Ligand-induced Closure of Inward Rectifier Kir6.2 Channels Traps Spermine in the Pore

L. Revell Phillips and Colin G. Nichols

Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63110

ABSTRACT Small organic amines block open voltage-gated K⁺ channels and can be trapped by subsequent closure. Such studies provide strong evidence for voltage gating occurring at the intracellular end of the channel. We engineered the necessary properties (long block times with unblock kinetics comparable to, or slower than, the kinetics of gating) into spermine-blocked, ATP-gated (N160D,L157C) mutant K⁺ channels, in order to test the possibility of “blocker trapping” in ligand-gated Kir channels. Spermine block of these channels is very strongly voltage dependent, such that, at positive voltages, the off-rate of spermine is very low. A brief pulse to negative voltages rapidly relieves the block, but no such relief is observed in ATP-closed channels. The results are well fit by a simple kinetic model that assumes no spermine exit from closed channels. The results incontrovertibly demonstrate that spermine is trapped in channels that are closed by ATP, and implicate the M2 helix bundle crossing, or somewhere lower, as the probable location of the gate.

KEY WORDS: spermine • ATP • inward rectifier • gating • K channel

INTRODUCTION

The location of the “gates” in K⁺ channels is of considerable topical interest. The static structures of several channels, as provided by crystallography (Doyle et al., 1998; Jiang et al., 2002a; Kuo et al., 2003), indicate two likely positions: in the selectivity filter or at the cytoplasmic end of the inner cavity that is formed by the four pore-lining helices (Fig. 1). Considerable evidence supports the notion that voltage gating of Shaker-like (Kv) channels occurs at the latter position (Holmgren et al., 1997, 1998; Liu et al., 1997; del Camino et al., 2000; del Camino and Yellen, 2001), as proposed for the gating of MthK and KcsA on the basis of the KcsA (closed) and MthK (open) crystal structures (Doyle et al., 1998; Jiang et al., 2002a,b). In ligand-gated channels, including those in the inward rectifier (Kir) channel family, the issue is currently much less clear. Consistent with a similar mechanism, mutations of residues at the lower end of M2 can “lock” Kir6.2 or Kir3 channels open (Enkvetchakul et al., 2001; Sajda et al., 2001; Yi et al., 2001), and introduction of prolines in this region can lock Kir3 channels essentially open or closed (Jin et al., 2002). In contrast, several studies indicate that accessibility of reagents or blockers to the inner cavity is not changed in the closed state (Bruening-Wright et al., 2002; Proks et al., 2003; Xiao et al., 2003), by default implicating the selectivity filter as the location of the gate. Our recent study of MTS-reagent accessibility in Kir6.2 channels indicates critical caveats to interpretation of such studies, and lead us to the conclusion that access to these reagents is gated by ATP and PIP₂ at or below the bundle crossing.

The notion of a gate at the cytoplasmic end of Kv channels is based on the classic “blocker trapping” experiments of Armstrong, who first examined the interaction of pore-blocking quaternary alkylamines with squid axon Kv channels (Armstrong, 1966, 1969, 1971; Holmgren et al., 1997; Liu et al., 1997). The present study was motivated to examine whether similar “blocker-trapping” occurs in ligand-gated Kir channels, and thereby test whether gating occurs above or below the blocker binding site. Such studies have not previously been performed on Kir channels, since in practice they require that blocker entry and exit rates be slower than, or at least on the same time scale, as gating itself. Such a situation rarely arises, since ligand gating tends to occur much more slowly than voltage gating. We took advantage of a mutant Kir6.2 (N160D) channel, in which the introduction of the negative charge in M2 renders the channel strongly rectifying, with measurable kinetics of spermine block and unblock (Shyng et al., 1997). At positive voltages, the spermine off-rate becomes significantly lower than rates of ATP gating, a necessary condition to reliably test for “trapping.” The results incontrovertibly demonstrate that spermine is indeed trapped in its binding site, in channels that are closed by ATP. Spermine is released when the channels are opened by removal of ATP, or by application of PIP₂. Since spermine is a pore blocker that likely binds in the inner cavity of the
Kir channel (Loussouarn et al., 2002), the data imply that the ligand (ATP- or PIP₂) -operated gate of Kir channels lies below the inner cavity, i.e., at or below the bundle crossing.

**MATERIALS AND METHODS**

**Expression of K\textsubscript{ATP} Channels in COS\textsubscript{m6} cells**

In all experiments Kir6.2 subunits were coexpressed with SUR1. COS\textsubscript{m6} cells were plated at a density of ~2.5 × 10⁶ cells per well (30-mm 6-well dishes) and cultured in Dulbecco’s modified eagle medium plus 10 mM glucose (DMEM-HG), supplemented with FCS (10%). On the first day after plating, cells were transfected using 1–2 μg each of pCMV6b-Kir6.2, pECE-SUR1, and pGreen-lantern (GFP) (GIBCO BRL) and 5 μl FuGene6 (Roche Co.). 2 d after plating, transfected cells were replated onto glass coverslips for patch-clamping.

The primary channel constructs used were Kir6.2[N160D, L157C, C166S,Δ36] and Kir6.2[N160D, L164C, C166S,Δ36], repectively. The N160D mutation induces potent, steeply voltage-dependent block by spermine (see Results). Similar trapping results were also seen in Kir6.2[N160D] channels without the C166S and L157C mutations, but the reported experiments were performed on the triple mutant subunits, as the L157C and C166S mutations raise the open state stability (Enkvetchakul et al., 2000), allowing patches to be studied for long periods of time without significant rundown of currents. These channel constructs also have a deletion of the last 36 amino acids from the COOH terminus (Δ36, Tucker et al., 1997). This removal of 36 amino acids has no effect on the function of channels coexpressed with SUR1 (as in all of the present experiments).

**Patch-clamp Measurements**

All experiments were performed at room temperature in a chamber that allowed the bathing solution exposed to the patch surface to be rapidly changed using an oil gate (Lederer and Nichols, 1989). Pipettes were pulled from Kimble 73813 soda lime glass using a horizontal puller (Sutter Instrument Co.). Electrode resistances ranged from 0.3 to 2.5 MΩ. Microelectrodes were sealed onto green fluorescing cells by applying light suction to the rear of the pipette. Inside-out patches were obtained by lifting the electrode and then passing the tip through the oil gate. Pipette and bath solutions contained a modified Spermine Blocker Trapping in Closed K\textsubscript{ATP} channels by spermine (see Results). Similar trapping results were also seen in Kir6.2[N160D] channels without the C166S and L157C mutations, but the reported experiments were performed on the triple mutant subunits, as the L157C and C166S mutations raise the open state stability (Enkvetchakul et al., 2000), allowing patches to be studied for long periods of time without significant rundown of currents. These channel constructs also have a deletion of the last 36 amino acids from the COOH terminus (Δ36, Tucker et al., 1997). This removal of 36 amino acids has no effect on the function of channels coexpressed with SUR1 (as in all of the present experiments).

**Results**

**Voltage-dependent Spermine Block of N160D/L157C Channels**

Classical “blocker-trapping” experiments, demonstrating that the blocker is trapped inside the pore by channel closure, require the blocker residency time to be measurable, and comparable to or slower than the rate of channel opening. Wild-type K\textsubscript{ATP} channels are “weak” inward rectifiers, and show only weak, fast block by polyamines and other organic blockers. However, the introduction of a negative charge (N160D) into the M2 helices that line the inner pore renders them strong rectifiers that are blocked potently, and with very “strong” voltage dependence, by polyamines (Shyng et al., 1997). Fig. 1 indicates the location of mutations considered in this study. We used primarily Kir6.2[L157C,N160D,C166S,Δ36] (referred to as N160D/L157C) mutant channels in the following experiments. The L157C mutation stabilizes the open state, providing channel activity with no rundown of excised patch currents for many min-
utes, allowing for the long protocols required in the following experiments.

Fig. 2 illustrates the voltage dependence and kinetics of spermine block in N160D/L157C channels. 10 μM spermine causes complete block of channel currents at positive voltages, with steep voltage dependence (Fig. 2 A and B). The relative conductance-voltage relationship is well fit by a single Boltzmann function (Fig. 2 C) and current relaxations are monoexponential, both for block and unblock (Fig. 2 D). Accordingly, we fitted exponential functions to current relaxations (Fig. 2 D) and estimated voltage-dependent on- and off-rates for a simple 2-state model (Fig. 2 E).

\[
\begin{align*}
& O \\ & \xrightarrow{k_{\text{in}}(v)} B \\ & \xleftarrow{k_{\text{out}}(v)} O
\end{align*}
\]

**SCHEME I**

The model reproduces the observed voltage dependence of spermine block, with \( k_{\text{in}}(0) = 1.30 \mu \text{M}^{-1} \text{s}^{-1} \), \( z_{\text{in}} = 1.95 \), and \( k_{\text{out}}(0) = 1.3 \text{s}^{-1} \), \( z_{\text{out}} = 2.1 \).

Measurements of off-rates using concentration “jump” experiments in inside-out membrane patches are always compounded by limited diffusion rates out of the recessed patch (Cannell and Nichols, 1991), but at positive voltages, the predicted spermine off-rate (Fig. 2 E) becomes extremely slow. It should thus be possible to determine the unidirectional spermine off-rate after removal of spermine from the solution (Fig. 3 A) with minimal contribution of diffusion. The measured time constant of spermine unblock at +40 mV of 10–30 s (Fig. 3 A) compares well to that predicted from the model (\( k_{\text{out}}(+40) = 0.05 \text{s}^{-1} \), \( \tau_{\text{out}} = 20.0 \text{s} \), Fig. 2 E). Brief (10 ms) pulses to a series of voltages more negative than −20 mV rapidly relieve the block (Fig. 3 A, expanded time scale to right). The relative recovery is plotted as a function of pulse voltage in Fig. 3 B. The data are well predicted by the voltage-dependent \( k_{\text{out}} \) estimated for the 2-state model (red line, Fig. 3 B). We have also examined the time dependence of current relief at −40 mV (Fig. 3 C). After removal of spermine (at +40 mV), the potential was stepped briefly back to −40 mV for variable times. The relative recovery is plotted as a function of pulse duration (Fig. 3 D) from multiple similar experiments. Again, the time course of recovery is well predicted by the simple 2-state model (red line) of spermine block, using rate constants as determined in Fig. 2. In the following experiments, we have collected data on the recovery from spermine block in channels closed by ATP, and have extended the analysis of this two-state model to incorporate channel gating.

**Spermine Is Trapped in ATP-closed Channels**

Fig. 4 A shows current records from a patch expressing N160D/L157C channels, with exposure to spermine and ATP as indicated. At +40 mV, channels are blocked rapidly and completely by 10 μM spermine. After spermine removal from the solution, unblock is extremely slow (tau ~30 s, trace 1). However, block is rapidly and completely relieved by a brief (300 ms) pulse to −40 mV (trace 2). N160D/L157C channels are >95% closed by 10 mM ATP (Enkvetchakul et al., 2000). As shown in trace 3, when spermine-blocked channels are subsequently exposed to ATP, a 300 ms pulse to −40 mV now completely fails to relieve spermine block, as evidenced by unaltered subsequent time course of spermine release, after ATP is removed (compare trace 3 to trace 1).

The above experiment indicates that ATP traps spermine in the pore, the simplest interpretation being...
that ATP does so by closing the channels. To exclude alternative possible consequences of ATP binding, the experiment was repeated on an additional mutant channel. In contrast to N160D/L157C channels, N160D/L164C channels, with a cysteine substituted at residue 164, bind ATP but have an extremely high open state stability and are not closed by 10 mM ATP (Enkvetchakul et al., 2000, 2001). In Fig. 4 B, the above experiment was repeated on a patch containing N160D/L164C channels. After the removal of spermine from solution, spermine block is rapidly and completely relieved by a brief (100 ms) pulse to −40 mV (trace 2). In contrast to N160D/L157C channels, block of N160D/L164C channels is still fully relieved when the 100-ms pulse is applied during exposure to 10 mM ATP (trace 3). That ATP traps spermine in
of gating provides a very unambiguous answer. Fig. 5 B shows the predicted recovery from spermine block in open channels and in partially ATP–closed channels for two extreme cases: (1) in which spermine can access its binding site in both open and closed channels (Scheme I), and (2) in which access is limited only to open channels (Scheme II):

**Scheme I**

![Scheme I Diagram]

**Scheme II**

![Scheme II Diagram]

O and C represent open and closed states, $O_B$ and $C_B$ represent open, blocked and closed, blocked states, and $k_x$ represents the relevant voltage-, [ATP]-, or [spermine]-dependent rate constants. To model the time course of recovery from block upon removal of spermine.
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Blocker Trapping in Closed KATP Channels

Superimposed in Fig. 5 B are the predictions of Schemes Iir and IIr, with the following rate constants (see Fig. 5 C): k\text{out} (−40) = 28 s\(^{-1}\), k\text{open} = 0.33 s\(^{-1}\). This opening rate approximates the lumped kinetics of KATP channel opening that are typically observed in excised patch recordings (Qin et al., 1989; Nichols et al., 1991). The closing rate (k\text{closed} = k\text{open}(1 − P_o)/P_o\)), was then calculated to give the appropriate open probability (P_o). In the particular patch illustrated in Fig. 5 B, the estimated relative open probability in 10 mM ATP was 0.10, and curves are predicted for the two schemes, with P_o = 1 (k_o = 0 s\(^{-1}\)) and 0.1 (k_o = 2.7 s\(^{-1}\)). The un-gated access model (Iir) predicts no slowing of recovery in partially closed channels, and the observed time course of recovery is well predicted by the fully gated access model (IIr).

Varying Open State Stability by Changing PIP\(_2\) Shifts the ATP-trapping Efficacy: PIP\(_2\) and ATP Act on the Same Gate

The above results argue that spermine cannot exit channels that are closed by ATP. Exogenous application of PIP\(_2\) leads to increased open state stability of K\(_{ATP}\) channels, and thereby increases open probability at any given [ATP] (Baukrowitz et al., 1998; Shyng and Nichols, 1998; Enkvetchakul et al., 2000, 2001). This is consistent with ATP and PIP\(_2\) acting on the same “gate,” and Fig. 5 C shows the results of an experiment designed to test whether PIP\(_2\) can similarly counter the spermine-trapping effects of ATP. Before treatment with PIP\(_2\), 10 mM ATP inhibited current in this particular patch to rP_o ~0.1, and on exposure to a 1-s pulse to −40 mV, trapping of spermine was essentially complete. The patch was subsequently exposed to PIP\(_2\), until rP_o in 10 mM ATP was increased to ~0.5. Upon repeating...
Figure 5. ATP traps spermine in closed channels. (A) Spermine (10 μM) was briefly applied to block N160D/L157C channels at +40 mV, and then spermine was removed ~2 s before the onset of the recorded traces and the patch was exposed to 10 mM ATP (rPo = 0.10) at the onset of each recording. Variable duration pulses to −40 mV were applied. ATP was subsequently removed, and the extent of recovery assessed as indicated (current was averaged for 1 s at dashed vertical line). Shown in gray is the response to a 100-ms pulse to −40 mV without exposure to ATP (rPo = 1.0). This completely relieves block in open channels. (B) Relative recovery of current is plotted versus pulse duration (from the experiment in A). The solid lines indicate the predicted recovery for Po = 1.0 and Po = 0.1 for Schemes I and II (see text). (C) Spermine (10 μM) was briefly applied to block N160D/L157C channels at +40 mV, and then spermine was removed ~2 s before the onset of the recorded traces. A 1-s pulse to −40 mV completely relieves block of open channels (rPo = 1.0, black trace). The protocol was repeated and, after removal of spermine, the patch was exposed to 10 mM ATP (blue trace, rPo ∼0.1) at the beginning of the recording. A 1-s pulse to −40 mV was applied and recovery assessed as indicated. The patch was then exposed to 5 mg/ml PIP2 for ~1 min to increase channel open state stability. Exposure to PIP2 increased rPo in 10 mM ATP to ∼0.5. The patch was again blocked by spermine (10 μM) and, after spermine removal, the patch was exposed to 10 mM ATP (orange trace, rPo = 0.50), and a 1-s step to −40 mV was applied and recovery assessed as indicated. (D) Relative recovery of current is plotted versus pulse duration. The solid lines indicate the predicted recovery for Po for Scheme II (see text). The trapping effect of ATP is antagonized by PIP2.

the trapping protocol, a 1-s pulse to −40 mV now resulted in much greater relief of spermine block (orange trace), consistent with PIP2-induced destabilization of the ATP-induced closure (Enkvetchakul et al., 2000). Fig. 5 D shows the predictions of Scheme IIr, with the rate constants used in Fig. 5 B, superimposed on the estimated fractional recovery from each of the traces in Fig. 5 C. Again, the dependence of the observed time course of recovery on open probability is well predicted by the fully gated access model (Scheme IIr).
From multiple experiments like those in Fig. 5, we were able to estimate the recovery timecourse (at ~40 mV) during exposure to different concentrations of ATP, with or without PIP_2 treatment (and hence at different open probabilities). Datasets were pooled by estimated relative open probability (rPo) in ATP. The data are plotted in Fig. 6, together with the predictions of Scheme II for each Popen. There is a biphasic recovery from block, and as Popen changes, the weighting of each phase shifts. The consistent quantitative agreement between data and model argues strongly that trapping of spermine is essentially complete in closed channels.

**DISCUSSION**

**Gated Access of both Cysteine Modifiers and Polyamines to the Kir Inner Cavity**

Our previous analysis of gating dependence of MTSEA modification of cysteines in the inner pore of Kir6.2 channels (Phillips et al., 2003) illustrated a complex voltage and gating dependence of overall modification rates that led us to propose ligand-gated access, i.e., that MTSEA cannot enter the ligand-closed channel, and hence that the ligand-operated gate lies below the inner pore (Jiang et al., 2002a,b). The present demonstration of spermine trapping in closed channels is a more direct demonstration of gated access to the pore, providing clear evidence that the polyamine cannot escape from its binding site, when channels are in the ligand-closed state. These experiments are similar to the classical blocker-trapping experiments of Armstrong (1966, 1969, 1971) that first led to the suggestion that voltage gating of Kv channels occurs by closure of a “gate,” located near the cytoplasmic end of the channel, now postulated to be at the bundle crossing of the S6 (M2) helices (Jiang et al., 2002a,b). Such experiments have not been performed previously in inward rectifier channels, since the necessary conditions (long block times providing unblock kinetics that are comparable to, or slower than, the kinetics of gating) are rarely encountered. These conditions are met in the present experiments by the very stable spermine block of a strong inward rectifying channel having relatively rapid ligand (ATP) gating.

There is currently some controversy regarding the binding site of polyamines in Kir channels. Based on the crystal structure of the cytoplasmic domain of Kir3 channels, Guo and colleagues (Guo and Lu, 2003; Guo et al., 2003) recently proposed that polyamines bind peripherally in the cytoplasmic portion of the pore and extend into the inner cavity. There is considerable evidence from other mutational analyses that, while polyamines may bind in the cytoplasmic pore to reduce single channel conductance, the high-affinity binding site responsible for classic strong inward rectification lies in or above the inner cavity (Lu and MacKinnon, 1994; Kubo and Murata, 2001; Xie et al., 2002). Either way, the present results argue that, to trap spermine, the ligand-operated gate would have to be at or below the inner cavity. Spermine block of N160D/L157C channels is well fit by a simple 2-state model with concentration- and voltage-dependent binding to a single site (Fig. 2). The overlapping, weakly voltage-dependent additional component of spermine inhibition that can be prominent in Kir2.1 and Kir2.3 channels (Lopatin et al., 1995; Xie et al., 2002, 2003) is not evident in these channels at low micromolar concentrations, greatly simplifying the present analysis. We can thus argue that because channel closure traps a single spermine molecule in the pore, the ligand-operated gate in Kir6.2 likely lies below the inner cavity. That the trapping by ATP closure of the channels is antagonized by PIP_2 is entirely consistent with the view that it is the same “gate” which is stabilized in the closed state by ATP, and in the open state by PIP_2 (Baukrowitz et al., 1998; Shyng and Nichols, 1998; Enkvetchakul et al., 2000, 2001).

**Comparison to other Studies**

There is now mounting structural and biophysical evidence that the voltage (or ligand) -operated gate of voltage-gated, or Ca-gated K^+ channels corresponds to
a pinching of the permeation pathway by the M2 helix bundle crossing, as a result of hinged motion of M2 at a conserved glycine (G156 in Kir6.2) residue in the center of M2 (Doyle et al., 1998; del Camino and Yellen, 2001; Jiang et al., 2002a,b; Rothberg et al., 2002). The structure of a bacterial Kir channel (KirBac1.1) pore demonstrates a similar structure in Kir channels (Kuo et al., 2003). These authors argue that hinged gating may not occur at the equivalent glycine residue, but there are parallel consequences of M2 (S6) mutations on open state stability of Kir and Kv channels (Holmgren et al., 1998; Enkvetchakul et al., 2000, 2001; Espinosa et al., 2001; Yi et al., 2001; Hackos et al., 2002).

We have now provided direct evidence for gated access to inner cavity residues in Kir6.2 (Phillips et al., 2003), but other recent studies have questioned the interpretation that the ligand-operated gate of Kir channels lies at or below the bundle crossing of the M2 helices, instead suggesting that gating must occur much higher, i.e., in the selectivity filter (Proks et al., 2003; Xiao et al., 2003). In particular, these latter studies reach conclusions that are not easily reconciled with those of either the present or our previous accessibility study. Proks et al. (2003) examined the rate of block of Kir6.2 channels by cytoplasmic Ba\(^{2+}\) after depolarizing pulses and provided evidence for a speeding up of the Ba\(^{2+}\) block rate in partially ATP-closed channels. We have repeated their experiment with wild-type Kir6.2+SUR1 channels in essentially identical conditions. After scaling the currents to the prepulse current (rather than the peak current which can be contaminated by large capacitance artifacts), we observe no significant change in the rapid phase of Ba\(^{2+}\) block. However, we do observe a significant, very slow (\(t \sim 1-3\) s), component of Ba\(^{2+}\) block that is only present in ATP. This slow phase of block might be due to a gated access (i.e., if a fraction of channels must open from long lived closed states before blocking). In another study examining gating dependence of pore access in Kir channels, Xiao et al. (2003) demonstrated no slowing of the MTSEA accessibility of M2 cysteines in Kir2.1 channels that were closed by reducing membrane PIP\(_2\) of the MTSEA accessibility of M2 cysteines in Kir2.1 channels, Xiao et al. (2003) demonstrated no slowing of the MTSEA accessibility of M2 cysteines in Kir2.1 channels, Xiao et al. (2003) demonstrated no slowing of the MTSEA accessibility of M2 cysteines in Kir2.1 channels, Xiao et al. (2003) demonstrated no slowing of the MTSEA accessibility of M2 cysteines in Kir2.1 channels, Xiao et al. (2003) reported

Conclusions

The present data demonstrate unequivocally that classic pore blocker trapping by channel closure can be observed in inward rectifier channels; trapping of spermine ions in closed channels is essentially complete. Although we cannot formally exclude a model whereby a closure at the selectivity filter somehow traps spermine in the filter itself, nor the possibility that the “gate” to spermine exit is distinct from the gate to K\(^{+}\) ions, the simplest interpretation is that ATP stabilizes the closing (and PIP\(_2\) stabilizes the opening) of a gate that is located intracellular to the spermine binding site. Since the spermine binding site is likely to be in the inner cavity, this would localize the gate to the M2 helix bundle crossing or below.

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