Paradoxical Activation of an Inwardly Rectifying Potassium Channel Mutant by Spermine: "(B)locking" Open the Bundle Crossing Gate

Yury Y. Vilin, John-Jose Nunez, Robin Y. Kim, Gregory R. Dake, and Harley T. Kurata

Department of Anesthesiology, Pharmacology, and Therapeutics (Y.Y.V., J.-J.N., R.Y.K., H.T.K.), and Department of Chemistry (G.R.D.), University of British Columbia, Vancouver, British Columbia, Canada

Received April 1, 2013; accepted July 25, 2013

ABSTRACT

Intracellular polyamines are endogenous blockers of inwardly rectifying potassium (Kir) channels and underlie steeply voltage-dependent rectification. Kir channels with strong polyamine sensitivity typically carry a negatively charged side chain at a conserved inner cavity position, although acidic residues at any pore-lining position in the inner cavity are sufficient to confer polyamine block. We have identified unique consequences of a glutamate substitution in the region of the helix bundle crossing of Kir6.2. Firstly, glutamate substitution at Kir6.2 residue F168 generates channels with intrinsic inward rectification that does not require blockade by intracellular polyamines or Mg2+2+. In addition, these F168E channels exhibit a unique "spiked" tail phenotype, whereby large decaying inward tail currents are elicited upon spermine unbinding. This contrasts with the time-dependent recovery of current typically associated with blocker unbinding from ion channels. Interestingly, Kir6.2[F168E] channels exhibit a paradoxical biphasic conductance-voltage relationship in the presence of certain polyamines. This reflects channel blockade at positive voltages, channel stimulation at intermediate voltages, and exclusion of spermine from the pore at negative voltages. These features are recapitulated by a simple kinetic scheme in which weakly voltage-dependent spermine binding to a "shallow" site in the pore (presumably formed by the introduced glutamate at F168E) stabilizes opening of the bundle crossing gate. These findings illustrate the potential for dichotomous effects of a blocker in a long pore (with multiple binding sites), and offer a unique example of targeted modulation of the Kir channel gating apparatus.

Introduction

Inwardly rectifying potassium (Kir) channels are regulated by diverse mechanisms including voltage-dependent block by Mg2+ and polyamines, and ligand-dependent gating (Hibino et al., 2010). These mechanisms underlie distinct channel functions and operate on significantly different time scales in the physiologic setting. Polyamine block rapidly alters channel activity on a "moment-to-moment" basis in response to changes in membrane voltage, most importantly during action potential firing (Lopatin et al., 1994; Lopatin and Nichols, 1996). This enables cells to overcome the electrical suppression generated by Kir channels around the K* reversal potential and transiently depolarize the membrane. Appropriate voltage-dependent inhibition of Kir channel activity is essential for normal function of excitable cells, as illustrated by the effects of gain-of-function (loss of polyamine sensitivity) mutations of Kir2.1 that underlie shortening of the QT interval and have been categorized as SQT3 (Priori et al., 2005; Patel and Pavri, 2009). In contrast, ligand-dependent gating involves conformational changes of the channel protein and alters cellular excitability over longer time scales in response to signaling cascades or changes in cellular metabolism (Wickman et al., 1998; Koster et al., 2000).

Interactions between blockers and ion channel gates were recognized in early descriptions of quaternary ammonium ion effects on voltage-induced closure of voltage-gated potassium (Kv) channels (Armstrong, 1966, 1971). A common observation, though not universal to all Kv channels (Holmgren et al., 1997), is that the presence of a blocking ion in the pore can decelerate kinetics of channel closure. This occurs when closure is unfavorable with a blocking ion occupying the pore, and so blocker unbinding delays or competes with gate closure. In these cases, a rising phase of tail currents is often observed upon repolarization, reflecting a sequence of blocker unbinding followed by channel closure (Stanfield, 1983). In contrast to Kv channels, Kir channels are not thought to exhibit marked gating-related conformational changes in response to membrane voltage. Rather, the observed voltage dependence of Kir channels in vivo results from polyamine interaction with a conserved negatively charged residue located

ABBREVIATIONS: bis-TEA-C10, bis-1,10-triethylaminodecane; hERG, human ether-a-go-go–related gene; Kir, inwardly rectifying potassium channel; Kv, voltage-gated potassium channel.
in the inner cavity, often referred to as the "rectification controller" residue (Lopatin et al., 1994; Wible et al., 1994; Nichols and Lopatin, 1997). Upon membrane hyperpolarization, blocker unbinding from the rectification controller region is favored, and time-dependent recovery of current is observed as blockers vacate the pore (Lopatin et al., 1995). Interactions of blockers with the Kir channel gating apparatus are not generally apparent, most likely because ligand-dependent mechanisms that control Kir channel conformation occur on a much slower time scale than voltage-dependent block.

In this study, we describe a unique interaction between polyamine blockade and the gating apparatus of Kir6.2 channels, the pore-forming component of the ATP-sensitive potassium channel complex (Inagaki et al., 1995; Shyng and Nichols, 1997). Specifically, introduction of acidic residues at certain positions in the Kir6.2 bundle crossing enables polyamines to paradoxically "prop open" channels. These findings illustrate a paradoxical combination of channel activation and inhibition by the same compound (but at different voltages), reflecting divergent effects associated with multiple binding sites along the Kir channel pore.

Materials and Methods

Kir6.2 Channel Constructs. Mouse Kir6.2 in the pcDNA3.1(−) vector was used as a background for all mutations characterized in this study. Mutations were generated by overlapping polymerase chain reaction methods and verified by Sanger sequencing (Genewiz, Inc., South Plainfield, NJ).

Electrophysiology. CosM6 cells were transfected with 500 ng of Kir6.2 cDNA (with mutations as described), 500 ng of hamster SUR1, and 250 ng of green fluorescent protein cDNA, using Fugene 6 (Roche, Basel, Switzerland) or Lipofectamine 2000 (Life Technologies, Carlsbad, CA) transfection reagents. Patch-clamp experiments were performed at room temperature, using a perfusion chamber that allowed rapid switching of solutions. Data were typically filtered at 2 kHz, digitized at 10 kHz, and stored directly on computer hard drive using pClamp v.10 software (Molecular Devices, Sunnyvale, CA). Symmetrical pipette and bath solutions were used in these experiments, with the following composition: 140 mM KCl, 1 mM K-EGTA, 1 mM K-EDTA, 4 mM K2HPO4, pH 7.3. Spermast and most polyamine analogs were purchased from Sigma-Aldrich/FLUKA Chemicals (St. Louis, MO), with the exception of bis-1,10-triethylamidoxycane (bis-TEA-C10), which was synthesized on-site at University of British Columbia.

Kinetic Model of Spermast Potentiation and Block of Kir6.2[F168E] Channels. Equilibrium properties of spermine interactions with Kir6.2[F168E] channels were fit with a modified version of previous kinetic models describing polyamine block of Kir channels (Shin and Lu, 2005; Kurata et al., 2007). A schematic outline of the kinetic model is presented in Figs. 8A and 9A. The model comprises a voltage-dependent gating step (describing the intrinsic voltage dependence illustrated in Fig. 1), followed by a weakly voltage-dependent binding step leading to the O* state, and lastly a steeply voltage-dependent entry of spermine into its terminal bound state OB.

The shallow binding step (O→O*) describes binding of spermine into a shallow site (weakly voltage-dependent) that we envision involves the F168E glutamate substitution. The key feature of the model is that although the O* state involves a spermine interaction in the bundle crossing apparatus, it retains a significant conductance. The deep binding step (O*→OB) describes entry of spermine into a stable, deep binding site and is steeply voltage-dependent. Thus, a strict physical interpretation of this model is that spermine interacts with Kir6.2[F168E] channels to achieve a partially conductive intermediate state (O*), and at more depolarized voltage can reach a deep, fully blocked state with little residual conductance.

Alternative physical interpretations of the model are touched on briefly in the Discussion section.

Each transition was described by a voltage-dependent equilibrium constant K, and effective valence z, in the form K(V) = K0(V = 0 mV) e^zV/RT. In the model described in Fig. 9, fractional conductance changes with voltage are described by the following equation:

\[
\frac{I}{I_0} = \frac{O + O^*}{C + O + OB} = \frac{K_0 + K_1 K_2}{K_0 + K_1 K_2 + K_3 K_4 K_5 K_6}
\]

where K0, K1, and K2 describe equilibrium constants as indicated in Fig. 8A, and c is the fractional conductance of the O* state (relative to O). Model parameters were initially minimized by fitting conductance-voltage relationships at multiple spermine concentrations (0, 30, 100, and 300 μM). Subsequently, parameters were refined manually to better account for the magnitude of the "spiked" spermine tail current and the kinetics of current recovery and relaxation after spermine unbinding. Essential model parameters are presented in Table 1. Kinetics of spermine block were simulated using the freely available IonChannelLab software (Santiago-Castillo et al., 2010), using the Q-matrix method (Colquhoun and Hawkes, 1995). The conductance ratio used for all presented model simulations was c = 0.9.

Results

Intrinsic Voltage Dependence of Kir6.2[F168E] Channels. Wild-type ATP-sensitive potassium channels and other Kir channels exhibit nearly linear macroscopic current-voltage relationships (Fig. 1C) when removed from the cellular environment and examined in the absence of contaminating polyamines or Mg2+ ions (Guo and Lu, 2000). Through a mutagenic scan of the Kir6.2 inner cavity, we recognized that intrinsic voltage dependence can be conferred upon Kir6.2 channels by substituting charged amino acid side chains at certain pore-lining positions (Kurata et al., 2004, 2010a). An additional example of this phenomenon is Kir6.2[F168E] channels, which exhibit intrinsic voltage dependence (Fig. 1). We determined current-voltage relationships and kinetics of current relaxation using a series of depolarizing pulses from −150 mV (Fig. 1A, depolarizing steps), and a series of hyperpolarizing pulses from +50 mV (Fig. 1B, repolarizing steps). Clear time-dependent channel opening of Kir6.2[F168E] channels is apparent with steps to negative voltages, while decay/closure is observed at depolarized potentials (Fig. 1, A and B), resulting in a pronounced inwardly rectifying current-voltage relationship (Fig. 1C). We describe this rectification as intrinsic to this channel mutant (i.e., not spermine-dependent), because it persists in cell-free patches in the absence of any blocking polyamines or divalent cations (recording solutions contained 1 mM EDTA). Single exponential fits to current changes elicited by voltage jumps (Fig. 1, A and B) were used to describe the kinetics of this voltage-dependent gating process. Depolarization-mediated channel closure was slightly more steeply voltage-dependent than channel reopening at negative voltages (Fig. 1D), although the overall voltage dependence was quite weak (z, ~0.3). Nevertheless, it is noteworthy that this voltage dependence arises in the absence of a canonical voltage-sensing domain.

Voltage-Dependent Gating of Kir6.2[F168E] Is Modified by a Diffusible Cellular Factor. Interestingly, the hypervarization-elicited activation of Kir6.2[F168E] channels observed in cell-free patches (Fig. 1A) differs significantly from kinetics observed in the on-cell (cell-attached) configuration, in which cytoplasmic constituents are still able to
interact with the channel. As described above, in cell-free conditions, hyperpolarizing voltage steps (e.g., from $+50 \text{ mV}$ to more negative voltages) of inside-out Kir6.2[F168E] patches generate a monotonic increase of inward current (Figs. 1B and 2B). In contrast, in cell-attached recordings, identical voltage protocols generate a large transient inward current that decays to a steady state (Fig. 2A). Steady-state on-cell currents were smaller ($I_{\text{on-cell}}/I_{\text{excised}} = 0.39 \pm 0.06$ at $-150 \text{ mV}$) relative to excised patch currents (Fig. 2), most likely due to the effects of ATP inhibition in the cell-attached recording mode. Similar manifestations in other channel types have been described as "resurgent" tails, or the well-known "hooked" tail behavior in human ether-a-go-go–related gene (hERG) channels (Smith et al., 1996; Wang et al., 2006). We have used the term "spiked" tail to distinguish this observation from the hooked tails characteristic of hERG channels, where a rising phase of the tail current is apparent upon repolarization. We could not convincingly detect a rising phase of the inward tail current after spermine block, suggesting that spermine unbinds from F168E channels extremely rapidly.

To better understand the difference between cell-attached and cell-free recordings, we tested several candidate intracellular compounds (ATP, spermine) known to interact with Kir6.2 and other Kir channels. Application of spermine to the bath solution (intracellular face) of inside-out Kir6.2[F168E] patches was necessary and sufficient to change the current behavior and recapitulate the spiked inward currents characteristic of on-cell recordings (Figs. 3A and 4). In contrast, ATP could not replicate these effects on tail current kinetics (not shown). These experiments indicated that intracellular spermine interactions with the Kir6.2[F168E] channel are likely responsible for the observed spiked inward current.

"Foot-in-the-door" effects of intracellular channel blockers have been reported, describing slowing of channel closure due to the presence of a blocker. However, the appearance of a spiked tail, reflecting an increased conductance that arises from blocker interactions with the channel, is fairly unique in the context of channel-blocker interactions. As described in the following sections, this observation implies that blocker interactions must transiently increase channel occupancy of a conductive state, which then returns to an equilibrium level after the blocker vacates the pore. This differs from the typical manifestation of spermine unbinding from inward rectifiers, where a gradual increase in current is observed as a blocker vacates the pore (this unbinding process has sometimes been referred to as "activation") (Lopatin et al.,

![Fig. 1. Intrinsic voltage dependence of Kir6.2[F168E] channels. Kir6.2[F168E] + SUR1 channels were expressed in CosM6 cells and characterized by inside-out patch recordings. Patches were subjected to a set of (A) depolarizing steps (10-mV increments starting from $-150 \text{ mV}$) or (B) repolarizing steps ($-10 \text{ mV}$ increments starting from $+50 \text{ mV}$) to observe the kinetics of channel closure/opening over a wide voltage range. (C) Steady-state current-voltage relationships for wild-type (WT) Kir6.2 ($n = 5$) or Kir6.2[F168E] ($n = 12$) (both coexpressed with SUR1), illustrating the intrinsic inward rectification of Kir6.2[F168E] channels. Currents in each patch were normalized to currents at $-150 \text{ mV}$. Outward currents illustrated in A–C are bona fide channel currents and can be fully blocked by spermine. (D) Current relaxation of Kir6.2[F168E] was fit at each voltage with a single exponential time constant ($n = 12$).](#)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Equilibrium Constant</th>
<th>Forward Rate</th>
<th>Forward $z\delta$</th>
<th>Backward Rate</th>
<th>Backward $z\delta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_g(0 \text{ mV})$</td>
<td>0.2</td>
<td>100 s$^{-1}$</td>
<td>-0.15</td>
<td>500 s$^{-1}$</td>
<td>0.15</td>
</tr>
<tr>
<td>$K_1(0 \text{ mV})$</td>
<td>25 mM$^{-1}$</td>
<td>250,000 s$^{-1}$ mM$^{-1}$</td>
<td>0.225</td>
<td>10,000 s$^{-1}$</td>
<td>-0.225</td>
</tr>
<tr>
<td>$K_2(0 \text{ mV})$</td>
<td>15</td>
<td>250,000 s$^{-1}$</td>
<td>1.2</td>
<td>10,000 s$^{-1}$</td>
<td>-1.2</td>
</tr>
<tr>
<td>$K_3(0 \text{ mV})$</td>
<td>15</td>
<td>250,000 s$^{-1}$</td>
<td>1.2</td>
<td>10,000 s$^{-1}$</td>
<td>-1.2</td>
</tr>
</tbody>
</table>

*These rates cannot be constrained by the experimental data. All that is required for adequate simulation of the experimental data is for these rates to be significantly faster than the intrinsic gating rates (forward and backward rates governing $K_g$).*
We explored the unique effects of spermine on Kir6.2[F168E] channels by examining blocker unbinding kinetics in a series of Kir6.2 pore-lining glutamate substitutions that are sensitive to spermine block (Fig. 3A). Although the kinetics of spermine unbinding vary somewhat between these mutant channels (Kurata et al., 2004), all pore-lining substitutions except F168E exhibit fairly conventional recovery from spermine block. To illustrate this, we parameterized blocker unbinding by calculating a tail recovery ratio (Fig. 3B), corresponding to the ratio of current 0.5 milliseconds posthyperpolarization (to −100 mV, after blockade in 300 μM spermine) to the steady-state current ($t_1/t_2$ in Fig. 3B, inset). Glutamate mutants with a slow monotonic recovery from spermine block (the best example is Kir6.2[N160E]) exhibit a tail recovery ratio less than 1, because very few channels have recovered from block at $t_1$ relative to the steady state ($t_2$). Kir6.2[F168E] channels differ substantially, as their spiked tail causes a large tail recovery ratio ($2.4 \pm 0.2$), reflecting a tail current magnitude at $t_1$ that is considerably larger than at $t_2$ (Fig. 3B). These channels appear to be briefly "propped open" during blocker unbinding and temporarily conduct larger currents than observed at steady state in the absence of spermine. Kir6.2[164E] channels exhibited a borderline tail recovery ratio close to 1, although this seems to reflect very rapid spermine unbinding kinetics rather than a prominent spiked tail as seen for Kir6.2[F168E] channels (compare exemplar currents in Fig. 3A).

Voltage Dependence of Spiked Tail Development Mirrors the Voltage Dependence of Spermine Block. The location of the F168E mutation at the putative ligand-controlled Kir channel gate, and our previous demonstration that Kir6.2 channel gating could be manipulated by targeted perturbations at this location (Khurana et al., 2011), led us to further investigate this unique behavior. To characterize the role of spermine binding in generation of the spiked tail, we measured the prepulse voltage dependence of tail current development (Fig. 4). We pulsed patches through a range of voltages in the absence and presence of spermine, followed by a repolarizing step to −150 mV. In the absence of spermine, Kir6.2[F168E] channels exhibit a monotonic current increase after the voltage step to −150 mV (Fig. 4A), reflecting the intrinsic gating behavior described in Fig. 1A. In the presence of spermine, Kir6.2[F168E] channels exhibit a border-line tail recovery ratio close to 1, although this seems to reflect very rapid spermine unbinding kinetics rather than a prominent spiked tail as seen for Kir6.2[F168E] channels (compare exemplar currents in Fig. 3A).
of spermine (Fig. 4B), intermediate prepulse voltages that do not elicit substantial channel block (e.g., –100-mV trace) generate tail currents that closely resemble currents observed in control conditions. However, more positive voltages, sufficient to cause steeply voltage-dependent polyamine block, markedly change the properties of the tail current and cause development of the large spiked inward tail. This observation indicates that development of the spiked tail requires spermine binding to the channel.

We quantified the development of the spiked tail current by normalizing tail current magnitude (after a step to –150 mV) generated by each prepulse voltage, relative to the steady-state current at –150 mV (Fig. 4C). In control conditions (no spermine), tail current magnitude becomes progressively smaller with depolarized prepulse potentials, again depicting voltage-dependent channel closure at depolarized voltages (as described in Fig. 1). In the presence of spermine, the large inward spiked tail causes significant deviation from the tail currents observed in control (Fig. 4C). At voltages at which spermine blocks the channel, very pronounced inward tail currents become evident. As would be expected for a spermine-mediated effect, the spiked tail currents appear at progressively more negative voltages (i.e., the tail current versus prepulse voltage curve is shifted left) as higher concentrations of spermine are applied (Fig. 4C). We also measured the kinetics of current relaxation after repolarization to numerous tail current voltages. Gating kinetics exhibited weak voltage dependence and were not significantly affected by the spermine concentration (Fig. 4D). These findings demonstrate that entry of spermine into the Kir6.2[F168E] channel generates a transient but significant increase in channel currents that appear briefly upon blocker unbinding.

**Spermine Blockade Promotes Channel Opening in Kir6.2[F168E] Channels.** A second unusual experimental feature that is consistent with paradoxical channel stimulation by spermine is the emergence of biphasic conductance-voltage relationships (Fig. 5). We generated families of step depolarizations from –150 mV to determine steady-state currents at different voltages and spermine concentrations (Fig. 5A). In the presence of spermine, currents elicited by intermediate voltages (below the onset of steeply voltage-dependent block) were significantly higher than currents in control conditions. When normalized to control conditions, this effect manifested as a distinct “hump” in the conductance-voltage relationship that was especially prominent at higher spermine concentrations (Fig. 5B). As more positive voltages were reached, spermine interactions with the channel changed modality, causing blockade rather than potentiation, resulting in complete inhibition of channel currents. This shape of the conductance-voltage relationship differs significantly from the sigmoidal voltage dependence typically observed for polyamine block of inward rectifiers (Lopatin et al., 1995; Lu, 2004), and cannot be well described by a Boltzmann function (see kinetic model later in text). Together with the development of large inward tails after the onset of spermine block (Fig. 4), these...
stimulation but at negative holding potentials (Fig. 6, B and C) current same patch, using the same spermine concentration jumps, spermine from the bathing solution. In contrast, in the very of spermine, followed by a slow recovery upon removal of rapid current inhibition is observed shortly after application mM spermine (Fig. 6). At positive holding potentials (Fig. 6A), illustrate the dual nature of spermine effects on Kir6.2\[F168E\] channels exhibit unusual biphasic relationships, reflecting results indicate that spermine occupancy in Kir6.2\[F168E\] channels generates a unique combination of channel stimulation superimposed on the canonical mechanism of spermine block.

This combination of channel stimulation and blockade by the same compound was made especially apparent with rapid solution jumps. At various holding potentials, we rapidly perfused Kir6.2\[F168E\] inside-out patches with 300 μM spermine (Fig. 6). At positive holding potentials (Fig. 6A), rapid current inhibition is observed shortly after application of spermine, followed by a slow recovery upon removal of spermine from the bathing solution. In contrast, in the very same patch, using the same spermine concentration jumps, but at negative holding potentials (Fig. 6, B and C) current stimulation is observed upon spermine application. In combination with the normalized steady-state G-V curves, these data illustrate the dual nature of spermine effects on Kir6.2\[F168E\] channels.

A Pharmacophore of the Spiked Tail Phenotype. We tested numerous spermine analogs to identify essential features that enable blockers to transiently prop open the Kir6.2\[F168E\] pore (Fig. 7). We examined the development of tails upon repolarization from +50 mV in the presence of various blockers (with differing alkylation and numbers of amines). Blockers with two or more amines (e.g., 1,10-diaminododecane, spermidine, and spermine) were sufficient to generate spiked tails reflecting a propped-open pore (Fig. 7, A–C). However, tail currents observed in the presence of spermidine and 1,10-diaminododecane were not as pronounced as the tail currents observed in spermine. In contrast, comparably long blockers with a single amine (e.g., 1-aminodecane) were unable to generate spiked tail currents (Fig. 7D). The chemical features of the terminal amines are also important for generating the spiked tail effect. We tested alkylated derivatives of 1,10-diaminododecane (with either methylated or ethylated terminal amines), but neither was able to reproduce the effects observed for spermine and 1,10-diaminododecane (Fig. 7E). Commonly used quaternary ammonium blockers [TEA\[+\], tetrabutylammonium (TBA\[+\])] did not recapitulate the effect of spermine (not shown). Overall, these observations indicate that blockers with at least two amines (nonalkylated) are sufficient to prop open the channel and generate the spiked tail effect.

These experiments also highlight that appearance of a spiked tail depends on very rapid blocker unbinding. For example, for blockers with slow unbinding rates (such as bis-TEA-C10), the slow rate of current recovery prevents the appearance of a rapid spiked tail (Fig. 7E). In this scenario, it is unclear whether the blocker can potentiates the channel or not, because the very slow blocker unbinding masks any current enhancement that might occur. Along the same line of thinking, an important observation is that the rate of current relaxation after hyperpolarization is very mildly affected by spermine over the range of concentrations used (Fig. 4D) and closely mirrors relaxation rates in zero spermine (although with opposite polarity). Thus, we suspect a plausible sequence of events upon hyperpolarization is that spermine unbinds rapidly from the pore, leaving an increased number of channels open, which then equilibrate to their steady-state open probability with rates similar to those observed in blocker-free conditions (Fig. 1).

Kinetic Model for Spermine Potentiation of Kir6.2\[F168E\] Channels. We aimed to generate a kinetic model that would account for the biphasic conductance-voltage relationships associated with spermine block (Fig. 5), development of spiked tails after the onset of spermine block (Fig. 4C), and the relatively invariant kinetics of channel closure after spermine unbinding (Fig. 4D). We hypothesized a kinetic model described schematically in Fig. 8A. In the model, the C↔O equilibrium (\(K_{eq}\)) describes a voltage-dependent gating mechanism observed in spermine-free conditions (see Fig. 1). In the presence of spermine, two binding steps are required to recapitulate the observed data. First, the O\(\ast\) state is accessed with relatively weak voltage dependence and preserves a significant K\(+\) conductance. Next, the system proceeds to a deep blocked state OB, with steep voltage dependence. Model states and simulation are described in Materials and Methods.

This simple kinetic model reasonably describes important features of the experimental data, including the intrinsic voltage dependence of channel gating (Fig. 8B), biphasic conductance-voltage relationships for spermine block at multiple concentrations (Fig. 8C), and the voltage dependence of spiked tails at multiple spermine concentrations (Fig. 8D). It is worth highlighting several aspects of the model that are essential to predict spermine-dependent potentiation and spiked tails:
1. Channels must exhibit a fairly low open probability in the absence of ligand (governed by $K_g$). This is most apparent when one considers the alternative possibility—if channels have a high intrinsic open probability, there is very little dynamic range for potentiation.

2. Channel interactions with the blocker must be able to promote a (at least partially) conductive state, before achieving the deeply blocked, nonconductive spermine-bound state typically associated with steep inward rectification. The absence of an intermediate conductive state will inevitably cause reduction of conductance as spermine is driven into the pore. We achieved good fits of the model with a conductance of the intermediate spermine-bound state ($O^*$) of 0.9 (relative to the open state).

3. Blocker unbinding must occur much more rapidly than channel closure. This enables the rapid rise in current upon hyperpolarization (as the blocker unbinds quickly from the deep site OB, and channels repopulate the $O$ and $O^*$ states), followed by a slower gating process governed by the $C\leftrightarrow O$ equilibrium with kinetics that mirror those observed in the absence of spermine. The consequences of slow blocker unbinding are highlighted in Fig. 9, recapitulating the effects of polyamine analogs such as bis-TEA-C10 (Fig. 7, D and E). In particular, as blocker unbinding kinetics are slowed, the appearance of the spiked tail disappears. This occurs because repopulation of $O$ and $O^*$ states from the OB state happens more slowly than the $C\leftrightarrow O$ equilibrium. Therefore, there is no transient increase...
Discussion

This study describes a unique paradoxical interaction between spermine and Kir6.2[F168E] channels, with a pore-lining glutamate substituted in the bundle crossing region. Glutamate substitution at most pore-lining positions in Kir6.2 is sufficient to confer high-affinity polyamine block, with typical monotonic unbinding kinetics (Kurata et al., 2004). However, glutamate substitution at F168, identified in Kir channel crystal structures as the narrowest constriction in the bundle crossing region (Kuo et al., 2003; Whorton and MacKinnon, 2011), results in channels with a biphasic response to spermine. These mutants exhibit a combination of potentiation of channel activity by spermine at intermediate voltages but blockade at depolarized voltages. This unique observation indicates that spermine and other polyamine blockers can exert significantly different effects (potentiation versus blockade) at different binding sites in the Kir channel pore, and also provides an example of a targeted modulation of the bundle crossing gating region of a potassium channel.

Mechanistic Basis for the Spiked Tail Phenotype.

The most obvious unique feature of spermine interactions with Kir6.2[F168E] channels is the development of large inward spiked tails upon repolarization (Fig. 4B). Based on our kinetic model, we envision that these large tails originate from very rapid unbinding of spermine, allowing for transient accumulation of channels in one or more open states (O and O* in the kinetic model), followed by a slower equilibration with the closed state of the channel (Kg ↔ C equilibrium in Fig. 8A). This accumulation of open channels can be detected when the blocker rapidly dissociates from the pore upon hyperpolarization, and implies that presence of spermine in the pore stabilizes an open conformation of Kir6.2[F168E] channels. This outcome differs considerably from conventional strong inward rectifiers (e.g., Kir2.1) and Kir6.2 channels that carry a negative charge at the conventional rectification controller.

in current upon unbinding of the blocker from the deep blocked state.
interactions with spermine that at position 168, in the vicinity of the bundle crossing, enables a time-dependent recovery of current upon hyperpolarization (Fig. 3A). Furthermore, in Kir6.2[N160D] channels, ATP application can readily close these channels and trap spermine in the inner cavity (Phillips and Nichols, 2003). In contrast, we suspect that introduction of a glutamate position (N160 in Kir6.2) in most cases, blocker unbinding manifests as a time-dependent recovery of current upon hyperpolarization (Fig. 3A). ATP application can readily close these channels and trap spermine in the inner cavity (Phillips and Nichols, 2003). In contrast, we suspect that introduction of a glutamate position (N160 in Kir6.2) channels. This effect is proposed to arise from deprotonation of the F168E residue and mutual repulsion of the M2 helices, suggesting an engineered/targeted mechanism to directly manipulate the bundle crossing gate (Khurana et al., 2011; Li et al., 2013). In the present study, we describe an additional modality for regulation of Kir6.2[F168E] channels, involving pharmacological modulation of this gating region by polyamine blockers. Taken together, these findings point toward the narrow constriction in the Kir6.2 bundle crossing region as a locus for control of channel gating and a target for extrinsic modulation of channel function. Although virtually all crystal structures of Kv and Kir channels have indicated a central importance of the bundle crossing region as the primary channel gate, other functional studies have suggested that Kir channels are gated by conformations in the selectivity filter region (Claydon et al., 2003; Proks et al., 2003; Xiao et al., 2003; Long et al., 2007; Hansen et al., 2011; Whorton and MacKinnon, 2011; Bavro et al., 2012). Our ability to modulate Kir6.2 channel gating with targeted disruption of the bundle crossing is consistent with this channel motif serving as a primary controller of the channel gating state.

Unexpected Functional Consequences of Shifting the Rectification Controller Position. A final noteworthy aspect of the data is the appearance of intrinsic voltage dependence in Kir6.2[F168E] channels (Fig. 1). Although inside-out patches sometimes contain residual polyamines causing the appearance of inward rectification, several observations confirm that the apparently intrinsic rectification features of Kir6.2[F168E] are indeed independent of contaminating polyamines. Firstly, it is readily apparent when Kir6.2[F168E] channels become blocked by spermine, because they generate clear spiked tail currents. However, these spiked tails are absent in control conditions (despite obvious rectification of currents) so long as the patch is well perfused (Fig. 1). Secondly, the voltage dependence of spermine block is considerably steeper than the voltage dependence of rectification observed in Kir6.2[F168E] in spermine-free conditions (Fig. 1). This is the second instance of intrinsic (i.e., not polyamine-mediated) voltage dependence that has emerged in Kir6.2 channel point mutants, despite the absence of a canonical voltage-sensing domain (Kurata et al., 2004). Specifically, we have reported intrinsic outward rectification in Kir6.2[L157E] channels (Kurata et al., 2010a), and now
intrinsic inward rectification in Kir6.2[Pi63E] channels. At present, it is unclear whether the mechanisms underlying voltage dependence in these two mutants are related. Although it is easy to speculate that charged residues in the transmembrane domain could act as voltage-sensing resi-
dues, this fails to explain why charged side chains at other nearby positions (such as the rectification controller residue) do not generate intrinsic voltage-dependent behavior.

**Conclusion.** We have described a unique biphasic interaction of spermine with a pore-lining glutamate-substituted Kir6.2 channel. Our findings illustrate that within a long channel pore, a compound can act as both a potentiator and a blocker, due to voltage-dependent interactions with multiple binding sites. These results also demonstrate in principle that channel modulators can be targeted toward the bundle crossing region to promote channel opening.

**Authorship Contributions**

**Participated in research design:** Kurata.

**Conducted experiments:** Vilin, Kim, Nunez, Kurata.

**Contributed new reagents or analytic tools:** Dake.

**Performed data analysis:** Vilin, Nunez, Kurata.

**Wrote or contributed to the writing of the manuscript:** Kurata, Nunez.

**References**


References