Mutations within the P-loop of Kir6.2 Modulate the Intraburst Kinetics of the ATP-sensitive Potassium Channel

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ABSTRACT The ATP-sensitive potassium (K\textsubscript{ATP}) channel exhibits spontaneous bursts of rapid openings, which are separated by long closed intervals. Previous studies have shown that mutations at the internal mouth of the pore-forming (Kir6.2) subunit of this channel affect the burst duration and the long interburst closings, but do not alter the fast intraburst kinetics. In this study, we have investigated the nature of the intraburst kinetics by using recombinant Kir6.2/SUR1 K\textsubscript{ATP} channels heterologously expressed in Xenopus oocytes. Single-channel currents were studied in inside-out membrane patches. Mutations within the pore loop of Kir6.2 (V127T, G135F, and M137C) dramatically affected the mean open time (\(\tau_o\)) and the short closed time (\(\tau_{cl}\)) within a burst, and the number of openings per burst, but did not alter the burst duration, the interburst closed time, or the channel open probability. Thus, the V127T and M137C mutations produced longer \(\tau_o\), shorter \(\tau_{cl}\), and fewer openings per burst, whereas the G135F mutation had the opposite effect. All three mutations also reduced the single-channel conductance: from 70 pS for the wild-type channel to 62 pS (G135F), 50 pS (M137C), and 38 pS (V127T). These results are consistent with the idea that the K\textsubscript{ATP} channel possesses a gate that governs the intraburst kinetics, which lies close to the selectivity filter. This gate appears to be able to operate independently of that which regulates the long interburst closings.

KEY WORDS: K\textsubscript{ATP} • Kir channel • gating • molecular dynamics simulations

INTRODUCTION

The crystal structure of the bacterial potassium channel KcsA (Doyle et al., 1998) has provided a framework for understanding the structure of related K\textsuperscript{+} channels, such as the mammalian voltage-gated (K\textsubscript{v}) and inwardly rectifying (Kir) K\textsuperscript{+} channels. It also provides insight into the mechanism of ion permeation through K\textsuperscript{+} channels. However, the crystal structure is essentially a static snapshot of the channel structure and does not reveal how the channel opens and closes. Traditionally, gating has been considered a physical process distinct from that of ion permeation (Hille, 1992), a view which is supported by a multitude of mutagenesis studies on the activation gate of voltage-gated K\textsuperscript{+} channels. These have revealed that the selectivity filter, which largely determines the single-channel conductance: from 70 pS for the wild-type channel to 62 pS (G135F), 50 pS (M137C), and 38 pS (V127T). These results are consistent with the idea that the K\textsubscript{ATP} channel possesses a gate that governs the intraburst kinetics, which lies close to the selectivity filter. This gate appears to be able to operate independently of that which regulates the long interburst closings.

channels (Lopez-Barneo et al., 1993; Liu et al., 1996; Starkus et al., 1997), and point mutations in the selectivity filter produce changes in the activation kinetics of Shaker K\textsubscript{v} channels (Heginbotham et al., 1994). Furthermore, there is accumulating evidence that both ligand activation of CNG channels (Sun et al., 1996; Liu and Siegelbaum, 2000) and the spontaneous opening of some types of Kir channels (Choe et al., 1998, 1999; Lu et al., 1999; So et al., 2001) may be associated with conformational changes in the selectivity filter or the outer pore. Kinetically (and possibly structurally) gating is a multi-component process; for example, the open state of many channels, including K\textsubscript{v} channels, is interrupted by short closings, known as flickers (Hille, 1992). The molecular basis of this fast gating process, and the location of the fast gate have not been identified. Therefore, we have used site-directed mutagenesis to study the fast gating properties of the \(\beta\)-cell ATP-sensitive K\textsuperscript{+} channel, a member of the Kir channel family that opens and closes spontaneously (Ashcroft and Gribble, 1998).

The ATP-sensitive K\textsuperscript{+} (K\textsubscript{ATP}) channel is an octameric (4:4) complex of two different types of protein subunits: Kir6.2 and SUR (for review see Ashcroft and Gribble, 1998). The pore is composed of four Kir6.2 subunits. Kir6.2 is a member of the Kir channel family and has cytosolic NH\textsubscript{2} and COOH termini and two transmembrane domains (TMs)*, linked by a P-loop that inserts

*Abbreviations used in this paper: K\textsubscript{ATP}, ATP-sensitive K\textsuperscript{+} channel; TM, transmembrane domain.
into the membrane to form the selectivity filter (Inagaki et al., 1995; Sakura et al., 1995). Binding of ATP to Kir6.2 results in channel closure (Tucker et al., 1998), whereas interaction of PIP2 with Kir6.2 enhances channel activity and relieves ATP inhibition (Shyng and Nichols, 1998). SUR is a member of the ATP-binding cassette transporter family (Aguilar-Bryan et al., 1995). It acts as a regulatory subunit, conferring multiple properties on the KATP channel, including stimulation by Mg nucleotides and K-channel openers, block by sulfonyleureas, and enhanced sensitivity to ATP (Tucker et al., 1997). It also influences the single-channel kinetics (Proks and Ashcroft, 1997; Babenko et al., 1999).

In the absence of channel modulators, KATP channel kinetics are characterized by spontaneous bursts of rapid openings and closings separated by long closed states. The frequency and duration of the bursts is markedly affected by mutation of residues at the intracellular end of TM2, or immediately after TM2, of Kir6.2 (Tucker et al., 1998; Loussouarn et al., 2000). Molecular models of Kir6.2, based on the structure of KcsA (Loussouarn et al., 1999; Capener et al., 2000), locate these mutations in a region corresponding to the helix bundle crossing of KcsA that has been postulated to function as a gate. However, none of these mutations influenced the mean short closed time within the burst (τC1), and single-channel analysis indicated that they affected only the interburst parameters (Tucker et al., 1998; Proks and Ashcroft, 1997, 1998). This has led to the suggestion that transitions between the bursts and the long closed states are governed by a “slow” gate that lies at the intracellular mouth of Kir6.2, and that there may be an additional “fast” gate that governs the rapid openings and closings within the burst. We define fast gating as the process that determines the rapid intraburst closings and slow gating as the process that is associated with the long intraburst closings.

The properties of fast gating differ in several respects from those of slow gating. First, most modulators of channel activity, like ATP, MgADP, sulfonyleureas and K-channel openers, primarily affect slow gating: all of these agents dramatically decrease the burst duration and the long intraburst closed times without substantially affecting τC1 (Alekseev et al., 1998; Trapp et al., 1998). Second, fast gating is influenced by both voltage and by extracellular potassium ions. Thus, at fixed external [K+]o, both τo and τC1 are voltage-dependent, increasing with membrane hyperpolarization (Alekseev et al., 1997; Benz et al., 1998; Trapp et al., 1998; Zilberter et al., 1988). Furthermore, both τo and τC1 shift with the reversal potential for potassium when [K+]o is reduced (Benz et al., 1998; Zilberter et al., 1988). These results have led to the suggestion that the fast gate may lie within the membrane voltage field. It has also been postulated that the fast gate may be coupled to the ion permeation pathway (Benz et al., 1998; Zilberter et al., 1988), as has been suggested for other types of ion channel (e.g., CIC-0; Pusch et al., 1995).

In this paper, we show that mutations within the pore loop of Kir6.2 produce marked effects on the fast, intraburst, gating kinetics without altering the burst duration or other parameters associated with slow gating. This would be consistent with the presence of a fast gate, linked to the pore loop, which operates essentially independent of the slow gate. These mutations can also account for much of the difference in the single-channel conductance and kinetics of Kir6.2 and the related Kir channel, Kir2.1.

**MATERIALS AND METHODS**

**Molecular Biology**

Synthesis of mRNA encoding rat SUR1 (Genbank Accession No. L40624; Aguilar-Bryan et al., 1995) and wild-type or mutant mouse Kir6.2 (Genbank D50581; Inagaki et al., 1995; Sakura et al., 1995) was performed as previously described (Tucker et al., 1998; Tucker et al., 1998). Site-directed mutagenesis of Kir6.2 was performed using the pALTER vector (Promega Corp.). The following mutations did not produce functional channels: V127K, V127S, V127A, V127D, E126Q, E126D, F133Y, and the double mutants F133Y + M137C, F133Y + G135F, and V127T + W83F.

**Electrophysiology**

Female *Xenopus laevis* were anesthetized with MS222 (2 g/liter added to the water). One ovary was removed via a mini laparotomy, the incision sutured, and the animal was allowed to recover. Once the wound had completely healed, the second ovary was removed in a similar operation, and the animal was killed by decapitation while under anesthesia. Immature stage V–VI *Xenopus* oocytes were incubated for 75 min with 1.0 mg/ml collagenase (Boehringer, type A) and manually defolliculated. They were co-injected with ~2 ng of SUR1 and ~0.1 ng wild-type or mutant Kir6.2, giving a 1:20 ratio (Trapp et al., 1998; Tucker et al., 1998). The final injection volume was ~50 nl/oocyte. Isolated oocytes were maintained in tissue culture and studied 1–4 d after injection at 18–24°C. Pipettes were pulled from either thin-walled (for 140-mM K+ solutions) or thick-walled (for 10-mM K+ solutions) borosilicate glass. Single-channel currents were measured at ~60 mV in excised inside-out patches using a patch-clamp amplifier (model Axopatch 200B; Axon Instruments Inc.). They were filtered at 2 kHz and digitized at 20 kHz using a Digidata 1200 Interface (Axon Instruments Inc.).

The external (pipette) and intracellular (bath) solutions contained the following (in mM): 140 KCl, 1 EGTA, 1 K2SO4, and 10 HEPES, pH 7.2 with KOH. This solution was chosen to complex stray divalent cations (Ba2+, Mg2+, etc.) that might produce rapid divalent cation block of inward or outward currents (Choe et al., 1998, 1999). P0/PK was measured under bi-ionic conditions by replacing intracellular K+ with Na+ and measuring the shift in the reversal potential of the current. The 10-mM K+ solution contained the following (in mM): 8 KCl, 1 EGTA, 1 K2SO4, 100 NMDG, and 10 HEPES, pH 7, with HCl.

**Single-channel Analysis**

Single-channel recordings were analyzed using a combination of pClamp (Axon Instruments Inc.) and in-house software (Smith
Proks et al., 1994). Single-channel current amplitudes were calculated from an all-points amplitude histogram. Single-channel conductance was measured by fitting a straight line to the data between −20 and −100 mV. Channel activity (NPo) was measured as the mean patch current (I) divided by the single-channel current amplitude (i), for segments of the current records of 1 min duration. Open probability (Po) was calculated from NPo/N, where N is the number of channels in the patch and was estimated from the maximum number of superimposed events.

For analysis of the single-channel kinetics, events were detected using the 50% threshold criterion with an imposed resolution for detecting events (tcrit) of 150 µs (Colquhoun, 1994). Open times were corrected for missed events according to Eq. 1 (Davies et al., 1992):

$$\tau_{OC} = \tau_{OM} \left( \sum_{j} a_{Cj} \exp\left(-t_{eo}/\tau_{Cj}\right) \right).$$  \hspace{1cm} (1)

where n is the number of exponentials in the closed time distribution, aCj is the fractional area of component j in the closed time distribution, τCj is the mean closed time of component j, and τOC and τOM are the corrected and the measured mean open times, respectively. Short closed times were not corrected because the fraction of open times that were not resolvable was very small. The burst distribution was derived using the Jackson criterion to define the interburst interval (Jackson et al., 1983).

The rate constants in Table II were calculated using the following kinetic scheme (Gillis et al., 1991; Alekseev et al., 1998; Trapp et al., 1998):

$$\tau_{OC} = \frac{\tau_{O1}}{k_{-1}} + \frac{\tau_{O2}}{k_{-2}} + \frac{\tau_{O3}}{1/(k_{1} + k_{2})} + \tau_{O4} = \frac{1 + k_{1}/k_{-1}}{k_{2}},$$

where τO1 is the mean open time, τO2 is the mean short closed time, τO3 is the mean long closed time, and τO4 is the mean burst time.

All data are given as mean ± SEM. The symbols in the figures indicate the mean and the vertical bars one SEM (where this is larger than the symbol). Statistical significance was tested using an unpaired t test or a paired t test, as appropriate.

RESULTS

To explore the molecular basis of fast gating, we measured single-channel currents in inside-out patches excised from Xenopus oocytes expressing the cloned β-cell KATP channel, Kir6.2/SUR1.

Effects of Lowering Potassium Concentration

We first investigated the effect of K⁺ on the single-channel kinetics within the burst. Previous studies on native KATP channels found that the mean open and short closed times increase when [K⁺]o is reduced (Benz et al., 1998; Zilberter et al., 1988). However, these investigators examined the effects of changing [K⁺] on the external side of the membrane only, and thus, it was not possible to discriminate the effects of K⁺ from those of voltage. Therefore, we tested the effect of symmetrical changes in [K⁺].

Figs. 1 and 2 show that, at negative membrane potentials, KATP channel openings occur in bursts, which are separated by relatively long closed intervals. When K⁺ was reduced from 140 to 10 mM on the both sides of the membrane concomitantly (Fig. 1), the mean open time (τO) at −130 mV increased from 0.84 ± 0.04 ms
Pore Mutations Affecting \(K_{\text{ATP}}\) Channel Gating

Kir channels show marked variations in both their single-channel conductance and their kinetics (Fig. 2 A). Thus, the mean conductance of Kir6.2/SUR1 channels in 140 mM \(K^+\) was 70 ± 2 pS \((n = 3)\), much higher than that observed for Kir1.1 channels (38 pS; Choe et al., 2000) or Kir2.1 channels (29 pS; Choe et al., 2000). This difference in conductance was associated with differences in the single-channel kinetics, with the mean open time \(\tau_o\) being much shorter for Kir6.2/SUR1 (2.3 ms at −60 mV) than for either Kir1.1 (23 ms; Choe et al., 1999) or Kir2.1 (280 ms, Choe et al., 1999).

There are four residues within the pore loop of Kir6.2 that differ from those of other Kir channels: V127, F133, G135, and M137 (Figs. 2 B and 7). Unusually, Kir6.2 has a phenylalanine (F133) rather than a tyrosine residue within the selectivity filter (GFG rather than GYG). Valine 127 is of also of interest, as it is conserved throughout all Kir channels with the exception of Kir6.1 and Kir6.2. Therefore, we examined the effect

\[K_{\text{ATP}}\]
of mutating the four different residues, one at a time, to those found in Kir2.1 (i.e., V127T, F133Y, G135F, and M137C). All mutations were coexpressed with SUR1.

When phenylalanine 133 was mutated to tyrosine, no currents could be recorded. Mutation of the other three residues, however, produced functional channels. In all cases, the mutations affected both the single-channel conductance and the channel kinetics, but the open probability remained unchanged (see Figs. 3 and 6). All mutants were blocked by 1 mM ATP with similar potency to the wild-type channel (unpublished data).

Our results suggest that V127 is largely responsible for the difference in the single-channel conductance ($\gamma$) of Kir6.2 and Kir1.1. Fig. 3 B shows that mutation of this residue to the isosteric amino acid threonine decreased $\gamma$ from $70 \pm 2$ pS ($n = 3$) to $38 \pm 3$ pS ($n = 3$), the same value as that found for Kir1.1 (Choe et al., 2000). The ability of the channel to discriminate against Na$^+$ ions was unaffected, however: the relative permeability to intracellular Na$^+$, $P_{Na}/P_K$, was $0.27 \pm 0.03$ ($n = 5$) and $0.28 \pm 0.02$ ($n = 5$) for the wild-type and V127T mutant channels, respectively. Mutation of...
Figure 4. (A) Open time distributions measured at $-60 \text{ mV}$ for wild-type (WT) and mutant Kir6.2/SUR1 channels, as indicated. The lines are fitted to either one exponential (WT, G135F) or the sum of two exponentials (V127T and M137C) with the time constants indicated. (B) Closed time distributions measured at $-60 \text{ mV}$ for wild-type (WT) and mutant Kir6.2/SUR1 channels, as indicated. The lines are fitted to the sum of three exponentials with the time constants indicated.
M137 to cysteine partially decreased \( \gamma \) (to 50 ± 3 pS, \( n = 3 \)), whereas the G135F mutation had a relatively small effect on conductance (62 ± 5 pS, \( n = 3 \)). In contrast to Kir1.1 and Kir2.1 channels (Choe et al., 1999), neither wild-type nor mutant Kir6.2/SUR1 channels showed any obvious substrate levels (Fig. 3 A, right).

Mutations within the P-loop of Kir6.2 also had a dramatic effect on the intraburst kinetics (Fig. 4). In both the wild-type channel and the G135F mutant, the open time histogram at −60 mV was best fit with a single exponential, whereas for both the V127T and M137C mutants, it was best fit by the sum of two exponentials (Fig. 4 A; see Fig. 6). There are two possible explanations for this finding. First, the V127T and M137C mutations may increase the number of open states. Second, it is possible that an additional short open time also exists for wild-type channels, but is too short to be resolved. In support of the latter hypothesis, a large number of studies have reported multiple open states for the native K<sub>ATP</sub> channel (Kakei et al., 1985; Nichols et al., 1991; Davies et al., 1992; Fan and Makielski, 1999). If the data for the pore mutants are fit with only a single exponential, for comparative purposes, the mean open time at −60 mV increased from 2.25 ± 0.15 ms (\( n = 5 \)) for the wild-type channel, to as much as 6.49 ± 0.93 ms (\( n = 5 \)) in the case of the V127T mutant. In contrast, the G135F mutation shortened \( \tau_c \) (to 1.00 ± 0.05 ms, \( n = 5 \)). These are the measured values of \( \tau_c \); the values corrected for missed events are given in Table I, as are the results of fitting the V127T and M137C pore mutants with two open times.

Closed time distributions for both wild-type and mutant channels were best fit by the sum of three exponentials, with \( \tau_{c1} \) governing the short closed time within a burst of openings, and \( \tau_{c2} \) and \( \tau_{c3} \) corresponding to the long closings between bursts. All three mutations significantly altered the short closed time (\( \tau_{c1} \)), with V127T and M137C increasing, and G135F decreasing, its duration (Fig. 4 B, 6). However, there was no significant effect on either \( \tau_{c2} \) or \( \tau_{c3} \). Furthermore, the mutations did not affect the mean burst duration (see Fig. 6). As a consequence of the change in \( \tau_c \) without alteration of the burst duration, the number of openings within a burst was modified. Thus, the V127T and M137C mutations produced longer \( \tau_c \) and fewer openings per burst; and, conversely, the G135F mutation reduced \( \tau_c \) and increased the number of openings per burst (see Fig. 6). The open probability was dominated by the slow gate parameters (frequency and duration of bursts and long closed times) and was unaffected by changes in fast gating (Fig. 6).

We attempted to explore the effect of mutating V127 to a range of other amino acids including both charged (V127K, V127D) and uncharged (V127S, V127A) residues, but none of these mutations expressed functional channels. Likewise, the reverse mutations in Kir2.1 (T139V) and Kir1.1 (T138V) also did not produce functional channels.

Effects of P-loop Mutations on Single-channel Kinetics

The intrinsic gating of the K<sub>ATP</sub> channel is usually represented by a linear scheme (Gillis et al., 1991, Alekseev et al., 1998; Trapp et al., 1998):

![Scheme II](image)

where O is the open state, C<sub>1</sub> is the intraburst short

### Table I

<table>
<thead>
<tr>
<th>Channel</th>
<th>Mean ( \tau_c ) (ms)</th>
<th>Mean ( \tau_{o1} ) (ms)</th>
<th>Mean ( \tau_{o2} ) (ms)</th>
<th>Percent ( \tau_{o1} )</th>
<th>Percent ( \tau_{o2} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>2.25 ± 0.15</td>
<td>1.64 ± 0.15</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>V127T</td>
<td>6.49 ± 0.93</td>
<td>5.56 ± 0.90</td>
<td>0.69 ± 0.07</td>
<td>7.44 ± 0.97</td>
<td>15.7 ± 3.1</td>
</tr>
<tr>
<td>G135F</td>
<td>1.00 ± 0.05</td>
<td>0.63 ± 0.08</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>M137C</td>
<td>5.28 ± 0.68</td>
<td>4.37 ± 0.66</td>
<td>0.71 ± 0.15</td>
<td>6.01 ± 0.72</td>
<td>16.5 ± 2.8</td>
</tr>
<tr>
<td>V127T + F133Y</td>
<td>7.45 ± 1.29</td>
<td>5.83 ± 1.40</td>
<td>1.67 ± 0.77</td>
<td>10.1 ± 1.7</td>
<td>29.4 ± 4.6</td>
</tr>
<tr>
<td>W83F</td>
<td>2.06 ± 0.18</td>
<td>1.55 ± 0.15</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Open time parameters for wild-type and mutant K<sub>ATP</sub> channels: \( \tau_c \) mean open time; mean corr. \( \tau_c \) mean open time corrected for missed events; \( \tau_{o1} \) and \( \tau_{o2} \) mean lifetimes of open states O<sub>1</sub> and O<sub>2</sub>, respectively; and percent \( \tau_{o1} \) and percent \( \tau_{o2} \) percentages of open states O<sub>1</sub> and O<sub>2</sub> in the open times distribution. Gating parameters were determined from five patches (wild type, V127T, G135F, M137C mutants) or four patches (V127T + F133Y, W83F mutants). All kinetic parameters were measured at −60 mV from recordings of several minutes.
closed state, and \( C_2 \) represents all the long closed states between bursts. The alternative scheme

\[
\begin{align*}
C_2 & \xrightleftharpoons[k_2]{k_1} C_1 \xrightleftharpoons[k_2]{k_1} O \\
\end{align*}
\]

(SCHEME III)

can be rejected on the grounds that ATP influences the open state but not the short closed state (\( C_1 \)). Gillis et al., 1991; Trapp et al., 1998). Although Kir6.2/SUR1 channels possess at least two long closed states (Figs. 4 B and 6), we have lumped them together as a single state (\( C_2 \)). This is because the P-loop mutations do not affect the duration of the long closed states, the burst duration, or the open probability. Furthermore, analysis of the long closed states is complicated by the possible presence of more than one channel in the patch, and the fact that long closings occur infrequently and are of variable duration. In Scheme I, the burst duration is given by \((1 + k_1/k_2)\), but in practice it is largely dominated by the rate constant \( k_2 \). The fact that the P-loop mutations do not affect the burst duration, therefore, indicates that they do not influence \( k_2 \). We calculated \( k_1 \) and \( k_1/k_2 \) at \(-60 \text{ mV} \) from \( \tau_{C_1} \) and \( \tau_O \) (Materials and Methods) for wild-type and mutant channels (Table II). It is evident that both rate constants are affected by the single mutations, consistent with the idea that they affect the fast gate.

We also calculated the free energy difference between the open and short closed state (\( \Delta G_{O,C_1} \)) using Eyring rate theory. Table II shows that \( \Delta G_{O,C_1} \) averaged \(-3.6 \text{ kJ/mol} \) for the wild-type channel, and was markedly altered by mutations in the P-loop. Thus, the G135F mutation reduced \( \Delta G_{O,C_1} \), whereas the other three mutations all enhanced it. This implies that the pore loop mutations influence the energy barrier between the open and short closed states. It is obvious from Table II that the calculated values of \( \Delta G_{O,C_1} \) are rather small—of the order of hydrogen bonds in proteins. This is consistent with the idea that openings and closings of the fast gate in both wild-type and mutant channels may correspond to small changes in the flexibility of the selectivity filter.

**Double Mutants**

Although mutation of F133 did not result in functional channels, we were able to record currents when the F133V mutation was combined with the V127T mutation. Fig. 5 compares single-channel currents and current-voltage relations for the double mutant V127T + F133V with those of the V127T mutant alone. The double mutant showed a further reduction in conductance, being \( 30.6 \pm 1.8 \text{ pS (} n = 3 \text{) as compared with } 38 \text{ pS for V127T and } 70 \text{ pS for the wild-type channel. The single-channel kinetics were also modified (Fig. 6). In particular, there was a further increase in the mean open time for V127T + F133Y mutant channels (\( \tau_O = 7.45 \pm 1.29 \text{ ms, } n = 4 \)). However, unlike the V127T mutant, \( \tau_C \) was not significantly different from that of the wild-type channel, suggesting that the F133Y mutation can compensate for the effect of the V127T mutation on the short closed time. Interestingly, the double mutant altered only the opening rate (\( k_1 \)), leaving the closing rate (\( k_1 \)) unaffected. The mean data is compared with that obtained for the single mutations in Fig. 6 and Table I. Neither of the double mutations M137C + F133Y or G135F + F133Y produced functional channel activity.

**Mutations Outside the Pore Loop Affect Conductance Not Gating**

Although the mutations reported above influence both the single-channel conductance and the intraburst kinetics, permeation and fast gating are not irrevocably coupled. We found that mutation of residues outside the pore loop were able to affect the former but not the latter. For example, mutation of tyrosine 83, which lies within TM1, to phenylalanine decreased the single-channel conductance (from 71 pS to 37.9 \( \pm 1.3 \text{ pS, } n = 3 \)), but did not alter the channel kinetics substantially. At \(-60 \text{ mV, } \tau_o = 2.06 \pm 0.18 \text{ ms, } \tau_{C1} = 0.44 \pm 9.4 \text{ ms,} \) the mean burst duration was \( 19.5 \pm 4.3 \text{ ms,} \) and the mean number of openings per burst was \( 12.9 \pm 2.5 \text{ (} n = 4; \text{ compare with Fig. 6).} \)

**DISCUSSION**

Our results are consistent with the idea that the \( \beta \)-cell \( K_{ATP} \) channel, Kir6.2/SUR1, has two modes of spontaneous gating that operate via different mechanisms. Slow gating governs the duration and frequency of bursts of openings, and the long interburst closed states, whereas fast gating determines the open and closed times within

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**Table II**

**Kinetic Model Parameters**

<table>
<thead>
<tr>
<th>Channel</th>
<th>( k_{-1} ) ( \times 10^3 )</th>
<th>( k_1 ) ( s^{-1} )</th>
<th>( \Delta G ) kJ/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>2.5 ( \pm 0.2 )</td>
<td>574 ( \pm 45 )</td>
<td>(-3.6 \pm 0.1 )</td>
</tr>
<tr>
<td>V127T</td>
<td>1.7 ( \pm 0.2^* )</td>
<td>127 ( \pm 34 )</td>
<td>(-6.5 \pm 0.3 )</td>
</tr>
<tr>
<td>G135F</td>
<td>3.3 ( \pm 0.2 )</td>
<td>1600 ( \pm 100 )</td>
<td>(-1.8 \pm 0.2 )</td>
</tr>
<tr>
<td>M137C</td>
<td>1.8 ( \pm 0.4 )</td>
<td>153 ( \pm 18 )</td>
<td>(-5.9 \pm 0.2 )</td>
</tr>
</tbody>
</table>

Rate constants \( k_{-1}, k_1 \) (Scheme I) and free energy difference between the open and short closed states (\( \Delta G \)) for wild-type and mutant channels were determined from the single-channel parameters described in Materials and Methods (\( n = 5 \)).
Previous studies have shown that mutations at the end of TM2 affect slow gating without substantially affecting fast gating (Tucker et al., 1998; Trapp et al., 1998; Loussouarn et al., 2000). We now show that the mutations within the P-loop disrupt fast gating without altering slow gating. They also influence the single-channel conductance. All of the P-loop mutants were blocked by ATP with similar efficacy to the wild-type channel, which agrees with current views that ATP inhibition involves closure of the slow gate and does not affect the fast gate (Trapp et al., 1998).

**Effects of P-loop Mutations on the Single-channel Conductance**

One explanation for the reduced single-channel conductance of the V127T, M137C, and W83F mutants is that the channel kinetics are too fast to be resolved, resulting in a time-averaged conductance of apparently smaller amplitude. This seems unlikely because the open channel noise was not affected by the mutation, and the kinetics of the W83F mutant, which also shows a reduced conductance, were unaltered. We can also exclude a block by external or internal cations (Choe et al., 1998) because our solutions contained no cations except for K⁺. A third possibility is that K⁺ acts as its own blocking ion; in a single-file selectivity filter, a slower flux of K⁺ might lead to both a reduced single-channel conductance and, via a “foot in the door” effect, to a longer $\tau_o$ (Swenson and Armstrong, 1981; Kiss and Korn, 1998). However, this idea is not supported by the G135F mutant, which has both a shorter $\tau_o$ and a reduced single-channel conductance. Furthermore, it cannot explain the fact that some mutations reduce the single-channel conductance (e.g., W83F) but do not alter the single-channel kinetics. However, it remains pos-

![Figure 5](image-url)
Pore Mutations Affecting $K_{\text{ATP}}$ Channel Gating

Sensible that $K^+$ serves as a gating ion, but that it binds in a position that does not impede $K^+$ flux.

The fact that the mutation F133Y did not produce functional channels indicates that the phenylalanine residue in the anomalous signature sequence GFG of the Kir6.2 subunit is crucial for the functional conformation of the P-loop. Threonine 127 appears to play a key role in the stabilization of the GYG sequence in other Kir channels because the additional replacement of valine 127 with threonine resulted in the functional expression of Kir6.2-F133Y channels. This effect is not unspecific, because changes at residues 137 and 135 did not produce functional channels. Thus, our results also suggest that the valine at position 127 in Kir6.2 may be important for the functional conformation of the anomalous GFG signature sequence. Interestingly, mutation of the tyrosine residue in the GYG signature sequence of Kir2.1 to phenylalanine did result in functional channels (So et al., 2001). The mutation did not affect the single-channel conductance and slightly reduced the mean open time.

Mechanism of Fast Gating

The molecular basis of the spontaneous fast gating characteristic of Kir6.2/SUR1 channels is not clear. The possibility that it results from a block by external or internal ions can be excluded, as our solutions contained no permeant ion other than $K^+$. It also seems unlikely that fast gating involves a narrowing of the inner mouth of the channel, as has been suggested for KcsA, because mutations in this region do not influence fast gating. Theoretically, conformational changes in any part of the channel could result in a transient constriction of the pore and thereby act as a gate. Moreover, the fact that mutation of a given residue alters gating does not necessarily imply that the wild-type residue either forms part of, or influences, a channel gate, as the effect of the mutation may be mediated allosterically. However, several pieces of evidence support the idea that the fast gate lies close to the selectivity filter. First, fast gating is voltage-dependent, implying that the gate itself, or a voltage sensor that controls the gate, lies within the membrane voltage field. Second, gating is influenced by $K^+$ ions. This is most simply explained by hypothesizing that $K^+$ ions interact with the permeation pathway; for example, $K^+$ binding might lower the energy barrier for channel opening. Third, P-loop mutations affect the fast gate (although we cannot exclude the possibility that these mutations may have allosteric effects).

Independence of Slow and Fast Gating

Several pieces of evidence suggest that fast and slow gating are distinct and are regulated by different mechanisms. First, many physiological (ATP and MgADP)
and pharmacological (sulphonylureas and K channel openers) modulators of channel activity primarily influence slow gating (Gillis et al., 1991; Alekseev et al., 1998; Trapp et al., 1998). Second, mutations in the P-loop that have a substantial effect on fast gating and do not alter kinetic parameters associated with slow gating, such as the burst duration, open probability, and long closed time durations. Third, fast gating, but not slow gating, is markedly affected by voltage. Taken together, these results are consistent with the idea that the KATP channel possesses two independent gates: (1) a fast gate that determines the openings and closings within the burst and (2) a slow gate that governs transitions between bursts of openings and interburst closings.

To what extent are the slow and fast gates physically distinct? Because channel modulators and mutations can affect one type of gating without the other, it appears that the two processes are independently regulated. What is less clear is whether they are associated with different regions of the channel. As discussed above, our results locate the fast gate close to the selectivity filter (Fig. 7). Because mutations at the end of TM2 (e.g., L164C, C166S) influence slow gating, it is possible that the slow gate might involve residues in this region (Fig. 7; Shyng et al., 1997; Trapp et al., 1998; Loussouarn et al., 1999). However, it is also possible that these mutations allosterically influence a gate that lies elsewhere.

Comparison with Other Kir Channels

In our experiments, removal of extracellular divalent cations did not appear to affect the gating of Kir6.2, in contrast to what was observed for Kir2.1 and Kir1.1 (Choe et al., 1999). This demonstrates that Kir channels differ in their response to divalent cations. Mutations at the cytosolic end of TM2 of Kir6.2, in positions corresponding to the proposed gate to the inner pore of KcsA, produced marked changes in burst duration but had no effect on the intraburst kinetics (Trapp et al., 1998; Loussouarn et al., 1999). In contrast, equivalent mutations in Kir2.1 did not significantly affect single-channel gating (Lu et al., 1999). This suggests that the molecular basis of fast gating of Kir6.2/SUR1 channels may be similar to that of Kir2.1 gating, and that slow gating is produced by a different mechanism. It seems probable that Kir6.2 may have evolved an additional gate because of the fact that, physiologically, this channel is gated by cytosolic substances.

Conclusions

In conclusion, our results demonstrate that mutations that perturb the P-loop of Kir6.2 affect the fast gating kinetics of the channel. This would be consistent with the location of the fast gate within the P-loop (Fig. 7). Mutations elsewhere in the protein (e.g., W83F in TM1) are able to influence conductance, but do not alter fast gating, and provide support for the idea that fast gating is associated with P-loop residues. Many other K+ channels, including voltage-gated K+ channels, exhibit a form of fast gating that is often referred to as flicker kinetics. Furthermore, the sequence of the P-loop is well conserved across K+ channel families. This suggests that other types of K+ channels may possess some form of fast gate that is associated with the selectivity filter.

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