Paradoxical activation of a Kir 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, University of British Columbia, Vancouver, BC, Canada (YYV, JIN, RYK, HTK)

Department of Chemistry, University of British Columbia, Vancouver, BC, Canada (GRD)
Running title: Engineered blocker-gate interaction in a potassium channel

To whom correspondence should be addressed: Harley T. Kurata, Room 2.352, 2350 Health Sciences Mall, Vancouver, BC, V6T 1Z3, e-mail: harley.kurata@ubc.ca; phone: 604-827-3634; fax: 604-822-6012; web: http://crg.ubc.ca/kurata

Number of text pages: 34
Number of tables: 1
Number of figures: 9
Number of references: 38
Abstract word count: 239
Introduction word count: 496
Discussion word count: 1222

Non-standard abbreviations:
Kir: Inwardly-rectifying potassium channel
Kv: Voltage-gated potassium channel
hERG: Human ether-a-go-go related gene
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 Mg$^{2+}$. 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 channels are regulated by diverse mechanisms including voltage-dependent block by Mg$^{2+}$ 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 physiological 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; Armstrong, 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 unfavourable 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 in the inner cavity, often referred to as the ‘rectification controller’ residue (Nichols and Lopatin, 1997; Lopatin et al., 1994; Wible et al., 1994). Upon membrane hyperpolarization, blocker unbinding from the ‘rectification controller’ region is favoured, 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 (K\textsubscript{ATP}) complex (Shyng and Nichols, 1997; Inagaki et al., 1995). Specifically, introduction of acidic residues at certain positions in the Kir6.2 bundle crossing enables polyamines to paradoxically ‘prop’ channels open. 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 PCR methods, and verified by Sanger sequencing (Genewiz, Inc).

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 GFP cDNA, using Fugene 6 (Roche) or Lipofectamine 2000 (Life Technologies) 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). 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 K$_2$HPO$_4$, pH 7.3. Spermine and most polyamine analogs were purchased from Sigma-Aldrich/FLUKA Chemicals, with the exception of bis-1,10-triethylaminodecane (‘bis-TEA-C10’) which was synthesized on site at UBC.

Kinetic model of spermine 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 Figure 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 $K_x$ and effective valence $z\delta_x$ in the form $K_x(V) = K_x(0 \text{ mV})e^{z\delta_FV/RT}$. In the model described in Scheme I, fractional conductance changes with voltage are described by the following equation:

$$\frac{I}{I_{(0 \text{ spm})}} = \frac{O + O^*c}{C + O + O^* + OB} = \frac{K_g + K_gK_1c}{1 + K_g + K_gK_1 + K_gK_1K_2}$$
where $K_g$, $K_1$, and $K_2$ 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 µM, 100 µM, 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 in 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 K\textsubscript{ATP} 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 polyamine or Mg\textsuperscript{2+} 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., 2010a; Kurata et al., 2004). 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] is apparent with steps to negative voltages, while decay/closure is observed at depolarized potentials (Fig 1A,B), resulting in a pronounced inwardly-rectifying current voltage relationship (Fig. 1C). We describe this rectification as ‘intrinsic’ to this channel mutant (ie. 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 (Figs. 1A,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 (\(z\bar{\delta} \sim 0.3\)). 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 hyperpolarization-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 (eg. from +50 mV to more negative voltages) of inside-out Kir6.2[F168E] patches generate a monotonic increase of inward current (Fig 1B, 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_{on-cell}/I_{excised} = 0.39±0.06 at-150 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 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, 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 and Nichols, 1996; Lopatin et al., 1994; Shin and Lu, 2005; Kurata et al., 2010b).

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 ms post-hyperpolarization (to -100 mV, after blockade in 300 µM spermine) to the steady-state current (t1/t2 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 t1 relative to the steady state (t2).
Kir6.2[F168E] channels differ substantially, as their ‘spiked’ tail causes a large tail recovery ratio (2.4±0.2), reflecting a tail current magnitude at t₁ that is considerably larger than at t₂ (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 (Fig. 4B), intermediate prepulse voltages that do not elicit substantial channel block (eg. -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 where 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 (ie. the tail current vs. 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 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 or 1 mM spermine (Figure 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. 6B,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 2 or more amines (eg. 1,10-diaminodecane, spermidine, spermine) were sufficient to generate ‘spiked’ tails reflecting a propped open pore (Fig. 7A,B,C). However, tail currents observed in the presence of spermidine and 1,10-diaminodecane were not as pronounced as the tail currents observed in spermine. In contrast, comparably long blockers with a single amine (eg. 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-diaminodecane (with either methylated or ethylated terminal amines) but neither was able to reproduce the effects observed for spermine and 1,10-diaminodecane (Fig. 7E). Commonly used quaternary ammonium blockers (TEA⁺, TBA⁺) did not recapitulate the effect of spermine (not shown). Overall, these observations indicate that blockers with at least 2 amines (non-alkylated) 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 potentiate 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 both 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 Figure 8A. In the model, the C↔O equilibrium (Kg) 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* 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 non-conductive 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↔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. 7D,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↔O equilibrium. Therefore, there is no transient increase in current upon unbinding of the blocker from the deep blocked state.
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 (C↔O 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’ position (N160 in Kir6.2). In most cases, blocker unbinding manifests as 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 at position 168, in the vicinity of the bundle crossing, enables interactions with spermine that prevent channel closure.

Overall, the ‘spiked’ tail observed upon spermine unbinding reflects that at depolarized voltages, when spermine has occupied the pore, channels are biased towards the open (spermine bound) state. From a model perspective, this origin of the large inward ‘spiked’ tails is analogous to the mechanism of tail current development upon repolarization in hERG potassium channels. Channels must first recover rapidly from a non-conducting state (OB in our model, or the inactivated state in typical hERG gating schemes) to an open state. This is followed by much slower equilibration between the open state and a closed channel state (Smith et al., 1996; Trudeau et al., 1995). In both cases, accumulation of channels in the open state results in a transient increase in conductance upon repolarization (Fig. 8B,D).

Mechanistic basis for biphasic conductance-voltage relationships

While accumulation of channels in the conductive open state (in a hERG-like kinetic scheme) can account for the observation of tail current potentiation, it is not sufficient to describe the biphasic conductance-voltage relationships generated in response to spermine (Fig. 5). To account for this experimental observation with a simple model, two conductive states are required (one that is spermine bound, O*, and one that is not, O). To account for the observation of spermine enhancement of Kir6.2[F168E] currents (Fig. 5), the model predicts...
enhanced occupancy of partially conductive spermine bound states at intermediate voltages (Fig. 8). An alternative scenario that should be considered is that spermine could interact with the closed state and promote channel opening to a spermine blocked state. The increase in K⁺ permeability of the open-blocked channel would then cause rapid spermine unbinding (due to interactions between K⁺ ions and spermine in the pore). In this scenario, one need not postulate a conductive spermine bound state, however, such a model is very difficult to build and constrain without a more concrete description of K⁺ permeation. In either case, the conceptual basis of the model is the same: interaction of spermine with the Kir6.2[F168E] channel at intermediate voltages must bias the channel towards the open state.

Structural implications of channel potentiation by spermine

The F168 position appears to be an essential locus for gating of Kir6.2 channels. Recent studies in our lab have demonstrated that intracellular alkalization can dramatically increase open probability of Kir6.2[F168E] 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). 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 towards 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. While 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 (Hansen et al., 2011; Whorton and MacKinnon, 2011; Long et al., 2007; Proks et al., 2003; Bavro
et al., 2012; Xiao et al., 2003; Claydon et al., 2003). 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 (ie. 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[F168E] channels. At present, it is unclear whether the mechanisms underlying voltage-dependence in these two mutants are related. While it is easy to speculate that charged residues in the transmembrane domain could act as voltage sensing residues, 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 towards 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.

John-Jose Nunez and Yury Vilin made equal contributions to the manuscript.
Reference List


MOL #86603


**FOOTNOTES**

†This work was supported by a CIHR operating grant [MOP-97998] to HTK, and NSERC Discovery Grants to HTK and GRD. HTK is a Heart and Stroke Foundation of Canada New Investigator, and a Michael Smith Foundation for Health Research Scholar. JJN was supported by an NSERC Undergraduate Student Research Award. RYK was supported by the UBC Faculty of Medicine Summer Student Research Program.

††Please address reprint requests to: Harley T. Kurata, Room 2.352, 2350 Health Sciences Mall, Vancouver, BC, Canada, V6T 1Z3, e-mail: harley.kurata@ubc.ca.
**FIGURE LEGENDS**

**FIGURE 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 mV) or (B) repolarizing steps (-10 mV increments starting from +50 mV), to observe the kinetics of channel closure/opening over a wide voltage range. (C) Steady-state current-voltage relationships for WT Kir6.2 (n=5) or Kir6.2[F168E] (n=12) (both co-expressed with SUR1), illustrating the intrinsic inward rectification of Kir6.2[F168E] channels. Currents in each patch were normalized to currents at -150 mV. Outward currents illustrated in panels 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).

**FIGURE 2. Extrinsic control of Kir6.2[F168E] tail current kinetics.** (A) Exemplar on-cell recording of Kir6.2[F168E] channels illustrating a ‘spiked’ tail upon repolarization to -150 mV. (B) Upon patch excision, identical voltage protocols fail to elicit the ‘spiked’ tail phenotype (note also the appearance of outward current due to washout of endogenous polyamines and Mg²⁺). Similar data was collected for 10 patches, but is consistently observed in all patches tested.

**FIGURE 3. Position-dependence of ‘spiked’ tail current development.** (A) Multiple pore-lining positions in Kir6.2 were substituted with glutamate, and spermine unbinding kinetics were characterized with a series of voltage steps (-10 mV increments from +50 mV, in 300 µM spermine). The structure illustrates pore-lining positions tested, on a molecular model of Kir6.2 constructed from the crystal structure of Kir2.2 (PDB: 3SPI). (B) Tail recovery ratios were calculated for spermine unbinding in each glutamate substituted mutant (n= 10 (129E), 8...
(157E), 6 (160E), 9 (164E), 16 (168E)). The inset panel illustrates the tail recovery ratio calculation, a ratio of current at $t_1$ (0.5 ms after the repolarizing voltage step to $-100$ mV), and $t_2$ (the steady state current at $-100$ mV). Kir6.2[F168E] channels had substantially larger tail recovery ratios than all other glutamate substituted mutants.

**FIGURE 4. Spermine blockade elicits development of the ‘spiked’ tail phenotype.** (A) Exemplar tail currents from Kir6.2[F168E] channels at $-150$ mV. Patches expressing Kir6.2[F168E] channels were pulsed through a range of voltages, followed by a repolarizing step to $-150$ mV. In the absence of spermine, prepulses to positive voltages elicit small tail currents due to the intrinsic rectification observed at depolarized voltage. (B) Exemplar ‘spiked’ tail currents from Kir6.2[F168E] channels elicited in the presence of 100 µM spermine, with identical voltage steps as in (A). Depolarized voltages that elicit spermine block cause development of unconventional large inward ‘spiked’ tail currents. (C) Tail currents (at $-150$ mV) elicited by different prepulse voltages were normalized to the steady state current magnitude at $-150$ mV ($n=8$ per concentration). (D) Tail current kinetics elicited through a range of repolarizing voltages were fit with a single exponential time constant, at different spermine concentrations as indicated ($n=8$ per concentration).

**FIGURE 5. Biphasic conductance-voltage relationships of Kir6.2[F168E] channels in the presence of spermine.** (A) Sample currents from a patch expressing Kir6.2[F168E] channels in control conditions and in the presence of 300 µM spermine, as indicated. Note the development of ‘spiked’ inward tails in the presence of spermine. (B) Currents in various spermine concentrations were normalized to control currents to generate conductance voltage relationships. Kir6.2[F168E] channels exhibit unusual biphasic relationships, reflecting
potentiation of currents by spermine at intermediate voltages (n=8 per concentration). No curve fitting is presented in this figure, data points are joined by straight lines. Biphasic conductance voltage relationships are fit with a kinetic model in Fig. 8.

**FIGURE 6. Rapid spermine jumps illustrate bimodal effects in Kir6.2[F168E] channels.** Inside-out patches expressing Kir6.2[F168E] channels were exposed to various spermine concentrations with a multi-barreled rapid perfusion apparatus (panel A, inset), at holding potentials of (A) +30 mV, (B) -50 mV, and (C) -80 mV. At negative voltages (B,C), spermine acts as a channel potentiator, increasing macroscopic currents. At positive voltages (A), spermine more closely resembles its canonical role as a Kir channel inhibitor. Similar data were recorded from 6 patches.

**FIGURE 7. Effects of various polyamine analogs on the development of ‘spiked’ inward tail currents.** (A-D) Tail currents from patches expressing Kir6.2[F168E] channels were elicited with voltage steps in -10 mV increments between +50 and -100 mV, in the presence of various polyamine analogs, as indicated (4-6 patches were tested with each blocker). (E) Structures of polyamine analogs tested in panels A-D.

**FIGURE 8. Kinetic model describing voltage-dependent interactions of spermine with Kir6.2[F168E] channels.** (A) Cartoon schematic of the kinetic model used to describe currents and polyamine interactions with Kir6.2[F168E] channels. The model comprises a closed state (C) in a shallow voltage-dependent equilibrium with the open state O. In the presence of spermine, a potentiated open state O* is accessed with weak voltage-dependence, and a fully
blocked open state OB is accessed with much steeper voltage dependence. Parameters for simulations are described in Table 1. (B) Currents simulated with the Kir6.2[F168E] model using stimulus voltages identical to Fig. 5, with a holding voltage of -150 mV, and voltage jumps in 10 mV increments up to +50 mV. (C) Simulated conductance-voltage relationships have been superimposed on the raw data from Fig. 5, at 3 different spermine concentrations. (D) The Kir6.2[F168E] model was also used to simulate tail current magnitudes after various prepulses (normalized to the -150mV prepulse), as described in Fig. 4. Model predictions are solid lines superimposed on the raw data.

**FIGURE 9. Perturbations of the Kir6.2[F168E] kinetic model duplicate the tail current effects of much slower channel blockers.** In Scheme I, we have highlighted the O*↔OB transition, using simulated currents to illustrate the effect of changing the kinetics of this transition. Observation of fast ‘spiked’ tails (left ‘Fast unbinding’ panel), as seen in the presence of spermine, requires rapid dissociation of the blocker from OB→O*. Slowing of this dissociation rate generates slower blocker unbinding, masking the re-equilibration between O and C states that underlies the falling phase of the ‘spiked’ tail (resulting in slow tail current kinetics, right panel).
**TABLE 1. Equilibrium and kinetic parameters for simulation of Kir6.2[F168E] currents**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Eq. 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</td>
<td>$^{†}$250000 s$^{-1}$mM$^{-1}$</td>
<td>0.225</td>
<td>$^{†}$10000 s$^{-1}$</td>
<td>-0.225</td>
</tr>
<tr>
<td>$K_2(0 \text{ mV})$</td>
<td>25</td>
<td>$^{†}$250000 s$^{-1}$</td>
<td>1.2</td>
<td>$^{†}$10000 s$^{-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 $K_g$).
Figure 1

(A) 'Depolarizing steps'

(B) 'Repolarizing steps'

(C) 

(D) 

Figure 1
Figure 2

(A) Kir6.2[F168E] on-cell

(B) Kir6.2[F168E] inside-out

+50 mV

-150 mV

1 nA

50 ms

*
Figure 4
Figure 5

Panel A shows the control tracing and the tracing with 300 μM spermine. The voltage values are indicated by the symbols: *-50 mV and ♦-80 mV. The scale for the current is 1 nA and the time scale is 250 ms.

Panel B presents a graph of normalized current against voltage (mV). The curves represent the responses at different spermine concentrations: 30 μM (open circles), 100 μM (gray circles), and 300 μM (black circles). The normalized current is plotted on the y-axis and the voltage on the x-axis.
Figure 6

A + 30 mV

300 μM spm  1 mM spm

 inhibition

100 pA

5 s

Inside-out patch

Spermine solutions

B -50 mV

300 μM spm  1 mM spm

400 pA

5 s

C -80 mV

300 μM spm  1 mM spm

800 pA

5 s

potentiation
Figure 7

(A) 1 mM spermine
(B) 1 mM spermidine
(C) 1 mM 1,10-diaminodecane
(D) 1 mM 1-aminodecane
(E) 1 mM bis-TEA-C10