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Molecular determinants of K_{ATP} channel inhibition by ATP

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ATP-sensitive K^+ (K_{ATP}) channels are both inhibited and activated by intracellular nucleotides, such as ATP and ADP. The inhibitory effects of nucleotides are mediated via the pore-forming subunit, Kir6.2, whereas the potentiatory effects are conferred by the sulfonylurea receptor subunit, SUR. The stimulatory action of Mg-nucleotides complicates analysis of nucleotide inhibition of Kir6.2/SUR1 channels. We therefore used a truncated isoform of Kir6.2, that expresses ATP-sensitive channels in the absence of SUR1, to explore the mechanism of nucleotide inhibition. We found that Kir6.2 is highly selective for ATP, and that both the adenine moiety and the β-phosphate contribute to specificity. We also identified several mutations that significantly reduce ATP inhibition. These are located in two distinct regions of Kir6.2: the N-terminus preceding, and the C-terminus immediately following, the transmembrane domains. Some mutations in the C-terminus also markedly increased the channel open probability, which may account for the decrease in apparent ATP sensitivity. Other mutations did not affect the single-channel kinetics, and may reduce ATP inhibition by interfering with ATP binding and/or the link between ATP binding and pore closure. Our results also implicate the proximal C-terminus in K_{ATP} channel gating.

Keywords: ATP inhibition/ATP sensitive potassium channel/K_{ATP}/Kir6.2

Introduction

ATP-sensitive potassium (K_{ATP}) channels play important functional roles in a variety of tissues by coupling cellular metabolism to electrical activity (Ashcroft and Ashcroft, 1990; Nichols and Lederer, 1991; Quayle *et al.*, 1996). Metabolic regulation is thought to be mediated by metabolically induced changes in the intracellular levels of the adenine nucleotides ATP and MgADP, which inhibit and activate the channel, respectively. Cloning of the K_{ATP} channel has revealed that it is an octameric complex of two proteins which assemble with a 4:4 stoichiometry (Clement *et al.*, 1997; Inagaki *et al.*, 1997; Shyng and Nichols, 1997). The pore-forming subunit, Kir6.2, is a member of the inwardly rectifying K^+ channel family (Inagaki *et al.*, 1995; Sakura *et al.*, 1995), while the other subunit is an ABC transporter, the sulfonylurea receptor (SUR) (Aguilar-Bryan *et al.*, 1995). The latter endows Kir6.2 with sensitivity to the inhibitory effects of sulfonylurea drugs and to the stimulatory effects of MgADP and K^+ channel openers (Nichols *et al.*, 1996; Gribble *et al.*, 1997a; Shyng *et al.*, 1997a; Trapp *et al.*, 1997; Tucker *et al.*, 1997). The genes encoding two closely related sulfonylurea receptors, SUR1 and SUR2, have been cloned, and further diversity is created by alternative splicing of SUR2 (Chutcow *et al.*, 1996; Inagaki *et al.*, 1996; Isomoto *et al.*, 1996). Comparison of the properties of cloned and wild-type K_{ATP} channels suggests that the β-cell K_{ATP} channel, and some types of brain K_{ATP} channel, are composed of Kir6.2 and SUR1, the cardiac type consists of Kir6.2 and SUR2A, and the smooth muscle type probably comprises Kir6.2 and SUR2B (Aguilar-Bryan *et al.*, 1998). Thus, Kir6.2 serves as a common pore-forming subunit for many types of K_{ATP} channel. Like other members of the inwardly rectifying K^+ channel family, it has two putative transmembrane domains (TM1 and TM2), which are linked by a pore loop (H5). The N- and C-termini are both cytosolic and comprise ~70 and ~220 amino acid residues, respectively.

Although both Kir6.2 and SUR subunits are normally required for functional expression of the K_{ATP} channel, we have shown that a mutant form of Kir6.2, in which either the last 26 (Kir6.2ΔC26) or 36 (Kir6.2ΔC36) amino acids are deleted, can express functional channel activity independently of SUR1 (Tucker *et al.*, 1997). These truncated isoforms of Kir6.2 retain intrinsic ATP sensitivity, but are not stimulated by MgADP. Despite the fact that Kir6.2 contains no obvious consensus sequences for nucleotide binding, a single mutation in this subunit (K185Q) markedly reduces the ability of ATP to inhibit channel activity (Tucker *et al.*, 1997). These data suggest that the site at which ATP mediates channel inhibition resides on Kir6.2 and that further site-directed mutagenesis of Kir6.2 should help define those regions involved in nucleotide inhibition.

The ability of non-hydrolysable analogues of ATP, and of ATP in the absence of Mg^{2+}, to inhibit channel activity suggests that ATP hydrolysis is not required for channel inhibition and that binding of the molecule is sufficient to cause channel closure (Ashcroft and Rorsman, 1989). Analysis of the inhibitory effects of ATP has been complicated, however, by the dual ability of nucleotides to both stimulate and inhibit wild-type K_{ATP} channels. This problem can be overcome by the use of the Kir6.2ΔC26 or Kir6.2ΔC36 isoforms, expressed in the absence of SUR1, since nucleotide stimulation is then absent. There is no significant difference in the ATP sensitivity of Kir6.2ΔC36 and Kir6.2ΔC26 currents (Tucker *et al.*, 1997).

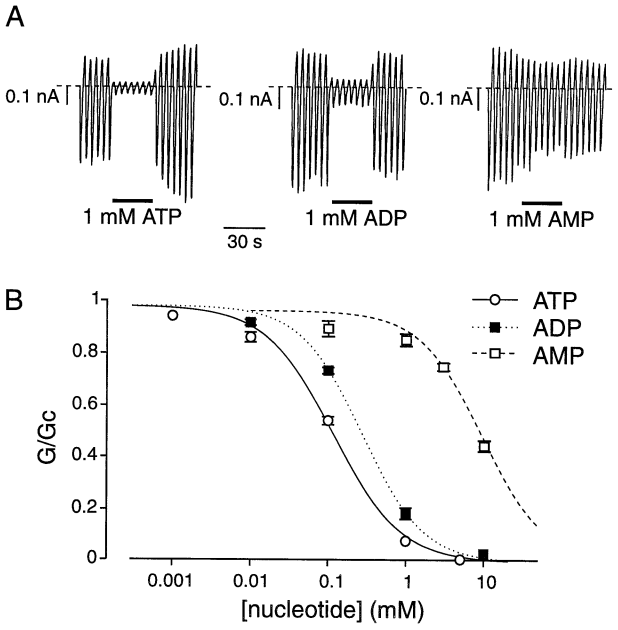

Fig. 1. Specificity of the phosphate moiety. (**A**) Macroscopic currents recorded from three different inside-out patches in response to a series of voltage ramps from –110 mV to +100 mV. Oocytes were injected with mRNA encoding Kir6.2ΔC36; 1 mM ATP, 1 mM ADP or 1 mM AMP were added to the internal solution as indicated by the bars. (**B**) Mean dose–response relationships for Kir6.2ΔC36 currents exposed to either ATP (n = 11), ADP (n = 5) or AMP (n = 6), as indicated. Test solutions were alternated with control solutions and 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 nucleotide. The lines are the best fit of the data to the Hill equation using the mean values for K_i and h given in the text.

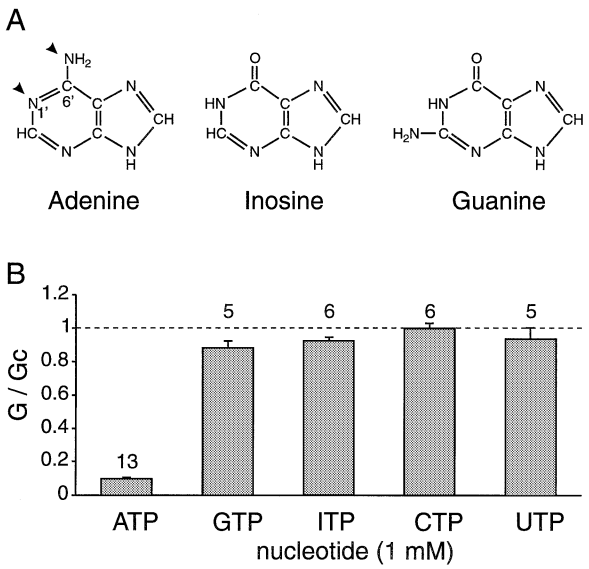

Fig. 2. Specificity for purines and pyrimidines. (**A**) Molecular structures of adenine, guanine and inosine. Arrows indicate differences between ATP and ITP. (**B**) Effect of 1 mM nucleotide triphosphate on macroscopic Kir6.2ΔC36 currents. Conductances are expressed as a fraction of their amplitude in control solution. The number of patches is indicated above the bars.

In the present study, we have defined structural features of the ATP molecule that are critical for K_{ATP} channel inhibition. By site-directed mutagenesis of the Kir6.2 subunit, we have also identified multiple residues that have profound effects on the ability of ATP to cause channel inhibition. These residues are found in two distinct regions of the channel: the N-terminus immediately preceding TM1 and the C-terminus immediately following TM2. Analysis of the single-channel currents suggests that the proximal C-terminus (following TM2) participates in K_{ATP} channel gating and that mutations in this region may alter the apparent ATP sensitivity of Kir6.2 indirectly, via their effects on gating. Other mutations reduced ATP sensitivity without altering the single-channel kinetics and may therefore primarily affect ATP binding and/or the link between ATP binding and gating. Thus mutations may influence the ATP sensitivity of Kir6.2 by a number of different mechanisms.

Results

Moieties of the ATP molecule critical for channel inhibition

We first explored the sensitivity of Kir6.2ΔC36 channels to adenine nucleotides. The effect of 1 mM ATP, ADP or AMP on the macroscopic currents is shown in Figure 1A, and the relationship between nucleotide concentration and the macroscopic conductance is given in Figure 1B. ADP inhibited Kir6.2ΔC36 currents almost as potently as ATP, the K_i for inhibition being 260 ± 22 μM (n = 5) compared with 115 ± 6 μM (n = 11) for ATP. By contrast, AMP was markedly less effective (K_i = 9.2 ± 0.5 mM, n = 6). Thus, at least two phosphate groups appear to be required for significant channel inhibition. The Hill coefficients were unaffected by the length of the phosphate tail, being 1.0 ± 0.1, 1.1 ± 0.1 and 1.1 ± 0.1 for ATP, ADP and AMP, respectively. Another group of adenine nucleotides that have been suggested to regulate K_{ATP} channel activity in response to changes in cell metabolism are the diadenosine polyphosphates (Ripoll *et al.*, 1996; Jovanovic *et al.*, 1998). These molecules consist of two adenosine molecules linked by three or more phosphate residues. AP_4A blocked Kir6.2ΔC36 currents with a potency similar to that of ATP: thus, 100 μM AP_4A produced 48 ± 2% inhibition (n = 4) compared with a 46 ± 1% block (n = 7) by 100 μM ATP.

Mean data for the inhibitory effects of different nucleotide triphosphates are given in Figure 2B and indicate that channel inhibition is highly specific for the adenine moiety. It has been shown previously that GTP has little inhibitory effect on Kir6.2ΔC26 currents, the K_i being 6 mM (Trapp *et al.*, 1997). From these results, it appears that elements unique to the adenine ring are critical for purine nucleotide inhibition of the K_{ATP} channel. Figure 2A illustrates the structure of the purine ring of ATP, GTP and ITP. Comparison of the structure of ATP with that of the most closely related nucleotide tested, ITP, suggests that nucleotide specificity may be determined by an interaction between Kir6.2 and either the nitrogen at the 1′ position, and/or the $–NH_2$ group at the 6′ position, of the purine ring (Figure 2A).

The effect of mutations in Kir6.2

We now consider which regions of Kir6.2 are critical for the inhibition of channel activity by ATP. Since ATP blocks only from the intracellular side of the membrane (Ashcroft and Rorsman, 1989), only those residues

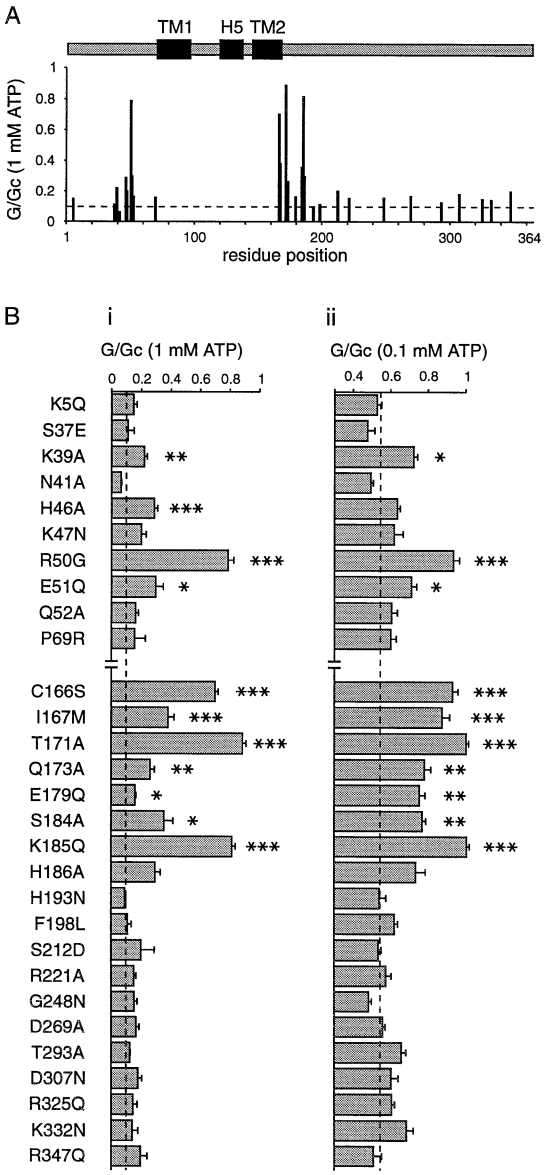

Fig. 3. Effect of mutations in Kir6.2ΔC26 on channel inhibition by ATP. (**A**) Schematic model of Kir6.2ΔC26 (above) and effect of mutations on the inhibitory effect of 1 mM ATP (below). The transmembrane domains (TM) and the pore loop (H5) are indicated. The dashed line indicates the level of inhibition observed for wild-type Kir6.2ΔC26. (**B**) Effect of mutations on Kir6.2ΔC26 upon the inhibitory effect of 1 mM ATP (i) and 100 μM ATP (ii). Macroscopic conductance is expressed as a fraction of that in the absence of ATP. The dashed line indicates the level of inhibition observed for wild-type Kir6.2ΔC26. **P* <0.001; ***P* <0.0001; ****P* <0.00001, compared with wild-type Kir6.2ΔC26. Amino acids are denoted by the single-letter code. The number of patches was between four and 10, except for K5Q ($n = 3$), S221D ($n = 3$) and R347Q ($n = 2$).

accessible to the cytosolic solution are likely to be involved, i.e. those that are located within the pore and the putative N- and C-termini (Figure 3A). By analogy with other K^+ channels, the H5 loop and part of TM2 may be expected to line the pore of Kir6.2 (Doyle *et al.*, 1998). However, it is doubtful that residues within these regions form part of the ATP-binding site because they lie within the membrane voltage field, and the block by ATP is not voltage sensitive (Figure 1). Amino acids involved in ATP inhibition are therefore likely to be located within the N- and C-termini, or at the cytosolic end of TM2, which is predicted to lie close to the inner mouth of the pore. As no classical consensus motifs for ATP binding can be discerned in the sequence of Kir6.2, we systematically mutated residues within these regions (Figure 3A). We targeted primarily residues capable of electrostatic interaction or hydrogen bonding as being the most likely to interact with ATP. In general, we neutralized charged residues and replaced other residues with an alanine or changed them to amino acids that were conserved throughout other Kir family members. For each mutation that produced significant current we tested the effects of 100 μM and 1 mM ATP, which inhibit wild-type Kir6.2ΔC26 currents by ~50% and ~90%, respectively. Figure 3 presents results for 29 mutations spread along the N- and C-terminal regions of the channel. The positions of these mutations, and the effect of 1 mM ATP, are summarized in Figure 3A, while Figure 3B illustrates the effects of 1 mM and 100 μM ATP in greater detail.

In addition to the mutation previously reported (K185Q), we identified four new mutations that significantly reduce the sensitivity to ATP: these were R50G, C166S, I167M and T171A (P <0.00001 against wild-type). Several other mutations, including K39A, Q173A, E179Q and S184A, caused smaller, but significant, shifts in the ability of 100 μM ATP to inhibit Kir6.2ΔC26 currents (P <0.001) (Figure 3B). It is clear from our results that the mutations which affect ATP sensitivity are located in two distinct regions of Kir6.2: one in the N-terminus and the other in the C-terminus, immediately following TM2 (Figure 3A).

Analysis of ATP-insensitive mutations

We analysed the properties of the least ATP-sensitive mutations in more detail. The effect of 1 mM ATP on Kir6.2ΔC26 containing the R50G, I167M and T171A mutations is compared with that of wild-type Kir6.2ΔC26 in Figure 4A, and the corresponding ATP dose–response curves are shown in Figure 4B. The values of K_i obtained for these and other mutations is given in Table I. None of the six mutations affected the Hill coefficient, which was close to unity in all cases: this indicates that a single ATP molecule is sufficient to cause inhibition of both wild-type and mutant channels. The values of K_i, however, were significantly lower for the mutant channels, varying between ~8 mM for Kir6.2ΔC26 carrying the T171A mutation to 300 μM for Kir6.2ΔC26-E179Q, compared with ~100 μM for the wild-type channel.

Analysis of single-channel currents

It has been shown previously for other ligand-gated channels that mutations that affect the apparent ligand sensitivity may do so by altering the gating of the channel rather than the ligand-binding site itself (Gordon and Zagotta, 1995; Varnum *et al.*, 1995; Zong *et al.*, 1998). If ATP stabilizes the closed state of the channel, then an apparent decrease in ATP sensitivity might be caused by an increase in the time spent in the open state. We therefore analysed the single-channel currents for a range of those mutations that produced shifts in ATP sensitivity. Wild-type Kir6.2ΔC26 channels are characterized by brief bursts of channel openings separated by long closed intervals (Figure 5). The single-channel kinetics and open probability of some of the ATP-insensitive mutations examined

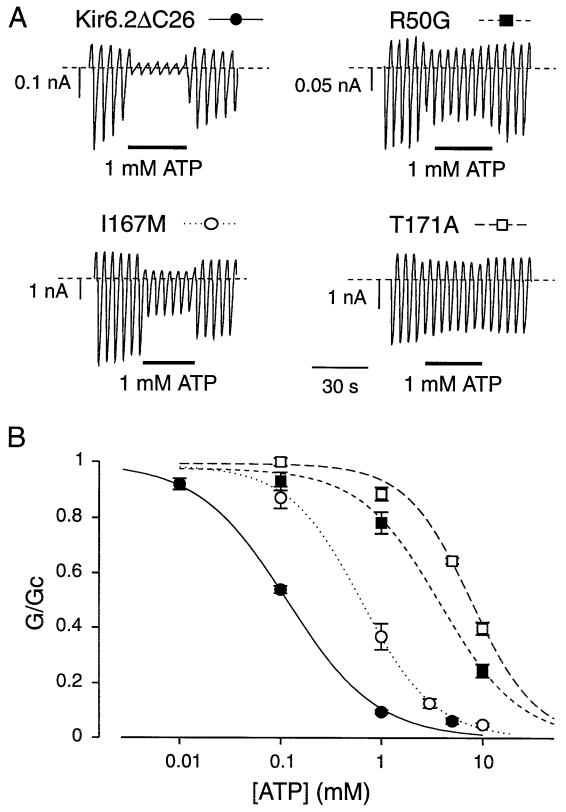

Fig. 4. ATP sensitivity of selected Kir6.2ΔC26 mutations. (**A**) Macroscopic currents recorded from inside-out patches in response to a series of voltage ramps from –110 mV to +100 mV. The oocytes were injected with mRNA encoding wild-type or mutant Kir6.2ΔC26 as indicated; 1 mM ATP was added to the internal solution as indicated by the bars. (**B**) Corresponding mean ATP dose–response relationships for wild-type Kir6.2ΔC26 and Kir6.2ΔC26 containing the mutations indicated by the symbols in (A). Test solutions were alternated with control solutions, and 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 using the mean values for K_i and h given in Table I. The number of patches is also given in Table I.

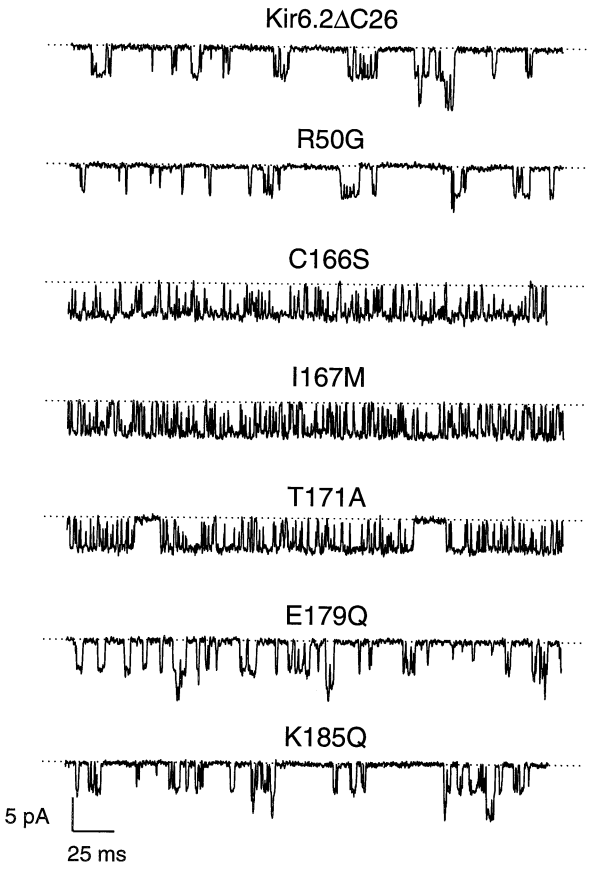

Fig. 5. Effects of mutations on single-channel currents. Single-channel currents recorded at –60 mV from inside-out patches excised from oocytes injected with mRNA encoding wild-type Kir6.2ΔC26 or Kir6.2ΔC26 containing the mutations indicated. The dotted line indicates the zero current level.

Table I. Mutations affecting ATP sensitivity

Mutation	K_i (μM)	P_o	i (pA)
Wild-type	106 ± 4 (n = 7)[a]	0.11 ± 0.03	–4.2 ± 0.3
R50G	3410 ± 720 (n = 6)	0.14 ± 0.07	–4.3 ± 0.3
C166S	2820 ± 340 (n = 10)	0.80 ± 0.02	–4.0 ± 0.3
I167M	639 ± 13 (n = 5)	0.45 ± 0.04	–4.0 ± 0.1
T171A	7710 ± 300 (n = 6)	0.43 ± 0.12	–4.6 ± 0.2
E179Q	296 ± 29 (n = 7)	0.10 ± 0.01	–4.2 ± 0.3
K185Q	4200 ± 210 (n = 6)[a]	0.09 ± 0.02	–4.2 ± 0.2

[a]Data from Tucker *et al.* (1997). The open probability (P_o) and single-channel current (i) were measured at –60 mV and n = 3 in all cases.

(R50G, E179Q and K185Q) were similar to those of wild-type Kir6.2ΔC26 (Figure 5, Tables I and II). By contrast, the C166S, I167M and T171A mutations exhibited a marked decrease in the long closed state and, as a consequence, a >4-fold increase in the channel open probability. Thus, the reduced ATP sensitivity of these mutations may, at least in part, reflect the change in the single-channel kinetics. It is noteworthy that the precise location of the mutation within the C-terminus markedly affects the channel open probability: the greater the linear distance from the cytosolic end of TM2, the smaller the effect on the single-channel kinetics. None of the mutations studied produced a marked effect on the single-channel current amplitude (Table I).

Discussion

The results we present here define the structural moieties of the ATP molecule that are critical for inhibition of K_{ATP} channel activity with greater accuracy. They also describe the effect of mutations in Kir6.2ΔC26 on the ability of ATP to inhibit channel activity. Mutations that alter the apparent ATP sensitivity of the channel are clustered in two distinct regions of Kir6.2: the N-terminus just preceding the first transmembrane domain and the proximal C-terminus that immediately follows the second transmembrane domain. These mutations appear to influence the apparent ATP sensitivity of the channel by different mechanisms. Our results also identify a region of Kir6.2 that is involved in channel gating.

Moieties of the ATP molecule required for channel inhibition

The ability of ATP, but not GTP or ITP, to cause high-affinity channel inhibition suggests that elements unique to the adenine ring are critical for the specificity of

Table II. Effect of mutations on single-channel kinetics

Mutation	Open time (ms)	Short closed time (ms)	Long closed time (ms)	% long closed time	Burst duration (ms)	Openings per burst
Wild-type	0.79 ± 0.06	0.31 ± 0.03	12.6 ± 2.9	41.3 ± 8.0	2.4 ± 06	2.4 ± 0.3
K185Q	0.97 ± 0.13	0.32 ± 0.02	5.6 ± 1.2	25.2 ± 6.7	3.1 ± 0.3	2.6 ± 0.3
T171A	2.03 ± 0.23	0.39 ± 0.02	28.0 ± 12.9	1.3 ± 0.45	176 ± 47	72 ± 19
I167M	1.41 ± 0.14	0.48 ± 0.04	25.2 ± 8.38	2.8 ± 1.4	88 ± 28	47 ± 15

We analysed two mutations in Kir6.2ΔC26 that obviously altered the single-chanel kinetics and one that did not. $n = 3$ in each case.

nucleotide binding and/or for the ability of nucleotide binding to induce closure of the pore. Comparison of the nucleotide structures suggests that these elements might include the nitrogen atom at the 1′ position, and/or the $–NH_2$ group at the 6' position of the purine ring (Figure 2A). It is clear that other structural features of ATP, in addition to the adenine ring, are also important, because AMP does not inhibit channel activity. The ability of ATP and ADP, but not AMP, to inhibit channel activity with high affinity demonstrates that interaction with more than the α-phosphate of the nucleotide is required. Furthermore, the fact that the potencies of ATP and ADP are not markedly different (115 μM compared with 260 μM) argues that the β-phosphate is essential for channel inhibition while the γ-phosphate is less important. The ability of AP_4A to inhibit K_{ATP} channel activity with a potency similar to ATP supports this idea, since the attachment of an additional phosphate group and an additional adenosine group to the γ-phosphate of ATP does not alter ATP sensitivity. Taken together, these data suggest that both the β-phosphate and moieties within the adenine ring are critical for K_{ATP} channel inhibition by ATP.

Regions of Kir6.2 that affect ATP sensitivity

We have found that mutation of residues in both the proximal N- and C-termini of Kir6.2 may affect the channel ATP sensitivity. This result is reminiscent of the interactive role of the N- and C-termini in the gating of two other distantly related types of cation channel. First, inactivation of the voltage-gated K^+ channel Kv4.1 requires the co-operative action of both the N- and C-termini (Jerng and Covarrubias, 1997). Secondly, residues in the proximal N-terminus of cyclic nucleotide-gated (CNG) channels are involved in channel activation by cyclic nucleotides, despite the fact that the cyclic nucleotide-binding site resides in the C-terminus (Zagotta and Siegelbaum, 1997). The N-terminus appears to regulate the gating of CNG channels by interacting physically with the C-terminus (Varnum and Zagotta, 1997). This leads us to suggest either that the N- and C-termini of Kir6.2 co-operate to form the ATP-binding site, or that their interaction may influence an ATP-binding site that is located in only one of these domains (either the N- or C-terminus).

Although Kir6.2 contains no classical consensus motif for ATP binding, a sequence in the C-terminus of the mouse protein shares homology with one type of nucleotide-binding motif GXGXXG (amino acids 243–248). This sequence is not, however, conserved between the different species from which Kir6.2 has been cloned (Takano *et al.*, 1996), and mutation of G248, which lies within this motif, does not affect channel inhibition by ATP (Figure 3). Thus it is unlikely to form part of the ATP-binding site.

Mutations may alter the apparent ATP sensitivity by different mechanisms

A mutation may alter the ATP-sensitivity of Kir6.2ΔC26 in one of several ways: for example, it may (i) impair the ability of the channel to close; (ii) interfere with the transduction mechanism by which ATP binding induces pore closure; or (iii) decrease the affinity of the ATP-binding site itself. Mutations that influence channel gating can be detected by their effects on the single-channel kinetics. We found that three mutations associated with a marked decrease in ATP sensitivity (C166S, I167M and T171A) also affected the intrinsic gating of the channel, producing a dramatic decrease in the frequency of the long closed state. One explanation for these results is that ATP binds preferentially to the long closed state of the channel: mutations that cause the channel to enter this state only rarely, will thereby also produce an apparent reduction in ATP sensitivity. A similar interpretation has been put forward to account for the decrease in ATP sensitivity (from ~10 μM to ~60 μM) observed when the asparagine at position 160, within TM2, was mutated (Shyng *et al*., 1997b). Although the altered kinetic behaviour of the C166S, I167M and T171A mutant channels may contribute to their reduced ATP sensitivity, it does not exclude the possibility that one or more of these mutations also affect ATP binding and/or transduction. Indeed, at least in the case of T171A, it is likely that additional effects on ATP binding and/or the transduction process are also involved because the I167M and T171A mutations caused a similar change in open probability, but had very different effects on the sensitivity to ATP inhibition (the K_i increased by 6- and 70-fold, respectively; Table I).

The mutations R50G and K185Q produced marked shifts in ATP sensitivity without noticeably affecting the single-channel kinetics. This result suggests that these mutations may affect either the affinity of the ATP-binding site or the mechanism by which ATP binding is transduced into changes in channel gating. Since a mutation may alter the ATP binding site directly or affect ATP binding allosterically, further work is required to determine if one or both of these residues actually lie within the ATP-binding site itself. It remains possible, for example, that a mutation may disrupt ATP binding or transduction allosterically, yet normally have no role in mediating channel inhibition.

Mutations at the cytosolic end of TM2 affect channel gating

The second transmembrane domain of Kir channels has been proposed to line the intracellular mouth of the pore, as mutations within this region affect the single-channel conductance, ion selectivity and the sensitivity to the blocking effects of intracellular cations such as Mg^{2+} and spermine (Nichols and Lopatin, 1997). Although it has not been studied as extensively as other Kir channels, there is evidence to support the idea that this is also the case for Kir6.2: mutation of N160 to aspartate, for example, enhances the sensitivity to spermine (Shyng *et al*., 1997b). The mutations C166S, I167M and T171A appeared to reduce markedly the frequency of the long closed state, suggesting that they impair the ability of the channel to close. The location of these residues, at the cytosolic end of TM2, would be consistent with the idea that they contribute to an intracellular gate that governs access to the channel pore. An equivalent region (the cytosolic end of S6) has been shown to participate in the gating of both voltage-gated K^+ channels and CNG channels (Liu *et al*., 1997; Loukin *et al*., 1997; Zong *et al*., 1998). Furthermore, mutations within this region of the yeast voltage-gated K^+ channel have a similar effect on the gating kinetics, markedly decreasing the frequency of a long closed state (Loukin *et al*., 1997). A gate located at the intracellular end of the pore may therefore be a common feature of K^+ channels.

Conclusion

In conclusion, we have identified two regions of Kir6.2 that appear to be involved in the inhibitory effect of ATP: the proximal N-terminus and proximal C-terminus. Our results suggest certain parallels with the CNG channels (Zagotta and Seigelbaum, 1996). Recent studies of these channels have identified three regions that are involved in channel activation by cyclic nucleotides: a cyclic nucleotide-binding domain, located in the C-terminus, a linker region connecting this motif to the sixth transmembrane domain (equivalent to TM2 of Kir6.2) and residues within the N-terminus that allosterically influence cyclic nucleotide activation (Gordon and Zagotta, 1995; Zong *et al*., 1998). Mutations within the C-terminal linker region, or in the N-terminus, alter the activating efficacy of cyclic nucleotides but do not affect the binding affinity. The involvement of several regions of the CNG channel in cyclic nucleotide activation is reminiscent of Kir6.2, where mutations in both N- and C-termini affect channel inhibition by ATP. Moreover, mutations within the C-terminal linker influence the gating of CNG channels, as do mutations in the same region of Kir6.2. The CNG channel may, therefore, provide a useful conceptual model for further studies of the mechanism by which ATP inhibits the K_{ATP} channel.

Materials and methods

Molecular biology

Mouse Kir6.2 (DDBJ/EMBL/GenBank accession No. D50581; Inagaki *et al.*, 1995; Sakura *et al.*, 1995) containing a deletion of the last 26 or 36 amino acids (Kir6.2ΔC26 or Kir6.2ΔC36; Tucker *et al.*, 1997) was used in this study. Site-directed mutagenesis of Kir6.2ΔC26 was carried out by subcloning the appropriate fragments into the pALTER vector (Promega, Madison, WI). For oocyte expression studies, constructs were subcloned into the pBF expression vector (B.Fakler, unpublished) which provides the 5′- and 3′-untranslated regions of the *Xenopus* β-globin gene. Synthesis of capped mRNA was carried out using the mMessage mMachine large-scale *in vitro* transcription kit (Ambion, Austin, TX).

Electrophysiology

Oocyte collection. Female *Xenopus laevis* were anaesthetized with MS222 (2 g/l 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 collagenase (Sigma, type V) and manually defolliculated. Oocytes were injected with ~2 ng of mRNA encoding wild-type or mutant Kir6.2ΔC. The final injection volume was ~50 nl/oocyte. Control oocytes were injected with water. Isolated oocytes were maintained in tissue culture and studied 1–4 days after injection (Gribble *et al.*, 1997b).

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*., 1997b). Patch electrodes were pulled from thick-walled borosilicate glass (GC150; Clark Electromedical Instruments) and had resistances of 250–500 kΩ when filled with pipette solution. Currents were evoked by repetitive 3 s voltage ramps from –110 mV to +100 mV and recorded using an EPC7 patch–clamp amplifier (List Electronik, Darmstadt, 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, CA). Single-channel currents were recorded from small inside-out membrane patches. They were filtered at 5 kHz using an 8-pole Bessel filter and sampled at 10 kHz.

The pipette solution contained (mM): 140 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 10 HEPES (pH 7.4 with KOH) and the internal (bath) solution contained (mM): 110 KCl, 1.4 $MgCl_2$, 30 KOH, 10 EGTA, 10 HEPES (pH 7.2 with KOH) and nucleotides as indicated. Solutions containing ATP were made up fresh each day and the pH was readjusted after addition of the nucleotide. 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 relationship between –20 mV and –100 mV: the average of five consecutive ramps was calculated in each solution. Nucleotide dose–response relationships were measured by alternating the control solution with a test solution, and the conductance was expressed as a fraction of the mean of the value obtained in the control solution before and after application of the test nucleotide. Dose–response curves were fitted to the Hill equation $G/G_c = 1/(1 + ([X]/K_i)^h)$ where [X] is the nucleotide concentration, K_i is the nucleotide concentration at which inhibition is half maximal and h is the slope factor (Hill coefficient).

Single-channel currents were analysed using a combination of pClamp and in-house software. Single-channel current amplitudes were calculated from an all-points amplitude histogram. Channel activity (NP_o) was measured as the mean current (I) divided by the single-channel current amplitude (i), for segments of the current records of ~1 min duration. Open probability (P_o) was calculated from NP_o/N, where N is the number of channels in the patch, and was estimated from the maximum number of superimposed events. In the case of mutants with high P_o, we only analysed patches that had no superimposed events, and thus we can be confident that only a single channel was present in the patch. For analysis of channel kinetics, unitary events were detected using a 50% threshold level method. Data are expressed as mean ± 1SEM.

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