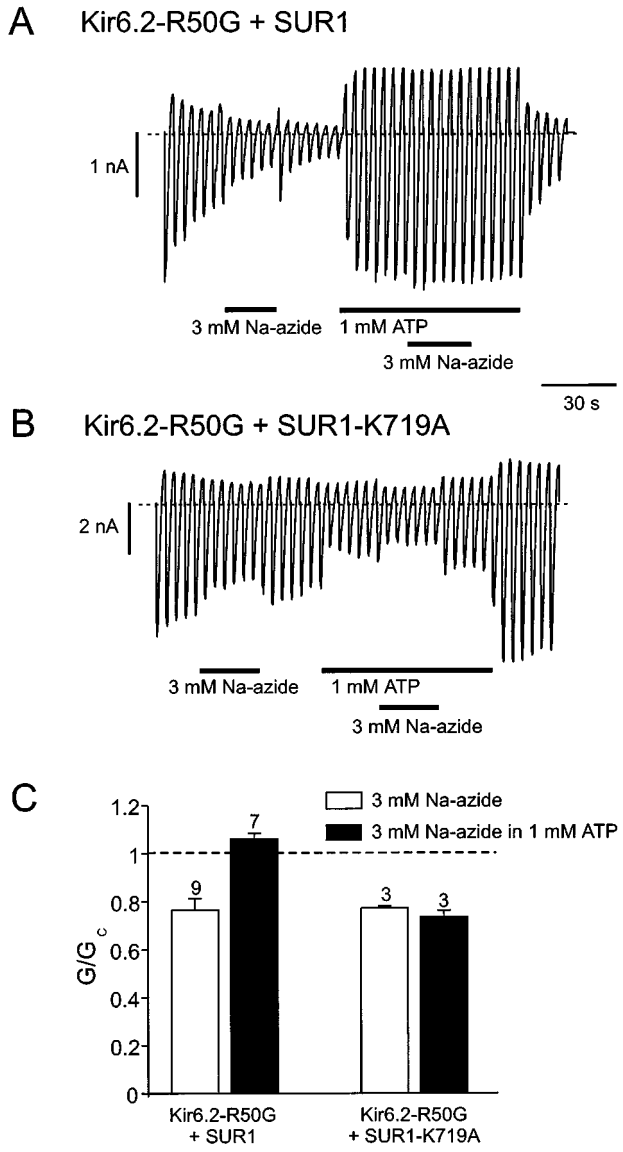

Figure 6 Effects of Na-azide on Kir6.2-R50G/SUR1 currents in the presence of ATP. (A) Macroscopic Kir6.2-R50G/SUR1 currents recorded from an inside-out patch in response to voltage ramps from −110 mV to +100 mV. The internal solution contained 2 mM $MgCl_2$. Na-azide (3 mM) and ATP (1 mM) were added to the internal solution as indicated by the bars. (B) Macroscopic Kir6.2-R50G/SUR1-K719A currents recorded from an inside-out patch in response to voltage ramps from −110 mV to +100 mV. Na-azide (3 mM) and ATP (1 mM) were added to the internal solution as indicated by the bars. (C) Mean slope conductance (G) in the presence of Na-azide or Na-azide plus ATP is expressed relative to the mean conductance in control solution (lacking Na-azide) before and after addition of the test solution (G_c). The dashed line indicates the control conductance. The number of patches is given above each bar.

inhibition is mediated solely by Na^+ ions and that N_3^- has no intrinsic effect on Kir6.2. Single-channel recordings further indicate that Na^+ does not act as an open channel blocker (of inward currents) but instead reduces channel activity.

When SUR1 was coexpressed with Kir6.2, the sensitivity of the resulting K_{ATP} channel to inhibition by NaCl remained unchanged, providing that intracellular Mg^{2+} was present. In the absence of Mg^{2+}, however, inhibition by both NaCl and Na-azide was abolished. The simplest explanation for these results is that conformational changes in the structure of Kir6.2 produced by association with a sulphonylurea receptor are responsible for a reduction in the efficacy of Na^+ inhibition. For example, the presence of SUR may interfere with the binding of Na^+ ions to their inhibitory site (on Kir6.2) or with the mechanism by which binding is transduced into channel inhibition.

Although Na-azide inhibition of Kir6.2ΔC36 currents was essentially indistinguishable from that of NaCl, Na-azide was significantly less potent at inhibiting Kir6.2/SUR1 currents in the presence of Mg^{2+}. One explanation for this finding is that azide has an additional stimulatory effect on Kir6.2/SUR, so that the current amplitude in the presence of Na-azide reflects a balance between activation and inhibition. Any stimulatory effect of azide must be conferred by SUR1, because it is not seen for Kir6.2ΔC36 currents.

The stimulatory effect of Na-azide appears to be potentiated by MgATP. Indeed, in the presence of 1 mM MgATP, the stimulatory effect of Na-azide on Kir6.2-R50G/SUR1 currents was large enough to be observed directly. The ability of MgATP to support Na-azide activation requires the functional integrity of NBD1 of SUR1. It is well established that Mg-nucleotides potentiate Kir6.2/SUR currents by interacting with the NBDs of SUR (Gribble *et al.*, 1997b; 1998; Trapp *et al.*, 1997; Shyng *et al.*, 1997). Thus, Na-azide may act by stabilizing the stimulatory effect of nucleotides at the NBDs of SUR; alternatively a stimulatory effect of Na-azide itself may be enhanced by nucleotide interaction with the NBDs.

Previous studies have shown that azide also interacts with other ATP-binding proteins, such as ATP-ases and ATP-

binding cassette transporters. For example, azide inhibits ATP hydrolysis at NBD1 of the cystic fibrosis transmembrane conductance regulator, which, like SUR, is an ABC transporter (Ko & Pedersen, 1995). In the case of the mitochondrial F_1/F_0 ATPase, azide appears to inhibit ATP turnover by preventing ADP from dissociating from the enzyme after ATP hydrolysis (Vasilyeva *et al*., 1982). Because MgADP is a more potent activator of Kir6.2/SUR1 currents than MgATP, it is thought that MgADP may be the active species and that MgATP must first be hydrolyzed to MgADP by the NBDs of SUR (Gribble *et al*., 1998). By analogy with other ABC transporters and ATP-ases, therefore, it is possible that Na-azide reduces ATP hydrolysis at NBD1 of SUR1; and that prolonged occupancy of NBD1 by ADP is responsible for azide-induced channel activation. Recent studies suggest that ATP may remain bound to NBD1 for several minutes after patch excision (Gribble *et al*., 2000), which may account for the small stimulatory action of Na-azide that is observed even in the absence of added nucleotide. Although our results are consistent with the idea that Na-azide may mediate a small stimulatory effect on the cloned β-cell K_{ATP} channel in the presence of MgATP, this was never sufficient to cause measurable activation of the wild-type channel, because it was masked by an additional inhibitory effect attributable to Na^+ ions. In contrast, Harvey *et al*. (1999), report activation of the native β-cell K_{ATP} channel by Na-azide in the presence of 100 μM MgATP. The reason for this difference is not clear.

Comparison to intact oocyte experiments

Azide blocks mitochondrial metabolism when applied to the extracellular solution, indicating that it readily permeates the cell. At pH 7.2, Na-azide is almost completely dissociated in solution: some azide (N_3^-) ions combine with protons to form the weak acid HN_3 that is able to cross the plasma membrane. Sodium ions, however, do not cross the membrane and therefore cannot be responsible for Na-azide inhibition of the whole-cell K_{ATP} current. Moreover, NaCl (30 mM) has no effect on the K_{ATP} current when applied to the extracellular surface of β-cells (unpublished observation). Thus, any inhibitory effect of Na-azide on the K_{ATP} current in intact cells (Gribble *et al*., 1997a; Trapp *et al*., 1997a) must be mediated by a different mechanism.

Physiological relevance

The ability of Na^+ to inhibit the β-cell type of K_{ATP} channel (Kir6.2/SUR1) suggests that Na^+ might be a physiological regulator of the activity of this channel. Kir6.2/SUR1 channels are found in pancreatic β-cells and in several different types of neurone, including dorsal vagal neurones and substantia nigra neurones (Inagaki *et al*., 1995; Sakura *et al*., 1995; Karschin *et al*., 1998; Liss *et al*., 1999). In β-cells, the intracellular Na^+ concentration is around 15 mM in both the presence and absence of glucose (Saha & Grapengeisser, 1995), a concentration that would be expected to produce around 30% inhibition of the K_{ATP} current. Resting $[Na^+]_i$ in substantia nigra neurones is around 10 mM in brain slice preparations (Guatteo *et al*., 1998) and rather higher (26 mM) in neurones cultured from foetal brain (Silver *et al*., 1997). Hypoxia produces an immediate increase in $[Na^+]_i$which can reach as much as 20 mM in substantia nigra neurones (Guatteo *et al*., 1998) and 35 mM after 60 min in foetal neurones (Silver *et al*., 1997). However, hypoxia also causes a fall in ATP (and a concomitant rise in MgADP), which would simultaneously enhance K_{ATP} channel activity. This explains why ischaemia or glucose removal cause membrane hyperpolarization, rather than membrane depolarization. It therefore seems probable that changes in intracellular Na^+ during metabolic inhibition do not markedly affect K_{ATP} channel activity.

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