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Nucleotide Modulation of Pinacidil Stimulation of the Cloned K_{ATP} Channel Kir6.2/SUR2A

FIONA M. GRIBBLE, FRANK REIMANN, REBECCA ASHFIELD, and FRANCES M. ASHCROFT

University Laboratory of Physiology, Oxford, United Kingdom

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ABSTRACT

ATP-sensitive K^+ channels are the target for K^+ channel openers such as pinacidil. These channels are formed from pore-forming Kir6.2 and regulatory sulfonylurea receptor (SUR) subunits. Pinacidil activates channels containing SUR2A (heart, skeletal muscle), but not those containing SUR1 (β cells). Surprisingly, binding of the pinacidil analog [^{3}H]P1075 is dependent on added nucleotides, yet in electrophysiological studies, pinacidil is effective in the absence of intracellular nucleotides. To determine the reason for this anomaly, we examined the functional interactions between pinacidil (or P1075) and nucleotides by expressing cloned Kir6.2/SUR2A channels in *Xenopus laevis* oocytes. Both pinacidil and P1075 activated macroscopic Kir6.2/SUR2A currents in the absence of added nucleotide, but the presence of intracellular ATP or ADP slowed the off-rate of the response. Mutation of the Walker A lysine in a single nucleotide binding domain (NBD) of SUR2A (K707A in NBD1, K1348A in NBD2), abolished this action of nucleotide. The K1348A mutation prevented stimulation by MgADP but had little effect on the amplitude of the pinacidil response. In contrast, Kir6.2/SUR2A-K707A currents were activated by MgADP, but only responded to pinacidil in the presence of Mg-nucleotide. Off-rates in the absence (or presence) of nucleotide were slower for the pinacidil analog P1075 than for pinacidil, consistent with the higher affinity of P1075. We suggest that slowing of P1075 dissociation by nucleotide enables binding to be detected.

ATP-sensitive K^+ channels (K_{ATP} channels) are found in a wide variety of tissues, including pancreatic β cells, cardiac, skeletal, and smooth muscles, and some neurons, where they couple the metabolic state of a cell to its electrical activity (Ashcroft and Ashcroft, 1990; Aguilar-Bryan and Bryan, 1999). This is considered to be mediated by changes in adenine nucleotide levels, particularly ATP (which blocks the channel) and MgADP (which activates the channel). K_{ATP} channels are also activated by K_{ATP}-channel openers, a structurally diverse group of drugs with a wide range of potential therapeutic applications, and they are inhibited by sulfonylurea drugs that are used to treat type 2 diabetes.

The K_{ATP} channel is a heteromeric complex of four Kir6.x and four sulfonylurea receptor (SUR) subunits: Kir6.2 subunits form the ATP-sensitive channel pore, and SUR acts as a regulatory subunit, endowing the K_{ATP} channel with sensitivity to the stimulatory effects of MgADP and K_{ATP}-channel openers and to the inhibitory actions of sulfonylureas (Aguilar-Bryan et al., 1995; Inagaki et al., 1995, 1996; Sakura et al., 1995; Isomoto et al., 1996; Clement et al., 1997; Tucker et al., 1997). Like other members of the ATP-binding cassette transporter family, SUR has two nucleotide-binding domains (NBDs). Mutagenesis studies have revealed that Mg-nucleotides promote channel activity by interacting with these NBDs (Nichols et al., 1996; Gribble et al., 1997b, 1998; Shyng et al., 1997).

Two SUR genes have been identified; one encodes the β cell isoform (SUR1), and the other encodes the cardiac/skeletal muscle (SUR2A) and smooth muscle (SUR2B) isoforms (Aguilar-Bryan et al., 1995, Inagaki et al., 1996; Isomoto et al., 1996). K_{ATP} channels containing different SURs show distinct sensitivities to K_{ATP}-channel openers. Thus, Kir6.2/SUR1 currents are activated by diazoxide but not by pinacidil (Gribble et al., 1997a), Kir6.2/SUR2A currents are strongly activated by pinacidil but only weakly responsive to diazoxide (Inagaki et al., 1996), and Kir6.2/SUR2B currents are activated by both drugs (Schwanstecher et al., 1998).

There is currently a discrepancy between the effects of pinacidil in electrophysiological experiments and that observed for the pinacidil analog P1075 in binding studies. Provided that the drug is tested soon after patch excision, pinacidil enhances cardiac K_{ATP} currents in the absence of added nucleotides (Fan et al., 1990; Terzic et al., 1995), although it is ineffective once channel activity has completely run down (Shen et al., 1991; Tung and Kurachi, 1991; Allard and Lazdunski, 1992; Terzic et al., 1995). In contrast, both nucleotide and Mg^{2+} ions are required for detection of

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ABBREVIATIONS: K_{ATP} channel, ATP-sensitive K^+ channels; SUR, sulfonylurea receptor; NBD, nucleotide-binding domain; W_A, Walker A.

[^{3}H]P1075 binding, both to native skeletal and cardiac muscle K_{ATP} channels (Dickinson et al., 1997, Löffler-Walz and Quast, 1998), and to cloned SUR2A and SUR2B subunits (Schwanstecher et al., 1998; Hambrock et al., 1998, 1999). It has not yet been determined whether this difference in nucleotide dependence is caused by differences in the mechanism of action of P1075 and pinacidil or has another explanation.

In this article, we explore the mechanism underlying the nucleotide modulation of pinacidil activity on Kir6.2/SUR2A currents and show that MgATP (and MgADP) can slow the off-rate of pinacidil through an interaction with the NBDs of SUR2. This may underlie the requirement for Mg-nucleotide in binding studies.

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) 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 SUR2A (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 a holding potential of 0 mV and 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 110 mM KCl, 1.4 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, pH 7.2 with KOH; final $[K^+]$ ~140 mM). 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) were prepared in ethanol and dimethyl sulfoxide, respectively. Nucleotides were dissolved directly in the bath solution and the pH was then readjusted as necessary. 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-sec voltage ramps from −110 mV to +100 mV. They were filtered at 0.5 kHz, digitised at 1 kHz using a Digidata 1200 Interface, and analyzed using pClamp software (Axon Instruments, Foster City, 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.

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 (Microcal Software, Northampton, MA). The decay in current after drug removal was best fit with a 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 a sum of two exponentials, with fast (τ_f) and slow (τ_s) time constants. 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. Exponentials faster than ~1 s could not be resolved because of the time taken to change solutions, and exponentials slower than 300 s could not be resolved accurately because other processes, such as rundown, start to influence the time course of the current. Statistical significance was tested using Student's *t* test. Data are presented as mean ± 1 S.E.

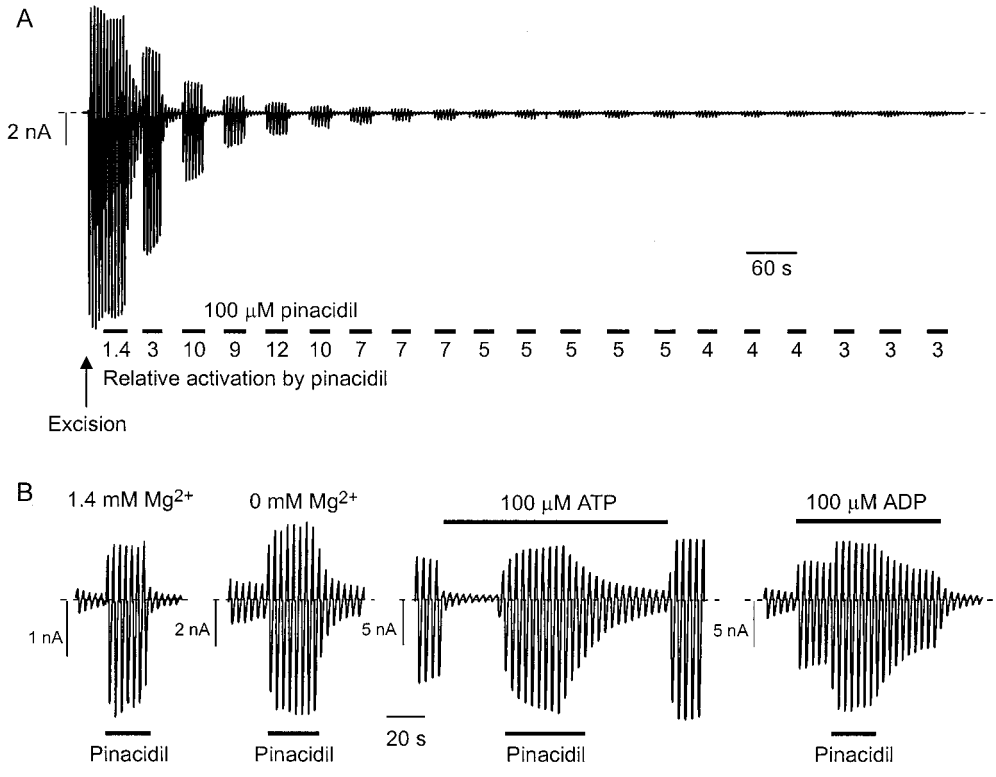

Fig. 1. Pinacidil activation of Kir6.2/SUR2A currents. A, macroscopic Kir6.2/SUR2A currents recorded in response to a series of voltage ramps from −110 to +100 mV. The patch was excised into nucleotide-free solution (1.4 mM Mg^{2+}) at the point indicated by the arrow. Pinacidil (100 μM) was applied repeatedly, as indicated by the bars; for each application, the conductance in the presence of the drug was expressed relative to the mean of the conductances measured in control solution before and after drug addition. This value is indicated below the bar (*x*-fold activation). B, effect of pinacidil (100 μM) applied in the presence of different intracellular solutions. Same voltage protocol as in A. Nucleotides (100 μM) and pinacidil (100 μM) were added as indicated by the bars. All solutions contained Mg^{2+} unless otherwise indicated. Each panel represents a different patch and the current was allowed to run down partially before pinacidil (and nucleotides) were added because there was little response to the drug immediately after patch excision (see A).

Results

Large currents were recorded immediately after excision of inside-out patches from oocytes coinjected with mRNAs encoding Kir6.2 and SUR2A. These subsequently declined with time (rundown) at a variable rate (Fig. 1A). Addition of 100 μM pinacidil in the absence of nucleotides to the intracellular (bath) solution increased the Kir6.2/SUR2A current (Fig. 1A). The extent of activation ranged from 2- to 9-fold, even within a single patch (mean 4.7 ± 0.4-fold; n = 7 patches). Responses were dependent on the time of drug application: they were small immediately after patch excision, increased after partial channel rundown, and then declined slowly over 10 to 15 min (Fig. 1A). The maximal extent of activation never exceeded the current amplitude measured immediately after patch excision. Channel activation did not require intracellular Mg^{2+} ions, because pinacidil enhanced Kir6.2/SUR2A currents even after ~10 min in Mg^{2+}-free solution (mean activation = 4.1 ± 0.6-fold; n = 4; Fig. 1B). These results contrast with the requirement for both ATP and Mg^{2+} for [^{3}H]P1075 binding to SUR2A (Schwanstecher et al., 1998; Hambrock et al., 1999).

We next examined the effect of pinacidil on Kir6.2/SUR2A currents in the presence of intracellular nucleotides. Figure 1B shows that application of 100 μM MgATP inhibited channel activity, and that subsequent addition of pinacidil (in the presence of ATP) restored the current to the control level. In the presence of MgATP, the mean activation by pinacidil was 8.1 ± 2.9-fold (n = 7; Fig. 2). Repeated drug applications produced stable responses (Fig. 3), consistent with the ability of MgATP to prevent channel rundown (Ohno-Shosaku et al., 1987). In contrast to ATP, 100 μM MgADP enhanced Kir6.2/SUR2A currents. The magnitude of this activation, like that produced by pinacidil, varied with the extent of channel rundown, and it was smallest immediately after patch excision. The subsequent addition of pinacidil to the MgADP-containing solution produced a further 1.5 ± 0.1-fold (n = 5) increase in channel activity (Figs. 1B and 2).

To investigate the role of the NBDs of SUR2A in Kir6.2/SUR2A channel activation by nucleotides and pinacidil, we introduced mutations into the Walker A (W_A) motifs of the NBDs. In other ATP-binding cassette transporters, a lysine residue in the W_A motif forms part of the nucleotide-binding site and is essential for ATP hydrolysis (Azzaria et al., 1989; Carson et al., 1995; Ko and Pedersen, 1995). Furthermore, mutation of a single W_A lysine in either NBD of SUR1 abolishes channel activation by Mg-nucleotides (Gribble et al., 1997b, 1998; Shyng et al., 1997). We therefore mutated each of the W_A lysines in SUR2A individually to alanine (K707A in NBD1 and K1348A in NBD2).

Fig. 2 shows that mutation K707A in NBD1 of SUR2A to alanine had no effect on channel activation by MgADP, in contrast to mutation of the equivalent residue in SUR1 (Gribble et al., 1997b; Shyng et al., 1997). Mutation of K1348A in NBD2 to alanine, however, abolished stimulation by MgADP and unmasked an inhibitory effect of the nucleotide (mediated by Kir6.2; Tucker et al., 1997). Figure 2 also shows the effects of the W_A mutations on the amplitude of the pinacidil response. Only the K707A mutation (in NBD1) prevented the stimulatory effect of pinacidil in the absence of Mg-nucleotide. Pinacidil was able to activate all mutant channels in the presence of MgATP, although the amount of activation of Kir6.2/SUR2A-K707A currents was very small (Fig. 2). In the presence of MgADP, pinacidil caused clear activation of wild-type, Kir6.2/SUR2A-K1348A and Kir6.2/SUR2A-K707A currents.

As is the case for pinacidil, P1075 stimulated Kir6.2/SUR2A currents in both the absence and presence of nucleotides (Fig. 3). The mean activation at −50 mV was 2.2 ± 0.3-fold (n = 6) in the absence of nucleotide, when expressed as a fraction of the mean current before and after drug addition (compared with 4.7-fold for pinacidil). In the presence of 100 μM MgATP, the off-rate of P1075 was very slow and we have expressed the extent of drug activation relative to the current recorded in MgATP solution before drug addition. This was 13 ± 4-fold (n = 6) (for comparison, pinacidil produced a 16 ± 4-fold increase in current when calculated in this way). Thus, despite the fact that binding has not been observed, P1075 is able to stimulate K_{ATP} currents in the absence of nucleotide.

Rate of Reversal of Pinacidil Activation. The marked increase in K_{ATP} current produced by pinacidil and P1075 reversed rapidly on removal of the drug. The presence of Mg-nucleotide seemed to slow the rate of reversal (off-rate; Figs. 1B and 3), suggesting that nucleotides might either slow the dissociation of the drug from its binding site or the rate at which activated channels relax back to the resting state. To explore this phenomenon more fully, we analyzed the off-rate of each drug by recording macroscopic currents at a fixed membrane potential of −50 mV (Fig. 3).

After pinacidil application in nucleotide-free solution, the off-rate of the response was best fit by a sum of three exponentials. One exponential (τ_r, 51 ± 8 s; n = 5) was caused by

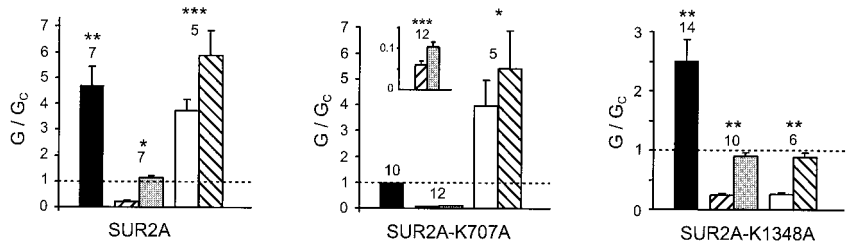

Fig. 2. Pinacidil activation of wild-type and mutant Kir6.2/SUR2A currents. Macroscopic currents recorded from oocytes coexpressing Kir6.2 and either wild-type or mutant (K707A, K1348A) SUR2A. Pinacidil (100 μM) was applied in the presence or absence (control) of the nucleotides indicated. Mean conductance in the presence of nucleotide, pinacidil or pinacidil plus nucleotide (G) is expressed relative to the control conductance (G_c). The control conductance is the mean of that measured in control solution (lacking both drug and nucleotide) before and immediately after removal of the drug and nucleotide. The dashed line indicates the control conductance. The number of patches is given above each bar. *P < .05; **P < .01; ***P < .001 (using the single sample t test of the percent increase by pinacidil). ■, 100 μM pinacidil; ▨, 100 μM MgATP; ▩, 100 μM MgATP + 100 μM pinacidil; □, 100 μM MgADP; ▧, 100 μM MgADP + 100 μM pinacidil.

the rundown of channel activity, as it was observed even in the absence of drug. Its contribution declined with time, in parallel with the rundown of the background current, and it was subtracted in subsequent analysis. The decay of the remaining current, which represents the pinacidil-activated component, was best fit by the sum of a fast and a slow exponential, with mean time constants of 1.3 ± 0.2 s (τ_f, n = 4) and 8.3 ± 1.4 s (τ_s, n = 4). The relative contributions of these two exponentials altered with time, although their time constants did not vary. Soon after patch excision (at ~75 s), the slow exponential comprised 54 ± 11% (n = 5) of the pinacidil-activated current, but after 3 min, its contribution had fallen to 26 ± 8% (n = 5). When K_{ATP} currents were refreshed by the application and subsequent removal of MgATP (0.1 to 1 mM), the amplitude of the slow component increased again, comprising 63 ± 7% (n = 4) of the pinacidil response ~80 s after MgATP removal.

When pinacidil was tested in the presence of MgATP or MgADP (100 μM), the off-rate of the drug followed a single exponential with a time constant of 12 ± 2 s (n = 11) in MgATP and 9.7 ± 0.6 s in MgADP (n = 5) (Fig. 4), rates which resemble the slow time constant (τ_s) observed in nucleotide-free solution. The presence of nucleotide thus seemed to enhance the proportion of the pinacidil-activated current that decayed slowly. The effect of MgATP required Mg^{2+} ions, because in Mg-free solution containing 100 μM ATP, the pinacidil response decayed rapidly, with a time constant of 1.3 ± 4 s (n = 5). In contrast to ATP and ADP, 200 μM AMP-PCP did not slow the off-rate of pinacidil in the presence of Mg^{2+} (τ_f = 1.6 ± 0.2 s, τ_s = 9.9 ± 0.7 s, amplitude τ_s = 10 ± 6%, n = 7; Fig. 4).

Fig. 3B illustrates the decay of the response to the pinacidil analog P1075 in the presence and absence of ATP. As for pinacidil, the off-rate was slowed by the presence of nucleotide: τ = 5.7 ± 0.6 s (n = 6) in control solution and 108 ± 17 s (n = 6) in the presence of 100 μM MgATP.

We next examined the off-rate of pinacidil for channels carrying mutations in the NBDs of SUR2A (Fig. 4). In the absence of nucleotide, most (>90%) of the pinacidil response of Kir6.2/SUR2A-K1348A currents reversed with a fast time constant of ~1.0 s (Fig. 4). Neither MgADP nor MgATP had a significant effect on this time constant. Thus, when 100 μM MgADP was present, the off-rate of the drug exhibited a single fast time constant of 1.7 ± 0.3 s (n = 5), whereas in the presence of 100 μM ATP, 83 ± 6% of the current decayed with a time constant of 1.3 ± 0.2 sec, and the remaining current declined with a time constant of 11 ± 2 s (n = 5). The off-rate of pinacidil on Kir6.2/SUR2A-K707A currents in the presence of MgADP followed a single exponential with a mean

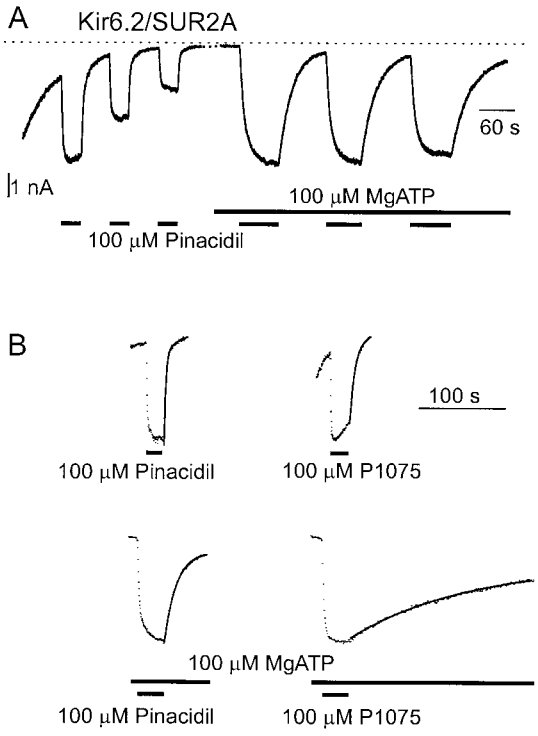

Fig. 3. Effect of nucleotides on the time course of pinacidil and P1075 reversal. Kir6.2/SUR2A currents were recorded at a holding potential of −50 mV. A, pinacidil (100 μM) and MgATP (100 μM) were added as indicated by the horizontal bars. The dashed line indicates the zero current level. The time constant of rundown in this patch was 62 s. B, currents recorded in ATP-free or 100 μM MgATP have been normalized to the same peak amplitude. Pinacidil (100 μM) or P1075 (10 μM) were applied as indicated by the bars. The dots indicate the data, the lines indicate fits of the data with one or two exponentials, and the following time constant and relative amplitudes (in brackets): Control, pinacidil: τ_f = 1.8 s, τ_s = 9.7 s (12%); P1075: τ_f = 5.5 s, τ_s = 106 s (38%). +MgATP, pinacidil: τ_s = 14 s; P1075: τ_s = 136 s.

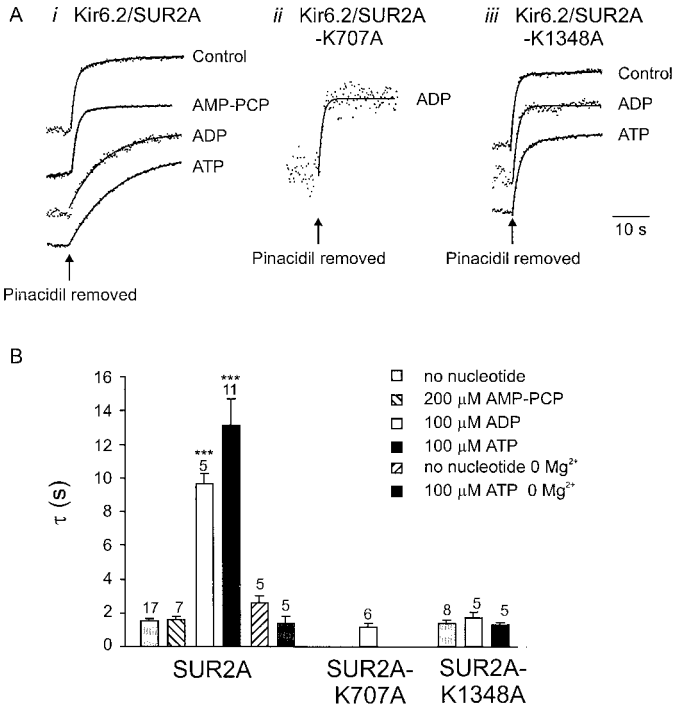

Fig. 4. Time course of pinacidil reversal. A, time course of pinacidil reversal for Kir6.2/SUR2A (i), Kir6.2/SUR2A-K707A (ii), or Kir6.2/SUR2A-K1348A currents (iii), at a holding potential of −50 mV. Pinacidil (100 μM) was applied in nucleotide-free solution (control) or in the presence of 100 μM MgATP, 100 μM MgADP, or 200 μM MgAMP-PCP, as indicated. The drug was removed at the arrow. The dots indicate the data, and the lines indicate fits of the data with one or two exponentials, and the following time constant and relative amplitudes (in brackets). i, Kir6.2/SUR2A: Control, τ_f = 1.0 s, τ_s = 8.2 s (16%); AMP-PCP, τ_f = 1.2 s, τ_s = 10.2 s (9%); ATP, τ_s = 13 s; ADP, τ_s = 9.3 s. ii, Kir6.2/SUR2A-K707A: ADP, τ_f = 1.0 s. iii, Kir6.2/SUR2A-K1348A: Control, τ_f = 1.2 s; ATP, τ_f = 1.7 s, τ_s = 12 s (17%); ADP, τ_f = 1.4 s. B, mean fast time constants for pinacidil (100 μM) reversal in the presence of Mg^{2+} and either no nucleotides (control) or 100 μM of the nucleotide indicated, for wild-type or mutant Kir6.2/SUR2A channels. The number of patches is given above each bar. *** $P < .001$.

time constant of 1.1 ± 0.3 s (n = 5); in MgATP solution, the activation was too small to measure the off-rate (Fig. 2).

Discussion

Our results show that both pinacidil and its analog P1075 can enhance Kir6.2/SUR2A currents in the absence of nucleotides. However, the off-rate of both drugs is slowed in the presence of MgATP or MgADP, which interact with the NBDs of SUR2A. The ability of Mg-nucleotide to slow the drug dissociation may account for the fact that [^{3}H]P1075 binding can only be detected in the presence of Mg-nucleotide.

Channel Activation by MgADP. Mutation of K707 in NBD1 of SUR2A did not prevent channel stimulation by MgADP, although the equivalent mutation in SUR1 abolishes MgADP activation (Gribble et al., 1997b). The amino acid sequence of NBD1 is not identical in SUR1 and SUR2A, so this difference may simply be because the mutation disrupts MgADP binding to NBD1 in SUR1 but not SUR2A. An alternative possibility is that NBD2 is essential for MgADP-activation in both SURs, whereas NBD1 plays a facilitatory role that differs between SUR1 and SUR2A. Indeed, binding studies (using SUR1) suggest NBD1 constitutes a high-affinity binding site for ATP, that is modulated by MgADP binding at NBD2 (Ueda et al., 1997, 1999).

Pinacidil Responses of Wild Type and Mutant K_{ATP} Channels. Consistent with studies on native cardiac and skeletal muscle K_{ATP} channels (Fan et al., 1990; Shen et al., 1991; Tung and Kurachi, 1991), pinacidil activated Kir6.2/SUR2A currents in both the absence and presence of added nucleotide, and the magnitude of the response varied with the degree of channel rundown.

Mutation of the W_A lysine in NBD1 of SUR2A abolished the pinacidil response in the absence of added nucleotide, indicating that NBD1 is critical for the action of the drug even in nucleotide-free solution. This result may be explained in two ways. First, NBD1 may play a key role in pinacidil activation even in the absence of bound nucleotide, and this role may be impaired by the W_A mutation. Secondly, interaction of nucleotide with NBD1 may be essential for pinacidil activation. In this case, the fact that pinacidil is effective in nucleotide-free solution would imply that ATP (or ADP) remains associated with NBD1 for some time after patch excision. The effect of the K707A mutation would be explained by a reduction in nucleotide binding and/or hydrolysis at NBD1.

The second hypothesis is supported by binding studies on SUR1, which suggest that 8-azido ATP takes 5–15 min to dissociate from NBD1 after exposure to nucleotide-free solutions (Ueda et al., 1997, 1999). The slow dissociation of nucleotide from NBD1 might also account for the gradual reduction in the effect of pinacidil after patch excision. Because the rate of dissociation of prebound nucleotide from SUR will depend on the experimental conditions, this hypothesis may explain why ATP-independent pinacidil responses have not always been reported for native skeletal muscle K_{ATP} channels or Kir6.2/SUR2B channels (Allard and Lazdunski, 1992; Schwanstecher et al., 1998). The idea that pinacidil sensitivity requires nucleotide binding to SUR is also consistent with reports that drug sensitivity can be restored after rundown of native cardiac and skeletal muscle K_{ATP} channels by the addition of MgADP or MgATP (Fan et al., 1990; Shen et al., 1991; Tung and Kurachi, 1991; Allard and Lazdunski, 1992; Terzic et al., 1995).

Addition of MgATP or MgADP to the intracellular solution partially restored pinacidil sensitivity to Kir6.2/SUR2A-K707A currents. This suggests that either the mutation does not completely abolish nucleotide binding (and/or hydrolysis) at NBD1, or that the interaction of nucleotide with NBD2 alone is sufficient to support the action of the drug.

Effects of Nucleotide on the Pinacidil Off-Rate. In the case of wild-type SUR2A, addition of MgATP or MgADP markedly slowed the off-rate of pinacidil. Mutation of the W_A lysine in a single NBD of SUR2A (either K707A or K1348A) largely abolished this effect. This suggests that binding and/or hydrolysis of nucleotide at both NBDs is necessary to slow the off-rate of the drug.

When pinacidil was tested on Kir6.2/SUR2A currents soon after the patch was excised into nucleotide-free solution, ~50% of the activated current reversed with a slow exponential whose time constant (τ_s) was similar to that observed in the presence of MgATP or MgADP. The magnitude of this component decreased with time, but was restored after refreshment of channel activity by MgATP. It is thus possible that after exposure to high concentrations of MgATP (either intracellular or in the bath solution), some SUR2A subunits have nucleotide bound at both NBDs, causing a proportion of the pinacidil response to reverse with a slow time constant. The time-dependent decrease in the magnitude of this exponential might then reflect the dissociation of nucleotide from a single NBD.

When K1348 was mutated in NBD2 of SUR2A, the slow component of pinacidil reversal was not completely eliminated. This component accounted for <10% of the response in the absence of nucleotide, but increased to ~20% in 100 μM MgATP. If the slow component is only observed when both NBDs are functional, as suggested above, the effect of the K1348A mutation may be explained by a reduction, but not complete abolition, of nucleotide binding and/or hydrolysis at NBD2.

Comparison of [^{3}H]P1075 Binding and Channel Activation. The ability of P1075 to activate Kir6.2/SUR2A currents in the absence of nucleotide contrasts with the MgATP-dependence of [^{3}H]P1075 binding to skeletal muscle membranes and to heterologously expressed SUR2A or SUR2B (Dickinson et al., 1997; Schwanstecher et al., 1998; Hambrock et al., 1998, 1999). One explanation for this anomaly might be that detection of [^{3}H]P1075 binding, and the functional response to P1075 (or pinacidil), require MgATP binding/hydrolysis by SUR. Association of nucleotide with SUR for 10 to 15 min after patch excision might support pinacidil activation in electrophysiological experiments but would play an insignificant role in binding studies that are performed much later after membrane isolation. One problem with this hypothesis, however, is that mutating the W_A lysine in NBD2 of SUR2B abolished binding of [^{3}H]P1075 (Schwanstecher et al., 1998), whereas the corresponding mutation in SUR2A did not prevent channel activation by pinacidil. An alternative idea, therefore, is that detection of [^{3}H]P1075 binding is only possible under conditions in which drug dissociation from SUR is slow. This idea is favored by the observation that maneuvers that prevent [^{3}H]P1075 binding to SUR2A or SUR2B (e.g., Mg^{2+} or ATP removal, mutation of the W_A lysine in NBD1 or NBD2 of SUR2B) also

abolished the slow reversal of pinacidil activation of Kir6.2/SUR2A currents (Schwanstecher et al., 1998; Hambrock et al., 1998, 1999). Although we have not attempted to interpret the on-rates for pinacidil or P1075, we speculate that the slow off-rate in the presence of Mg-nucleotides may be associated with an increase in the affinity of drug binding. This could account for the inability to detect P1075 binding in the absence of nucleotide, because [^{3}H]P1075 binding studies are performed at lower drug concentrations (1 to 10 nM) than that used in the current study (10 μM).

The time course of the off-rate of P1075 in the presence of MgATP (τ =108 s) was ~10 times slower than that of pinacidil (τ =12 s). This difference is similar to the ~10-fold higher affinity of P1075 (17 nM) in binding studies, compared with that of pinacidil (~150 nM: 78 nM for the active enantiomer (−)pinacidil, Hambrock et al., 1999). The off-rate of P1075 is also similar to the rate of dissociation of [^{3}H]P1075 from SUR2A in binding studies (τ = 95 s) (Hambrock et al., 1999). Although the binding studies were performed at 37°C and the electrophysiology at 20 to 22°C, the similarity between these measurements suggests that the time course of the current decline is determined by drug unbinding rather than by the rate at which the activated channel relaxes back to the resting state.

Although it is widely recognized that the activity of many K_{ATP}-channel openers is dependent on the nucleotide environment, the mechanisms behind these interactions have remained elusive. It has been proposed, for example, that ATP hydrolysis is essential for binding of [^{3}H]P1075 to SUR2B (Schwanstecher et al., 1998). The roles of the individual NBDs of SUR have not, however, been addressed. Our results suggest that nucleotides may modulate pinacidil efficacy by interacting with the NBDs and that when a single NBD of SUR2A is mutated, pinacidil activation reverses rapidly in the presence of nucleotide. The cooperative effect of nucleotide binding at both NBDs, however, seems to stabilize a pinacidil-activated state, resulting in a slow reversal of the drug response.

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Send reprint requests to: Prof. Frances M. Ashcroft, University Laboratory of Physiology, Parks Rd., Oxford OX1 3PT, UK. E-mail: frances.ashcroft@physiol.ox.ac.uk