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Differential Response of K_{ATP} Channels Containing SUR2A or SUR2B Subunits to Nucleotides and Pinacidil

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ABSTRACT

ATP-sensitive K-channels (K_{ATP} channels) are the target for K_{ATP}-channel openers (KCOs), such as pinacidil and P1075. These channels are formed from pore-forming Kir6.2 and regulatory sulfonylurea receptors (SUR2A in heart and skeletal muscle; SUR2B in smooth muscle). The two isoforms of SUR2 differ only in their final 42 amino acids, a region that includes neither the Walker A and B nucleotide binding motifs nor the proposed KCO binding site, yet channels containing SUR2A or SUR2B respond differently to both nucleotides and KCOs. We explored the basis for this difference by expressing Kir6.2/SUR2A and Kir6.2/SUR2B currents in *Xenopus laevis* oocytes. Kir6.2/SUR2B but not Kir6.2/SUR2A currents were activated by the Mg-nucleoside triphosphates MgATP and MgGTP, whereas both channel types responded to the diphosphates MgADP and MgGDP. This activation of Kir6.2/SUR2B currents by MgATP explains how the ATP concentration-response curve is shifted to the right in the presence of Mg^{2+}. In the absence of nucleotide, pinacidil and P1075 activated Kir6.2/SUR2B and Kir6.2/SUR2A currents, but the presence of nucleotide slowed the drug off-rates. In the presence of MgATP, the response to pinacidil reversed ~14 times more slowly with SUR2B than SUR2A. The EC_{50} for ATP, measured by its ability to slow the pinacidil off-rate, was also ~20 times higher for channels containing SUR2A than SUR2B. Our findings suggest that nucleotide binding and/or hydrolysis is enhanced in SUR2B compared with SUR2A, and that the greater KCO-affinities of SUR2B compared with SUR2A may be a consequence of this altered nucleotide handling.

K_{ATP} channels couple the metabolic state of a cell to its electrical activity. They are found in a variety of cell types, including smooth, cardiac, and skeletal muscle, pancreatic β-cells and some neurons (Ashcroft and Gribble, 1999). In cardiac muscle, they are involved in the response to ischemia and ischemic preconditioning (Nichols and Lederer, 1991), in vascular smooth muscle they regulate vessel tone (Quayle et al., 1997), in skeletal muscle they may be activated during fatigue (Davis et al., 1991), and in pancreatic β-cells, they play a key role in glucose-stimulated insulin secretion (Ashcroft and Gribble, 1999). K_{ATP} channels are the target for a range of therapeutic drugs, including the sulfonylureas used in the treatment of type 2 diabetes, and the KCOs such as diazoxide and pinacidil (Edwards and Weston, 1993; Ashcroft and Gribble, 1999).

The pore of the K_{ATP} channel consists of a tetramer of Kir6.2 subunits, each of which is associated with a regulatory SUR (Aguilar-Bryan et al., 1995; Sakura et al., 1995; Clement et al., 1997). The SUR subunit endows the channel with sensitivity to sulfonylureas and to the stimulatory actions of MgADP and the K_{ATP}-channel openers (Tucker et al., 1997). SUR is a member of the ABC transporter family, and like other members of this group, has multiple transmembrane domains and two intracellular NBDs, each containing a Walker A and a Walker B motif. It is believed that a conserved aspartate in the Walker B motif coordinates the Mg^{2+} ion of MgATP and that a conserved lysine residue in the Walker A motif is involved in the binding and/or hydrolysis of ATP (Azzaria et al., 1989; Carson et al., 1995; Ko and Pedersen, 1995; Ueda et al., 1997, 1999). The sulfonylurea receptors found in cardiac (SUR2A) and smooth (SUR2B) muscles are splice variants of a single gene, differing in their final exon (Inagaki et al., 1996; Isomoto et al., 1996). The splice site is distal to the Walker A and B motifs in NBD2, and alters only the final 42 amino acids of the C terminus.

Several drug and nucleotide binding sites have been localized on the sulfonylurea receptor. Sulfonylurea binding involves the C-terminal group of transmembrane helices of SUR1 (Ashfield et al., 1999), and a neighboring, but not exactly overlapping, region in the homogous domain of SUR2 has been implicated in binding of the K_{ATP}-channel openers pinacidil, P1075, and cromakalim (D'hanan et al., 1999a; Uhde et al., 1999; Babenko et al., 2000). A question that has remained unanswered, however, is why many KCOs bind

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F.R. and F.M.G. contributed equally to this article.

ABBREVIATIONS: K_{ATP}, ATP-sensitive potassium (channel); KCO, K_{ATP} channel opener; SUR, sulphonylurea receptor; Kir, inwardly rectifying potassium (channel); ABC, ATP binding cassette (transporter); NBD, nucleotide binding domain; AMP-PCP, β-γ-methylene-ATP.

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with 3- to 4-fold higher affinity to SUR2B than to SUR2A even though the splice variation does not alter the sequence of the proposed KCO-binding site (Schwanstecher et al., 1998; Shindo et al., 1998; Hambrock et al., 1999). Based on earlier data, we suggested that the potency of pinacidil on Kir6.2/SUR2A channels is modified by the presence of Mg-nucleotides (Gribble et al., 2000). One possibility, therefore, is that the greater KCO-binding affinity of SUR2B compared with SUR2A is related to a difference in Mg-nucleotide handling between the two SUR2 isoforms. In favor of this idea, lower concentrations of ATP are required for [^{3}H]P1075 binding to SUR2B than to SUR2A (Hambrock et al., 1999). In addition, Kir6.2/SUR2B channels respond to diazoxide in the presence of MgATP, whereas Kir6.2/SUR2A channels require the additional presence of ADP (D'hanan et al., 1999b).

In this article, we explore this idea by comparing the functional responses of Kir6.2/SUR2A and Kir6.2/SUR2B channels with Mg-nucleotides and with K_{ATP} channel openers. We show that channels containing SUR2B, but not those containing SUR2A, show marked activation by nucleoside triphosphates even in the absence of KCOs. In the presence of MgATP, both channel types are activated by pinacidil and P1075 but the drugs reverse more slowly with SUR2B than with SUR2A. The results indicate that the C-terminus of SUR2 influences how the channels respond to Mg-nucleotide, and suggest that this difference may itself determine the binding affinity for KCOs.

Materials and Methods

Molecular Biology. Mouse Kir6.2 (Genbank accession no. D50581; Sakura et al., 1995) and rat SUR2A (Genbank accession no. D83598; Inagaki et al., 1996) and SUR2B (Genbank accession no. D86038; Isomoto et al., 1996) cDNAs were subcloned into the pBF vector. Mutagenesis of individual amino acids was performed using the altered sites II System (Promega, Madison, WI). Synthesis of mRNA for oocyte expression was carried out using the mMessage mMachine large-scale in vitro transcription kit (Ambion, Austin, TX).

Electrophysiology. Oocytes were prepared from female *Xenopus laevis* and coinjected with ~0.1 ng Kir6.2 mRNA and ~2 ng of mRNA encoding wild-type or mutated SUR2 (Gribble et al., 1997a). The final injection volume was 50 nl/oocyte. Isolated oocytes were maintained in Barth's solution and studied 1 to 4 days after injection. Macroscopic currents were recorded from giant excised inside-out patches at 20 to 24°C (Gribble et al., 1997a). The pipette (external) solution contained 140 mM KCl, 1.2 mM $MgCl_2$, 2.6 mM $CaCl_2$, 10 mM HEPES, pH 7.4, with KOH. The intracellular (bath) solution contained either 110 mM KCl, 1.4 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, pH 7.2, with KOH (final [K^+] ~140 mM) or 110 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES, pH 7.2 with KOH (final [K^+] ~140 mM). Results were similar using the two solutions, and data were therefore combined. The Mg-free solution contained 107 mM KCl, 2.6 mM $CaCl_2$, 10 mM EDTA, 10 mM HEPES, pH 7.2 with KOH (final [K^+] ~140 mM). Stock solutions of pinacidil (Sigma, Poole, UK) and P1075 (Leo Pharmaceuticals, Ballerup, Denmark) were prepared in ethanol and dimethyl sulfoxide, respectively. Nucleotides were dissolved directly in the bath solution and the pH then readjusted as necessary. $MgCl_2$ was added to maintain the free Mg^{2+} concentration. 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. In some experiments, currents were recorded in response to repetitive 3-s voltage ramps from −110 mV to +100 mV, with a holding potential of 0 mV. They were filtered at 0.5 kHz, digitized at 1 kHz using a Digidata 1200 Interface, and analyzed using pClamp software (Axon Instruments, Burlingame, CA). The slope conductance was measured by fitting a straight line to the current-voltage relation between −20 mV and −100 mV: the average response to five consecutive ramps was calculated in each solution. ATP concentration-response curves were fit to the Hill equation: $G/G_c = 1 / (1 + ([ATP] / K_i)^h)$, where [ATP] is the ATP concentration, K_i is the ATP concentration at which inhibition is half-maximal, and h is the slope factor (Hill coefficient).

To measure the time course of the pinacidil (or P1075) response, patches were held at −50 mV and macroscopic currents were recorded in response to repeated drug applications. Currents were sampled at 20 Hz and analyzed using Microcal Origin software (Microcal Software, Northampton, MA). The decay in current after drug removal was best fit with the sum of three exponentials, one of which (τ_r) is attributable to channel rundown. This was subtracted in further analysis. The remaining current, representing the drug-activated component, was fit by the sum of two exponentials, with fast (τ_f) and slow (τ_s) time constants. Exponentials with time constants <1 s could not be detected because of the time required to exchange solutions. The relative contribution of the slow component was calculated from $(A_s/(A_s + A_f)) \times 100\%$, where A_s is the amplitude of the slow component and A_f that of the fast component. The results obtained from repeated drug applications on a single patch were averaged for each test condition.

Statistical significance was tested using Student's *t* test. Data are presented as mean ± S.E.M.

Results

Macroscopic currents were recorded from *X. laevis* oocytes expressing either Kir6.2/SUR2A or Kir6.2/SUR2B channels. In both cases, the currents were small in the cell-attached patch, but increased on patch excision. Current amplitudes were similar for Kir6.2/SUR2A and Kir6.2/SUR2B channels. We first examined the effects of nucleotides alone, and then in conjunction with pinacidil, on the macroscopic K_{ATP} current in inside-out membrane patches.

Effects of Nucleotides. Application of ATP in the presence of Mg^{2+} to the intracellular membrane surface inhibited both Kir6.2/SUR2A and Kir6.2/SUR2B currents. Inhibition of Kir6.2/SUR2B currents became weaker over the course of 10 to 20 s but remained stable in the case of Kir6.2/SUR2A currents (Fig. 1A). Concentration-response relationships for ATP inhibition of Kir6.2/SUR2B currents were constructed by measuring the steady-state extent of block. Half-maximal inhibition (K_I) was produced by 29 ± 3 μM ATP (n = 13) for Kir6.2/SUR2A currents and by 117 ± 22 μM ATP (n = 11) for Kir6.2/SUR2B currents (Fig. 1B).

We have shown previously that Mg-nucleotides exert both stimulatory and inhibitory effects on the β-cell type of K_{ATP} channel, Kir6.2/SUR1, and that the current amplitude in the presence of Mg-nucleotides reflects the balance between these two opposing actions (Gribble et al., 1998). Inhibition is mediated by a direct interaction of the nucleotide with Kir6.2, and is independent of Mg^{2+} ions, whereas activation occurs when Mg-nucleotides interact with the NBDs of SUR1 (Tucker et al., 1997; Gribble et al., 1998). A further complication is that the presence of SUR1 enhances the sensitivity of Kir6.2/SUR1 currents to the inhibitory effect of ATP (Tucker et al., 1997). Thus, although the different responses of Kir6.2/SUR2A and Kir6.2/SUR2B currents to MgATP must be conferred by the sulfonylurea receptor, it is not clear whether it results from a difference in the degree of inhibition or of activation.

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To distinguish between these possibilities, we tested the effect of ATP in the absence of Mg^{2+}, a procedure that selectively abolishes the stimulatory action of nucleotides. In contrast to what we observed in the presence of Mg^{2+}, ATP (100 μM) blocked Kir6.2/SUR2A and Kir6.2/SUR2B currents to similar degrees in Mg-free solution (Fig. 1C). The extent of block was 90 $\pm$ 4% (n = 4) for Kir6.2/SUR2A channels and 94 $\pm$ 2% (n = 11) for Kir6.2/SUR2B channels. This indicates that Kir6.2/SUR2A and Kir6.2/SUR2B are blocked by ATP to similar extents and suggests that the different ATP sensitivities observed in the presence of Mg^{2+} are the consequence of an additional stimulatory action of MgATP on Kir6.2/SUR2B currents. If this activation takes time to develop, it could also account for the slow increase in Kir6.2/SUR2B currents in the maintained presence of MgATP. Our results are consistent with previous reports that the ATP concentration-response curve of vascular smooth muscle K_{ATP} channels and cloned Kir6.2/SUR2B currents is shifted to the right in the presence of Mg^{2+} (Kamouchi and Kitamura 1994; Isomoto et al., 1996), unlike that of cardiac K_{ATP} channels and cloned Kir6.2/SUR2A currents (Lederer and Nichols 1989; Okuyama et al., 1998).

In contrast to MgATP, 100 μM MgADP activated Kir6.2/SUR2A and Kir6.2/SUR2B currents to similar extents (Fig. 1C) [by 1.8 $\pm$ 0.2-fold (n = 11) and 1.7 $\pm$ 0.1-fold (n = 9), respectively]. This suggests that the stimulatory effect of MgADP is similar for both types of channel, whereas that of MgATP differs.

To determine whether this difference between nucleoside di- and tri-phosphates also holds for other nucleotides, we tested the effects of MgGTP and MgGDP. These nucleotides have been shown to activate Kir6.2/SUR1 currents via the SUR subunit (Trapp et al., 1997) but have very little inhibitory effect on Kir6.2 (Trapp et al., 1997; Tucker et al., 1998). Because the degree of activation is greater when the channel open probability has first been reduced by nucleotide inhibition, currents were blocked with 200 μM AMP-PCP. Figure 2, A and C, shows that MgGDP activated both Kir6.2/SUR2A and Kir6.2/SUR2B currents. In contrast, MgGTP activated Kir6.2/SUR2B currents but produced a small inhibition of Kir6.2/SUR2A currents (Fig. 2, A and B). The diphosphate was more effective than the triphosphate at activating Kir6.2/SUR2B, as previously reported for SUR1 (Trapp et al., 1997; Gribble et al., 1998).

Effect of the Walker A Mutations on Nucleotide Activation. To investigate the roles of the individual NBDs of SUR2A and SUR2B, we mutated the lysine residue in the Walker A motif of either NBD1 or NBD2 to alanine (K707A and K1348A, respectively). In other ABC transporters, these mutations have been shown to abolish ATP binding and/or hydrolysis (Azzaria et al., 1989; Carson et al., 1995; Ko and Pedersen, 1995; Ueda et al., 1997, 1999), and in SUR1 they prevent activation by Mg-nucleotides (Gribble et al., 1997b, 1998; Shyng et al., 1997).

When the K1348A mutation was made in SUR2A or SUR2B, channel activation by 100 μM MgADP was abolished and the inhibitory effect of MgADP, mediated through Kir6.2, was unmasked (Fig. 3B). Mutating the Walker A lysine in NBD1 had different effects in SUR2A and SUR2B. Whereas 100 μM MgADP activated Kir6.2/SUR2A-K707A currents, the nucleotide produced partial inhibition of Kir6.2/SUR2B-K707A currents (Fig. 3B). However, the extent of

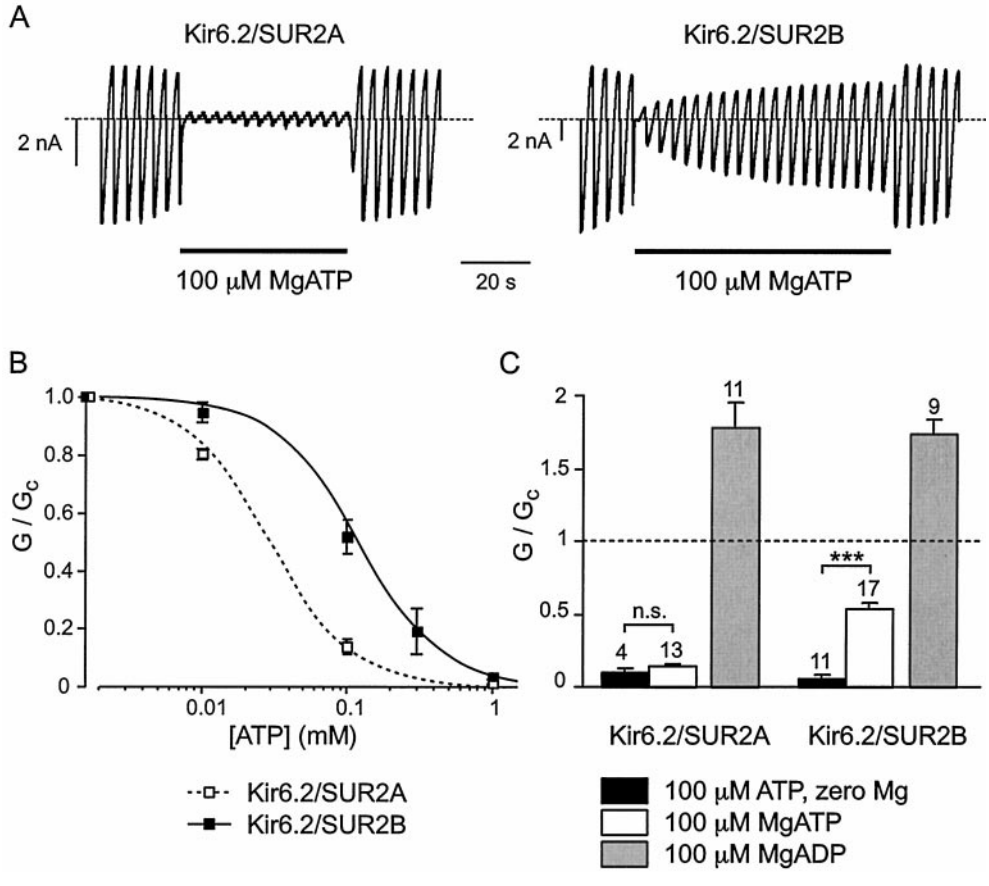

Fig. 1. ATP-sensitivity of Kir6.2/SUR2A and Kir6.2/SUR2B currents. A, macroscopic currents recorded from oocytes coexpressing Kir6.2 and either SUR2A or SUR2B in response to a series of voltage ramps from -110 to $+100$ mV. ATP (100 μM) was added to the intracellular solution as indicated by the bars. All solutions contained Mg^{2+}. B, mean ATP concentration-response relationships for Kir6.2/SUR2A (□, n = 13) and Kir6.2/SUR2B (■, n = 11) currents, in the presence of Mg^{2+}. The slope conductance (G) is expressed as a fraction of the mean (G_c) of that obtained in control solution before and after exposure to ATP. The lines are the best fit of the data to the Hill equation. For Kir6.2/SUR2A currents, K_i = 29 and h = 1.5. For Kir6.2/SUR2B currents, K_i = 117 and h = 1.5. C. Macroscopic currents recorded from oocytes coexpressing Kir6.2 and either SUR2A or SUR2B in the presence of ATP, ADP, and Mg^{2+} as indicated. Mean conductance in the presence of these agent(s) (G) is expressed relative to the mean of the conductance in the control solution before and after their addition (G_c). The dashed line indicates the control conductance. The number of patches is given above each bar. Statistical significance is indicated by: n.s., not significant; ***$P < .001$.

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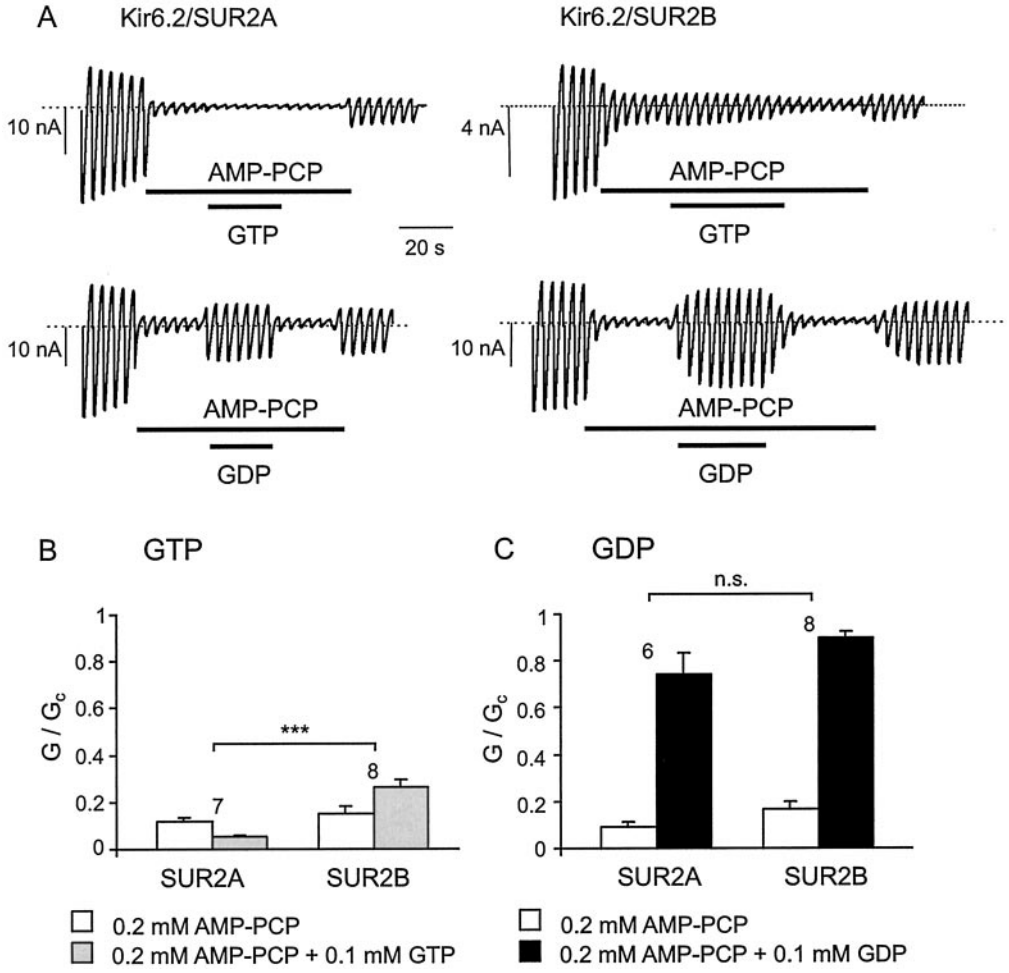

Fig. 2. Guanine nucleotide activation of Kir6.2/SUR2A and Kir6.2/SUR2B currents. A, macroscopic currents recorded from oocytes coexpressing Kir6.2 and either SUR2A or SUR2B in response to a series of voltage ramps from −110 to +100 mV. AMP-PCP (200 μM), GTP (100 μM), and GDP (100 μM) were added to the intracellular solution as indicated by the bars. All solutions contained Mg^{2+}. B and C, macroscopic currents recorded from oocytes coexpressing Kir6.2 and either SUR2A or SUR2B in the presence of 200 μM AMP-PCP plus either 100 μM GTP (B) or 100 μM GDP (C), in the presence of Mg^{2+}. Mean conductance in the presence of nucleotide(s) (G) is expressed relative to the mean conductance in control solution before and after nucleotide addition (G_c). The dashed line indicates the control conductance. The number of patches is given above each pair of bars. The percentage change in conductance caused by the addition of GDP or GTP (relative to AMP-PCP alone) was compared statistically between Kir6.2/SUR2A and Kir6.2/SUR2B currents: n.s., not significant; $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$.

this inhibition was not as great as that for Kir6.2/SUR2B-K1348A, suggesting that the stimulatory effect of MgADP may not have been abolished completely by the SUR2B-K707A mutation. The extent of activation of Kir6.2/SUR2A-K707A channels (2.2-fold) was similar to that found for wild-type Kir6.2/SUR2A channels (1.7-fold), indicating that the mutation did not reduce the extent of activation.

Mutation of either K707A or K1348A in SUR2B also abolished the stimulatory action of MgATP (Fig. 3A), as has been reported previously with SUR1 (Gribble et al., 1998).

Effects of K_{ATP}-Channel Openers. Both pinacidil (100 μM) and P1075 (10 μM) activated Kir6.2/SUR2A and Kir6.2/SUR2B currents in the absence of nucleotide (Table 1; Fig. 4, 5A). Mutation of the Walker A lysine in NBD1 (K707A), but not in NBD2 (K1348A), of either SUR2A or SUR2B abolished the nucleotide-independent action of pinacidil. Addition of 100 μM MgATP fully restored the response of Kir6.2/SUR2B-K707A currents to pinacidil (Table 1), but only partially restored the response of Kir6.2/SUR2A-K707A currents.

Rates of Reversal. As reported previously (Gribble et al., 2000), the activation of Kir6.2/SUR2A by pinacidil or P1075 reversed rapidly in the absence of added nucleotide. This was also found to be the case for Kir6.2/SUR2B currents (Fig. 4, 5A). The off-rate of pinacidil could be fit by a single exponen-

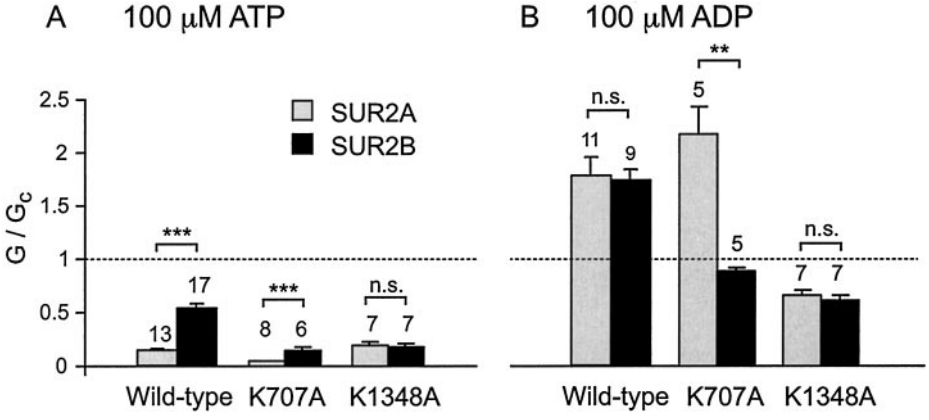

Fig. 3. Activation of wild-type and mutant Kir6.2/SUR2A and Kir6.2/SUR2B currents by nucleotides. Macroscopic currents recorded from oocytes coexpressing Kir6.2 and either wild-type or mutant SUR2A or SUR2B in the presence of 100 μM ATP (A) or 100 μM ADP (B). Mean conductance in the presence of nucleotide (G) is expressed relative to the mean conductance in control solution before and after nucleotide addition (G_c). All solutions contained Mg^{2+}. The dashed line indicates the control conductance. The number of patches is given above each bar. Statistical significance is indicated by: n.s., not significant; $^{**}P < .01$; $^{***}P < .001$.

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tial with a mean time constant of 1.8 ± 0.2 s (n = 5) for Kir6.2/SUR2A currents and of 3.3 ± 0.5 s (n = 6) for Kir6.2/SUR2B currents. The off-rates of P1075 were slightly slower: τ = 5.7 ± 0.6 s (n = 6) for Kir6.2/SUR2A currents and τ = 5.7 ± 0.9 s (n = 7) for Kir6.2/SUR2B currents. The C-terminus does not, therefore, appear to have a marked effect on the off-rate in the absence of added nucleotide.

Addition of ATP in the presence of Mg^{2+} slowed the off-rate of both drugs. For Kir6.2/SUR2A currents, the off-rate of pinacidil in the presence of 100 μM ATP could be described by a single exponential with a time constant of 13 ± 1 s (n = 13) (Fig. 5A). For Kir6.2/SUR2B currents, the off-rate of pinacidil was too slow to measured in 100 μM ATP and, in 10 μM ATP, reversed with a time constant of 108 ± 25 s (Fig. 5A; n = 6). Even slower off-rates were measured when P1075 was removed in the continued presence of 100 μM ATP: the time constant of the off-rate was ~110 s for Kir6.2/SUR2A currents (from Gribble et al., 2000) and the activation was effectively irreversible for Kir6.2/SUR2B currents (Fig. 4).

At lower ATP concentrations, the off-rate of the pinacidil response was best described by the sum of two exponentials: a fast exponential, with a time constant similar to that measured in the absence of nucleotide, and a slow exponential (Fig. 5A). The contribution of the fast exponential declined as the ATP concentration increased, being reduced to zero with 100 μM ATP (Fig. 5B). The ATP concentration at which 50% of the pinacidil-activated current reversed rapidly was 1.4 μM for Kir6.2/SUR2A currents and 0.09 μM for Kir6.2/SUR2B currents. The fast time constant was essentially independent of the ATP concentration. However, the slow time constant for Kir6.2/SUR2B currents increased at higher ATP concentrations (Fig. 5C).

Discussion

Different Responses of SUR2A and SUR2B to Nucleotides. In the absence of Mg^{2+}, Kir6.2/SUR2A and Kir6.2/SUR2B currents were inhibited to similar degrees (~90%) by 100 μM ATP, suggesting that there is little difference in the ability of the two SUR2 isoforms to enhance the sensitivity of the ATP inhibitory site on Kir6.2. Thus, although the C-terminal 42 amino acids of SUR have been implicated in conferring the different ATP sensitivities of K_{ATP} channels containing SUR1 and SUR2A (Babenko et al., 1999), our results suggest that the splice variation between SUR2A and SUR2B does not have a marked effect on Mg-independent ATP inhibition. In the presence of Mg^{2+}, however, the inhibitory effect of ATP on Kir6.2/SUR2B currents was greatly reduced. Taken together with the effects of GTP, our results suggest that Mg-nucleoside triphosphates have a much greater stimulatory action on Kir6.2/SUR2B currents than on Kir6.2/SUR2A currents. This additional stimulation accounts for the apparent lower ATP sensitivity of Kir6.2/SUR2B currents when measured in the presence of Mg^{2+}.

In contrast to the nucleoside triphosphates, the diphosphates MgADP and MgGDP activated Kir6.2/SUR2A and Kir6.2/SUR2B currents to similar extents. One possible explanation for our results, therefore, is that only Mg-nucleoside diphosphates mediate channel activation, and that the stimulation of Kir6.2/SUR2B currents by nucleoside triphosphates results from greater Mg-dependent nucleotide hydrolysis by SUR2B. ATP hydrolysis has been measured in other ABC transporters (Senior and Gadsby, 1997), and recent azido-ATP binding studies on SUR1 have suggested that the second NBD may be able to hydrolyze ATP (Matsuo et al., 1999). An alternative explanation, however, is that the different effects of Mg-nucleoside triphosphates on Kir6.2/SUR2A and Kir6.2/SUR2B currents are caused by a lower nucleotide potency on SUR2A compared with SUR2B. In support of this idea, it has been shown previously that the sensitivity of Kir6.2/SUR2A channels to activation by UDP is lower than that of Kir6.2/SUR2B channels [K_i values of ~240 μM and ~70 μM, respectively (Isomoto et al., 1996; Okuyama et al., 1998)].

Responses to K_{ATP} Channel Openers. Pinacidil and P1075 activated Kir6.2/SUR2A and Kir6.2/SUR2B currents in the absence of added nucleotide, and under these conditions the effect of both drugs was rapidly reversible. The off-rate of P1075 was similar for channels containing SUR2A and SUR2B, suggesting that the C terminus does not influence the rate of reversal in the absence of nucleotide.

In the presence of MgATP, however, the activation of Kir6.2/SUR2B currents by pinacidil and P1075 reversed more slowly, as shown previously for Kir6.2/SUR2A currents (Gribble et al., 2000). In fact, the off-rate of pinacidil was ~14 times slower for Kir6.2/SUR2B currents than for Kir6.2/SUR2A currents when measured in the presence of 10 μM MgATP (τ = ~110 s for SUR2B and ~8 s for SUR2A). A similar difference was also observed with P1075: in the presence of 100 μM MgATP, activation of Kir6.2/SUR2A currents reversed with a time constant of ~110 s, whereas the rate of reversal of Kir6.2/SUR2B currents was too slow to be measured.

Binding studies have shown half-lives for the dissociation of [^{3}H]P1075 that are comparable with the off-rates we measured electrophysiologically ($\tau_{0.5}$ of ~10 min for SUR2B and ~1.1 min for SUR2A, in 3 mM MgATP at 37°C: Hambrock et al., 1999). Our data suggest that the greater potency of pinacidil and P1075 on Kir6.2/SUR2B currents compared with Kir6.2/SUR2A currents is caused by differences in the ability of MgATP to slow the off-rate of these drugs. This may

TABLE 1

Activation of wild-type and mutant K_{ATP} currents by nucleotides and pinacidil

Currents in the presence of pinacidil (100 μM) and/or ATP (100 μM) are given as a percentage of the current in control solution, lacking both drug and nucleotide. Data are presented as mean ± S.E.M. (n). Channels were composed of Kir6.2 and the sulfonylurea receptor indicated.

		Pinacidil	ATP	ATP + pinacidil
SUR2A	Wild-type	349 ± 61 (12)	14 ± 4 (7)	118 ± 9 (7)
	K707A	94 ± 3 (10)	4 ± 1 (12)	10 ± 1 (12)
	K1348A	250 ± 37 (14)	19 ± 4 (10)	89 ± 7 (10)
SUR2B	Wild-type	233 ± 19 (8)	43 ± 7 (5)	84 ± 6 (5)
	K707A	88 ± 3 (5)	10 ± 3 (6)	113 ± 11 (6)
	K1348A	198 ± 35 (7)	10 ± 2 (5)	86 ± 5 (5)

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explain why the potencies of the drugs on channels containing SUR2A and SUR2B seem to differ even though the SUR splice variation does not form part of the proposed KCO-binding site (D'hanan et al., 1999a; Uhde et al., 1999; Babenko et al., 2000). Indeed, because Kir6.2/SUR2A and Kir6.2/SUR2B currents behaved differently even in the absence of pinacidil, it is most likely that the principal difference between Kir6.2/SUR2A and Kir6.2/SUR2B channels is in either the binding and/or hydrolysis of nucleotide at the NBDs or in coupling these events to the opening of Kir6.2.

At intermediate MgATP concentrations, the pinacidil off-rate was best fit by the sum of two exponentials, one fast and one slow. The concentration of ATP at which half the current decayed rapidly (IC_{50}) was lower for Kir6.2/SUR2B (0.1 μM) than Kir6.2/SUR2A (1.4 μM) currents. The time constant of the slow exponential found for Kir6.2/SUR2B currents was also ATP-dependent, and increased with ATP concentration. There are at least two possible explanations for these results. First, there may be a real difference in the ATP affinity of SUR2A and SUR2B, and the ATP-dependence of the slow exponential for Kir6.2/SUR2B currents might be caused by enhanced ATP binding at higher concentrations. In support of this idea, we measured different ATP IC_{50} values for Kir6.2/SUR2A and Kir6.2/SUR2B currents in the presence of pinacidil (1.4 μM versus 0.1 μM, respectively), and differences in ATP sensitivity have also been reported from [^{3}H]P1075 binding studies [110 μM versus 3 μM, for SUR2A and SUR2B, respectively (Hambrock et al., 1999)]. Secondly, nucleotide hydrolysis might slow the pinacidil off-rate more than nucleotide binding alone. If more hydrolysis occurs with SUR2B than with SUR2A, this might cause the longer time constants found for Kir6.2/SUR2B currents. The enhancement of hydrolysis at higher ATP concentrations might account for the ATP-dependence of the slow exponential.

Effect of the Walker A Mutations. Mutation of the Walker A lysine in NBD2 abolished the stimulatory effect of MgADP on both Kir6.2/SUR2B and Kir6.2/SUR2A currents. Similar results have been described previously for SUR1 and SUR2A (Gribble et al., 1997b, 2000; Shyng et al., 1997; D'hanan et al., 1999b). Our data are consistent with the idea that the K1348A mutation reduces MgADP binding to NBD2 of SUR2, and thereby impairs Mg-nucleotide stimulation of channel activity.

In contrast, the corresponding mutation in NBD1 had different effects in the two SUR2 isoforms. In SUR2B, mutating the Walker A lysine in NBD1 (K707A) significantly impaired MgADP-activation [as observed previously for SUR1 (Gribble et al., 1997b)], whereas the identical mutation in SUR2A did not prevent stimulation by MgADP (Gribble et al., 2000). This finding is slightly curious because the sequence of NBD1 is identical in SUR2A and SUR2B. The results suggest either that the mutation affects nucleotide binding at NBD1 differently in SUR2A and SUR2B, or that nucleotide binding at NBD1 is critical for MgADP activation of Kir6.2/SUR2B but not Kir6.2/SUR2A channels. In either case, our data suggest that the alternatively spliced C terminus of SUR2 modifies the properties of NBD1. Interactions between the NBDs of SUR1 have been demonstrated in binding studies using azido-ATP (Ueda et al., 1997, 1999) but have not yet been studied biochemically for SUR2. The finding that SUR2B behaves more like SUR1 when NBD1 is mutated is interesting because the alternatively-spliced final exon of SUR2B is more homologous to that of SUR1 (75% identity) than to that of SUR2A (36% identity) (Isomoto et al., 1996).

Effect of the Walker Mutations on Pinacidil Activation. We suggested previously that pinacidil may only be effective on wild-type Kir6.2/SUR2A channels when Mg-nucleotide is bound at NBD1 and that the activation observed in nucleotide-free solutions is caused by the presence of pre-bound ATP or ADP, which takes several minutes to dissociate from its binding site (Gribble et al., 2000). In agreement with this idea, mutation of lysine 707 in SUR2B, as in SUR2A, abolished the effect of pinacidil in the absence of added nucleotide. Unlike what was found for Kir6.2/SUR2A-K707A currents, however, addition of MgATP fully restored pinacidil activation of Kir6.2/SUR2B-K707A currents. One possible interpretation of this result is that the C terminus of SUR2 influences the nucleotide binding site at NBD1, consistent with the idea that the tail of SUR is involved in mediating interactions between the NBDs. Alternatively, the result might be caused by a difference in the nucleotide handling of SUR2B compared with SUR2A.

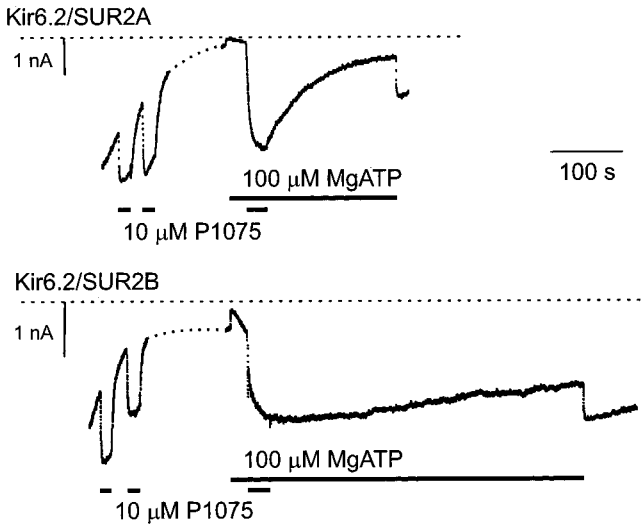

Fig. 4. Effect of nucleotides on the time course of P1075 reversal. Kir6.2/SUR2A and Kir6.2/SUR2B currents recorded at a holding potential of −50 mV. P1075 (10 μM) and MgATP (100 μM) were added as indicated by the horizontal bars. The dashed line indicates the zero current level.

Conclusions

Our results demonstrate two principal differences between SUR2A and SUR2B. First, Mg-nucleoside triphosphates stimulated Kir6.2/SUR2B but not Kir6.2/SUR2A currents. Secondly, MgATP was more effective at slowing the off-rate of K_{ATP} channel openers on Kir6.2/SUR2B compared with Kir6.2/SUR2A currents. It thus seems that SUR2B can make use of nucleoside triphosphates where SUR2A cannot, consistent with the report that Mg-ATP is sufficient for the activation of Kir6.2/SUR2B currents by diazoxide, whereas Kir6.2/SUR2A currents only responded to the drug in the presence of Mg-ADP (D'hanan et al., 1999b). The different effects of nucleoside triphosphates on channels containing SUR2A or SUR2B suggest the possibility that MgATP and MgGTP may stimulate channel activity because of their hydrolysis to the corresponding nucleoside diphosphate and that the rate of this hydrolysis

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may be faster for SUR2B than SUR2A. Our results are consistent with a model in which the C terminus of SUR influences interactions between the two NBDs and so accounts for the different nucleotide sensitivities and KCO off-rates of Kir6.2/SUR2A and Kir6.2/SUR2B channels. The marked difference in pinacidil off-rates for Kir6.2/SUR2A and Kir6.2/SUR2B currents in the presence of MgATP probably accounts for the different binding affini-

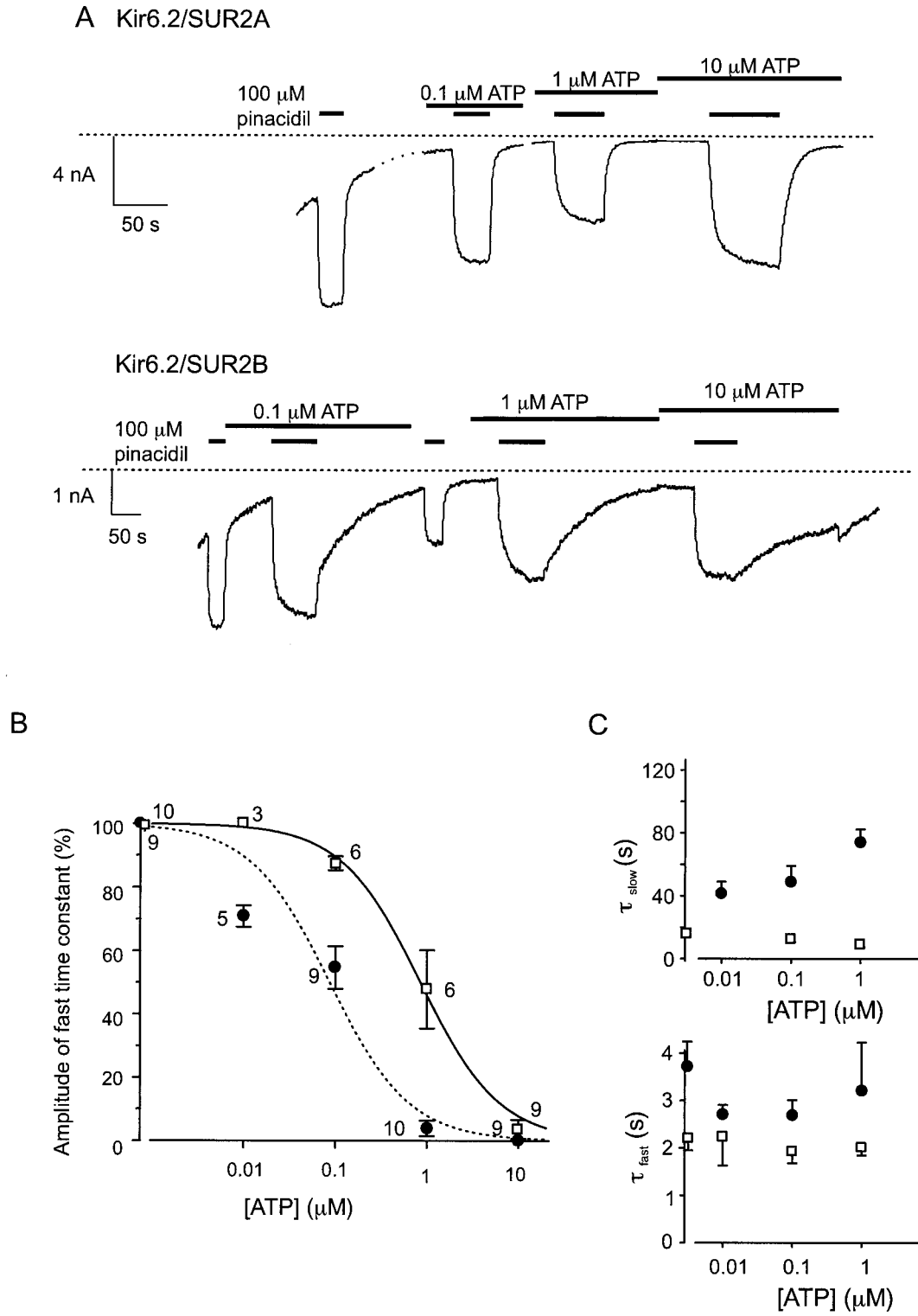

Fig. 5. Effect of nucleotides on the time course of pinacidil reversal. A, Kir6.2/SUR2A and Kir6.2/SUR2B currents recorded at a holding potential of −50 mV. Pinacidil (100 μM) and MgATP were added as indicated by the horizontal bars. The dashed line indicates the zero current level. B, relationship between ATP concentration and the fraction of the current that decays with a fast exponential on removal of pinacidil for Kir6.2/SUR2A (□) and Kir6.2/SUR2B (●) currents. The lines drawn through the points are fitted with the equation $y = 100/[1 + ([ATP]/IC_{50})]$, with IC_{50} = 1.4 μM for Kir6.2/SUR2A currents and 0.09 μM for Kir6.2/SUR2B currents. The numbers adjacent to the symbols indicate the number of patches. C, ATP-dependence of the time constants of the fast (above) and slow (below) exponentials for the data shown in B.

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ties of SUR2A and SUR2B to this KCO. A similar effect may occur with other K_{ATP} channel openers.

Note Added in Proof

As this article was being readied for publication, studies of nucleotide binding to the NBDS of SUR2A and SUR2B have been reported (Matsuo et al., 2000).

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