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Recommended Citation

Kucheryavykh, Yuri V.; Pearson, Wade L.; Kurata, Harley T.; Eaton, Misty J.; Skatchkov, Serguei N.; and Nichols, Colin G., "Polyamine permeation and rectification of kir4.1 channels." *Channels*. 1, 3. 172-178. (2007).

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Research Paper

Polyamine Permeation and Rectification of Kir4.1 Channels

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Original manuscript submitted: 03/20/07

Revised manuscript submitted: 05/02/07

Manuscript accepted: 05/03/07

This manuscript was previously published online as a *Channels* E-publication.

KEY WORDS

glia; Kir4.1; spermine; spermidine; philanthotoxin; polyamine; voltage dependence

ACKNOWLEDGEMENTS

This work was supported by NIH NINDS CNS S11 NS48201 (to Misty J. Eaton) and NIH NINDS and NCRR SNRP NS39408 (to Serguei N. Skatchkov). Harley T. Kurata is supported by a Fellowship from CIHR.

ABSTRACT

Inward rectifier K⁺ (Kir) channels are expressed in multiple neuronal and glial cells. Recent studies have equated certain properties of exogenously expressed Kir4.1 channels with those of native K⁺ currents in brain cells, as well as demonstrating the expression of Kir4.1 subunits in these tissues. There are nagging problems however with assigning native currents to Kir4.1 channels. One major concern is that in many native tissues, the putatively correlated currents show much weaker rectification than typically reported for cloned Kir4.1 channels. We have now examined the polyamine-dependence of Kir4.1 channels expressed at high density in Cosm6 cells, using inside-out membrane patches. The experiments reveal a complex and variable rectification that can help explain the variability reported for candidate Kir4.1 currents in native cells. Most importantly, rectification seems to be incomplete, even at high polyamine concentrations. In excised membrane patches, with high levels of expression, and high concentrations of spermine, there is ~15% residual conductance that is insensitive to spermine. From a biophysical perspective, this is a striking finding, and indicates either that a bound spermine fails to completely block permeation or that significant spermine permeation (i.e. 'punchthrough') is occurring. To examine this further, we have examined block by philanthotoxin (PhTx, essentially spermine with a bulky tail). PhTx block, while less potent, is more complete than spermine block. This leads us to propose that spermine 'punchthrough' may be significant in Kir4 channels, and that this may be a major contributor to the weak rectification observed under physiological conditions.

INTRODUCTION

Inward rectifier K⁺ (Kir) channels are so called because of their properties of inward rectification—reduction of conductance with depolarization. This property is striking in certain family members, especially those encoded by the Kir2.x and Kir3.x sub-family members.¹ Others, for example Kir1.x and Kir6.x sub-family members, normally show very little inward rectification. Channels formed by Kir4.x sub-family members were initially reported to show essentially strong rectification,² this rectification being conferred by polyamines, and dependent on the presence of a negative charge in the second transmembrane helix,² just as in the Kir2.x and Kir3.x sub-family members. Kir4.1 (KCNJ10) is an important component of brain Kir channels, and mutations are associated with deafness, epilepsy and seizure³⁻⁷ in animals and humans. Recent studies have demonstrated the expression of Kir4.1 subunits in various brain cells, including cortical astrocytes, retinal Müller cells, spinal cord and brainstem,⁸⁻¹⁴ and have also attempted to equate certain properties of exogenously expressed Kir4.1 currents with those of native K currents in these tissues.^{10,13}

There are nagging problems however with assigning native currents to Kir4.x channels. One major concern is that in many native tissues, the putatively correlated currents show much weaker rectification than reported for cloned Kir4.1 channels,¹⁵ such as variable or multi-phasic rectification in Müller glial cells¹⁶⁻²⁰ and spinal cord cells,¹³ and with the strength and voltage-dependence decreasing as external [K⁺] (K_{out}) is reduced. However, there have been relatively few studies of the effects of K_{out} on Kir4 channel rectification under controlled conditions in membrane patches, and both the underlying mechanism, and implications for the physiology of Kir4-expressing cells has not been studied. A major caveat to any whole-cell voltage-clamp experiments is the unreliability of controlling intracellular conditions. To circumvent this problem, we have now examined the polyamine and divalent ion-dependence of Kir4.1 channels expressed at high density in

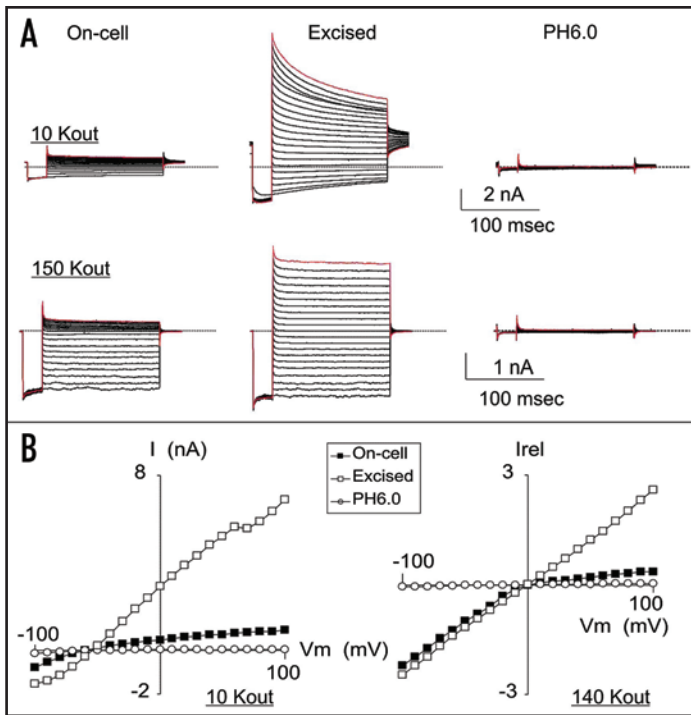


Figure 1. (A) Representative patch-clamp recordings of Kir4.1 currents expressed in Cosm6 cells, in response to voltage steps from 0 (hold) to voltages between -100 and +100 mV in the on-cell configuration (left), after excision into 150 mM Kout, pH 7.0 (center) and after switching to bath pH 6.0 (right). Kout = 10 mM (above) or 150 mM (below). (B) Steady state current-voltage relationships from (A). Rectification is quite weak at low (normal) Kout. At high Kout, rectification is stronger, but still incomplete at positive voltages. Similar results were obtained in five other patches.

Cosm6 cells, using inside-out membrane patches. The experiments reveal complexities of rectification that can help explain the variability reported for Kir4.x candidate currents in native cells and that may be explained by a relatively high rate of polyamine 'punch-through' to the extracellular side of the membrane in this channel.

MATERIAL AND METHODS

Experimental methods are described in detail in previous publications.²¹⁻²³ Briefly, COSm6 cells were transfected with pCMV-Kir4.1 (with insertion of Kir2.1 trafficking sequence²⁴ "NSFCYENEVALT" immediately after residue P272, to increase expression density). Patch-clamp experiments were made at room temperature, in a chamber that allowed the solution bathing the exposed surface of the isolated patch to be changed rapidly. Data were normally filtered at 0.5–2 kHz, signals were digitized at 5 kHz and stored directly on computer hard drive using Clampex software (Axon Inc.). The standard pipette (extracellular) and bath (cytoplasmic) solution used in these experiments had the following composition: 150 mM KCl, 1 mM K-EGTA, 1 mM K-EDTA, 4 mM K₂HPO₄, pH 7.²⁵ All polyamines and diamines were purchased from Fluka AG. Off-line analysis was performed using Fetchan, pSTAT (Axon Inc.) and Microsoft Excel programs. Wherever possible, data are presented as mean ± s.e.m. (standard error of the mean). Microsoft Solver was used to fit data by least-square algorithm. Currents in the presence of polyamine were expressed relative to the current in the

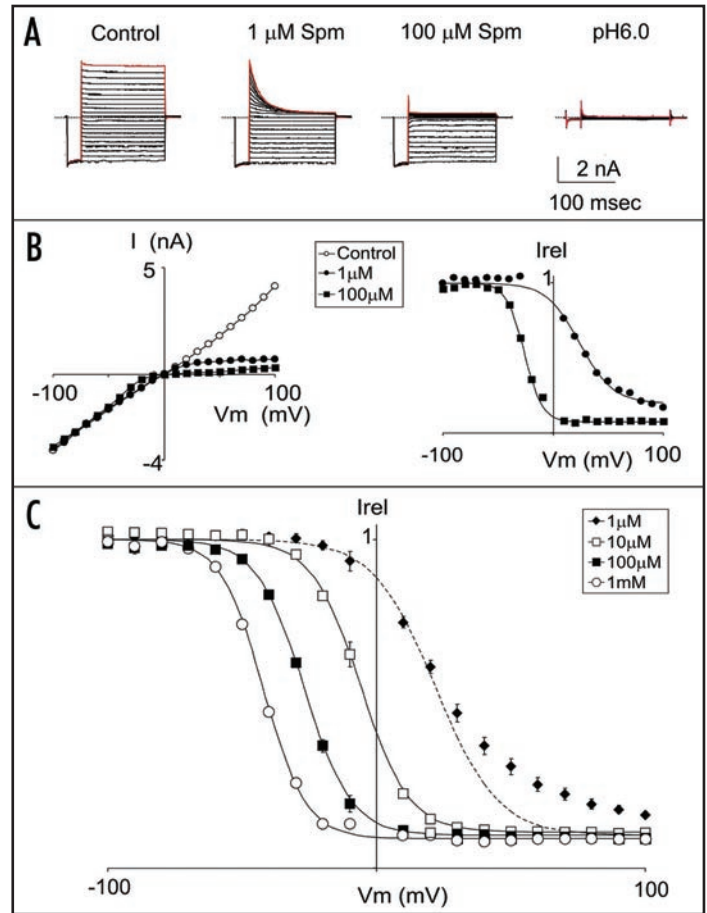


Figure 2. (A) Representative excised patch-clamp recordings of Kir4.1 with 150 mM K⁺ in the pipette, in response to voltage steps from 0 (hold) to voltages between -100 and +100 mV, with spermine at concentrations indicated, or following exposure to pH 6.0. (B) Steady state current-voltage relationships from (A) (left) and Irel-voltage relationship (right), after subtraction of pH6.0 currents. (Note: pH6.0 subtraction was routinely used in subsequent experiments in Figures 2–5, but for clarity, original records are not shown). (C) Mean Irel-voltage relationships from n = 5 experiments as in (A), fitted with eqn1 (z = 2.2, 2.8, 2.9, 3.2, V_{1/2} = +22, -6, -27, -42 mV, and offset = 0.10, 0.11, 0.10 and 0.09, for spermine = 1, 10, 100 μM and 1 mM, respectively, mean ± s.e.m., n = 5–8).

absence of polyamines (Irel). Irel-voltage relationships were fit by a Boltzmann function plus offset (Figs. 2, 4 and 5):

$$I_{rel} = (1 - \text{offset}) * (1 - 1/[1 + \exp(zF/RT) * (V_m - V_{1/2})]) + \text{offset} \quad (\text{Eqn. 1})$$

where R, T and F have their usual meanings, z is the effective valence of block, V_m and V_{1/2} are the membrane voltage and the voltage at half maximal block, or were fit by the sum of two Boltzmann functions (Fig. 3):

$$I_{rel} = \text{Amp1} * (1 - 1/[1 + \exp(z_1/RT) * (V_m - V_{1/2,1})]) + (1 - \text{Amp1}) * (1 - 1/[1 + \exp(z_2/RT) * (V_m - V_{1/2,2})]) \quad (\text{Eqn. 2})$$

where Amp1 is the fractional amplitude of the high affinity component, z_x and V_{1/2,x} are the effective valence of block, and voltage at half maximal block for each component.

Figure 3. (A) Representative excised patch-clamp recordings of Kir4.1 with 150 mM K^+ in the pipette, in response to voltage steps from 0 (hold) to voltages between -100 and $+100$ mV, with spermidine at concentrations indicated. (B) Steady state current-voltage relationships from (A). (C) Relative Irel-voltage relationships from (B). Relationships are fitted with the sum of two Boltzmann functions (Eqn. 2) ($z_1 = 2.3, 2.2, 2.3, z_2 = 1.2, 0.8, 1.2, V_{1/2,1} = +37, -12, -20$ mV, $V_{1/2,2} = +150, +65, +44$ mV, and $Amp1 = 0.74, 0.74, 0.70$, for spermidine = 10, 100 μ M and 1 mM, respectively. Dashed line indicates best fit of Eqn. 1 to 1 mM spermidine data.

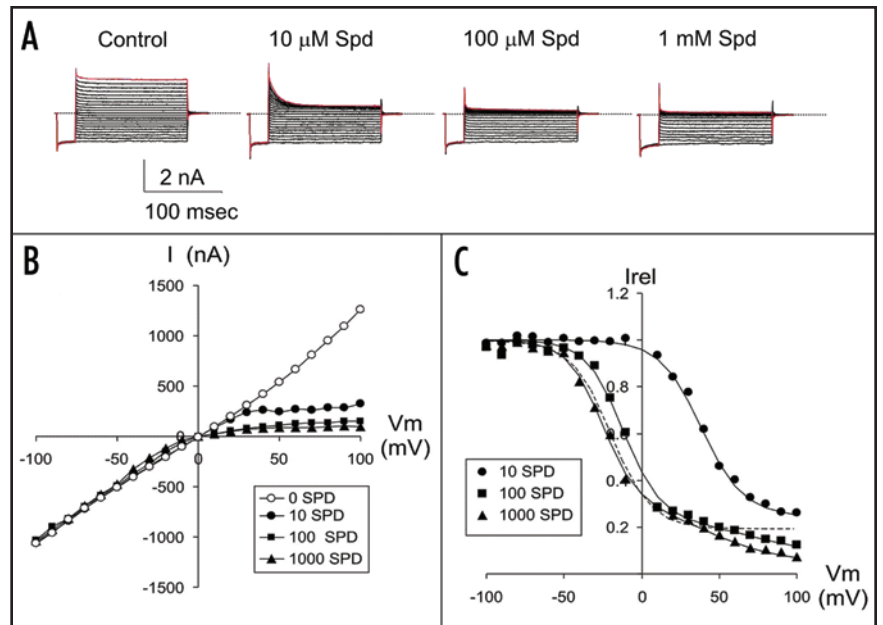


Figure 4. (A) Representative excised patch-clamp recordings of Kir4.1 with 150 mM K^+ in the pipette, in response to voltage steps from 0 (hold) to voltages between -100 and $+100$ mV, with Mg^{2+} at concentrations indicated. (B) Steady state current-voltage relationships from (A). (C) Irel-voltage relationships from (B), fitted with Eqn. 1 ($z = 1.8, 1.3, 1.0, V_{1/2} = +61, +42, +18$ mV and offset = 0.49, 0.35 and 0.24, for $Mg^{2+} = 10, 100 \mu$ M, and 1 mM, respectively). Rectification is shallow and incomplete, even at 1 mM Mg^{2+} at $+100$ mV.

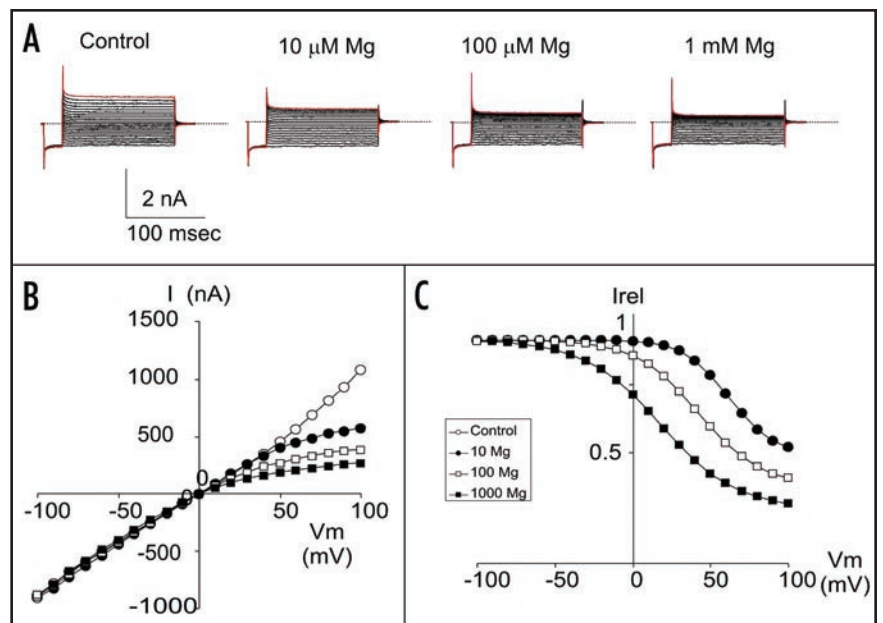
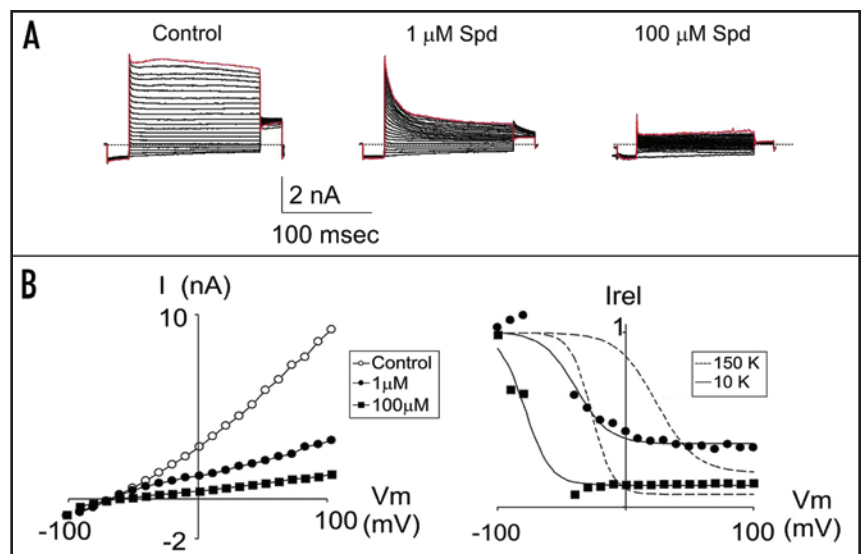


Figure 5. (A) Representative excised patch-clamp recordings of Kir4.1 with 10 mM K^+ in the pipette, in response to voltage steps from 0 (hold) to voltages between -100 and $+100$ mV, with spermine at concentrations indicated. (B) Steady state current-voltage relationships from (A) (left) and relative Irel-voltage relationship (right). Rectification is steep ($z = 2.0, 2.5, V_{1/2} = -39, -79$ mV, for spermine = 1, 100 μ M, respectively), but incomplete, with offset of 0.36 and 0.13, at 1 and 100 μ M spermine, respectively. For comparative purposes, Irel-V relationships obtained under the same spermine concentrations with 150 mM K_{out} from Figure 2 are shown in dashed lines.



As an empirical measure of the degree of rectification, we measured the 'rectification ratio' (RR), i.e. the absolute ratio of currents at 30 mV positive to the reversal potential ($I_{\text{Erev}+30}$) to the current at 30 mV negative to the reversal potential ($I_{\text{Erev}-30}$):

$$\text{RR} = I_{(\text{Erev}+30)} / I_{(\text{Erev}-30)} \quad (\text{Eqn. 3})$$

RESULTS

Kir4.1 channels show strong rectification in high external $[\text{K}^+]$, but weak rectification in physiological $[\text{K}^+]$. Figure 1 shows representative currents in on-cell and excised membrane patches from Cosm6 cells expressing recombinant Kir4.1 channels, with high (150 mM), and low, pseudo-physiological (10 mM) $[\text{K}^+]$ in the pipette (Kout). Prior to excision, on-cell currents clearly show significantly less rectification at low Kout than at high Kout. As an empirical measure of the degree of rectification, the rectification ratio (Eqn. 3) was considerably higher in on-cell patches with 10 Kout (RR = 0.47 ± 0.08 , n=5) than with 150 Kout (RR = 0.21 ± 0.02 , n = 5). It was not possible to assess these parameters at different Kout on the same patch. However, with similar electrodes, the absolute level of outward current tends to be quite high at low Kout, compared to currents at high Kout, suggesting that the 'cross-over' phenomenon that is typical of 'classical' Kir2-like channels¹ will be absent (Fig. 1B). Following patch excision, rectification is substantially lost in both cases, although complete linearization of outward current was not achieved in low Kout (Fig. 1A). Kir4.1 channels are very sensitive to block by even slightly acidic pH.²⁶⁻²⁸ In patches from non-transfected cells, basal currents were unaffected by switching from pH7.4 to pH6.0 (data not shown). Therefore to assess complete block of Kir4.1 channels, patches were routinely exposed to polyamine-free solution at pH6.0 (as in Fig. 1). Importantly, exposure to pH6 inhibits all current through Kir4.1-expressing membrane patches (Fig. 1), indicating that the small outward conductances measured in the on-cell condition are also through Kir4.1 channels.

Spermine and spermidine block underlies physiological rectification. Inward rectification of Kir channels results from block by cytoplasmic polyamines and Mg^{2+} ions.¹ As shown in Figures 2–5, excision of Kir4.1 channels into polyamine- and Mg^{2+} -free solutions caused complete loss of rectification, although 'wash-out' was frequently slow, with loss of rectification taking seconds to minutes for completion, as is typically observed with Kir2.x channels.²⁹ Upon exposure of Kir4.1 channels to 1–100 μM spermine or spermidine (Figs. 2 and 3), rectification was restored. Putrescine (not shown) and Mg^{2+} (Fig. 4), at concentrations up to 1 mM caused only minimal rectification of Kir4.1. Rectification induced by spermine or spermidine is concentration dependent, with ~ 20 mV shift in the mid-point of rectification for a 10-fold decrease in polyamine concentration. At very low spermine concentrations, however, the time course of block becomes very long, and at 1 μM , the steady state Irel-voltage relationship is difficult to ascertain (Fig. 2).

By comparison with Kir2.1, a striking and previously unappreciated feature of polyamine block of Kir4.1 currents is the incomplete nature. There is a clear pedestal of conductance, even at saturating [spermine] or voltage (Fig. 2). A similar pedestal is apparent in the presence of spermidine, although a secondary phase of channel block is now also evident (Fig. 3), and a single Boltzmann function

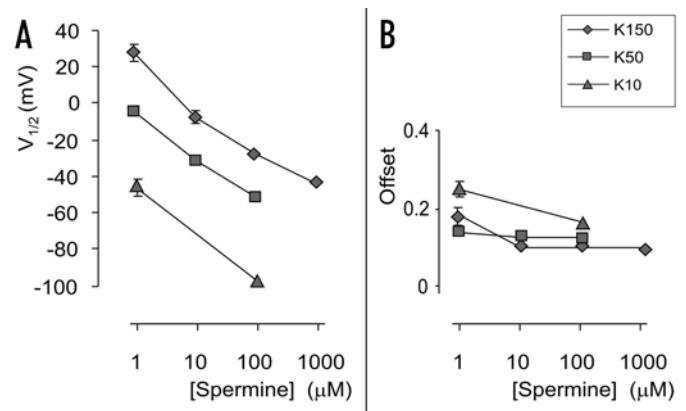


Figure 6. (A) $V_{1/2}$ and (B) plateau offset versus [spermine] for excised patches (mean, n = 3–7), with different Kout (10, 50, 150 mM, Kin = 150 mM).

is inadequate to fit the data (dashed line Fig. 3C), requiring Irel-V relationships to be fit by the sum of two Boltzmann functions.

Initially, we had assumed the pedestals of unblocked conductance in both on-cell conditions and in the presence of spermine or spermidine to be a reflection of unsubtracted "leak" current, but it is present in every patch (Fig. 3C) and, like the pedestal conductance seen in the intact cell (Fig. 1), it is inhibited by switching to pH6.0. The question of whether this results from an incomplete block of the channel when spermine is present, or from spermine permeation is considered below. Nevertheless, the similarity of the pedestal conductance in the intact cell, and in excised patches exposed to spermine or spermidine, together with the very low sensitivity to Mg^{2+} block, indicates that spermine and spermidine are the likely physiologically relevant blockers of the channel.

Kout dependence of rectification is due to Kout dependence of polyamine sensitivity. Importantly, exposure of patches containing low Kout to spermine induced only weak and incomplete rectification (Fig. 5); even in 100 μM spermine, prominent outward currents are apparent with 10 mM Kout. Figure 6 summarizes mean fitted parameters to Irel-voltage relationships for Kir4.1 channels exposed to different [spermine] at different Kout values. As Kout is lowered, rectification shifts to more negative voltages, with $V_{1/2}$ shifting in proportion to E_K (Fig. 6A). In addition, the plateau conductance tends to become more prominent (Fig. 6B). This plateau conductance, and increasing prominence at low Kout, will contribute to the apparent lowering of the degree of rectification in intact cells, but moreover has important implications for the mechanism of inward rectification itself in these channels.

Pedestal of unblocked current at saturating (polyamine) and voltage: Comparative effects of spermine and philanthotoxin. Most analyses of rectification in other Kir channels, and mechanistic models of channel block, predict essentially complete block at saturating [polyamine].³⁰ A sizeable (~ 10 to 15%) pedestal of non-blocked current at positive voltages in Kir4 channels has significant implications for the mechanism of channel block. It implies one of two possibilities: (1) that when the polyamine binding site(s) are saturated, $[\text{K}^+]$ ions can still pass through the channel (i.e. past the polyamine), or (2) that occupancy of a completely occluding site by polyamine is not complete, even at the highest [polyamine]. This possibility could be accounted in, for example, a model in which polyamines first bind in a concentration-dependent way, at a shallow,

Figure 7. (A) Structures of philanthotoxin (PhTx) and natural polyamines. (B) Hypothetical model for spermine block and permeation that could account for a plateau conductance. The model predicts no plateau for philanthotoxin, since blocker permeation is obviated. (C) Representative patch-clamp recordings of Kir4.1 in an excised patch (140 Kout), in response to voltage steps from 0 (hold) to voltages between -100 and +100 mV, with spermine or PhTx at concentrations indicated.

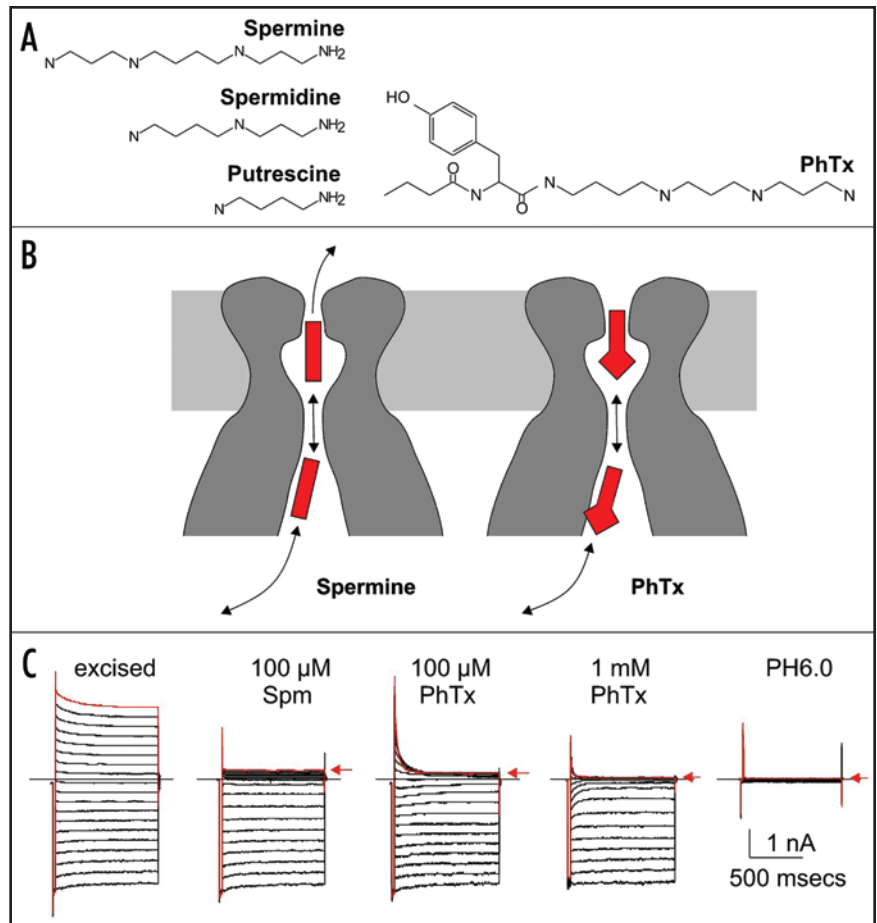
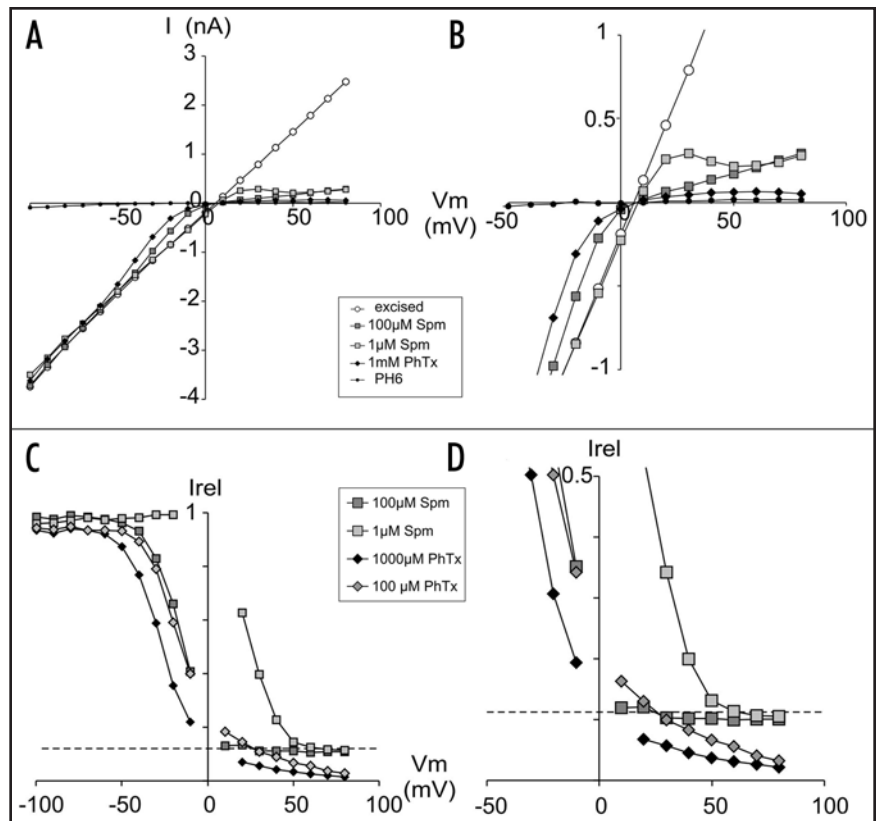


Figure 8. (A, expanded in B) Steady state current-voltage relationships from Figure 7, at concentrations indicated. (C, expanded in D) Irel-voltage relationships from (A). Although a plateau offset is prominent with Spm block at 1 or 100 μ M, rectification is essentially complete with 1 mM PhTx block.



incompletely, blocking site, and from there enter a deeper completely blocking site from which there is a significant rate of exit to the outside (Fig. 7B). In an attempt to distinguish these possibilities, we have examined channel block by philanthotoxin (PhTx), a spider toxin that is chemically essentially spermine with a bulky tail group (Fig. 7A). As shown in Figure 7C, PhTx blocks Kir4.1 channels with similar *potency* to spermine, and with similar *voltage-dependence*, although the kinetics of block are considerably slower. Importantly, however, at high concentrations and positive voltages, there is a distinct difference in the blocking profile: At concentrations giving a comparable voltage range of block, PhTx block crosses-over spermine block. PhTx is more complete at positive voltages and does not attain a plateau (Figs. 7C and 8). As discussed below, the bulky spermine analog is unable to permeate the channel and suggests that spermine permeation may underlie the 'pedestal' of current that is observed in Kir4 channels.

DISCUSSION

Variable rectification in Kir4 channels and physiological relevance. Several studies have attempted to assign native currents to Kir4.x/5.x channels. However, in many native tissues apparent Kir4.x/5.x channels show rather weak physiological rectification (examples in refs. 10, 13 and 16–20). The present results help to reconcile these findings and suggest that native Kir4.1 currents will underlie weakly rectifying currents under physiological conditions.

Unlike classical strong inward rectifiers of the Kir2 sub-family, which typically show increasing rectification with K_{out} and 'crossover' of current-voltage relationships,¹ Kir4.1 currents rectify more weakly at low K_{out} than at high K_{out}, to the extent that outward current is greater at all voltages in 10 mM K_{out} than at 150 mM K_{out} (Fig. 1). Most biophysical analyses of Kir4 channels have been performed at high (150 mM) K_{out}, and the relatively weak rectification at physiological K_{out} has been largely unappreciated. The pedestal conduction in spermine seems to reach a minimum at about 10% (Fig. 2), as [spermine] is saturated, but the plateau is slightly higher at lower [spermine] and at lower K_{out} (Fig. 6). It is unclear what the free spermine concentration really is in cells, and may well vary under different physiological conditions. The finding that the plateau conductance tends to be higher as K_{out} is reduced (Fig. 5) provides a mechanistic explanation for the relatively weaker rectification that is observed in the intact cell under such conditions.

The relevance of a weak and K_{out}-dependent rectification of Kir4 currents to the physiology of cells in which these channels are present is unclear. In electrically active cells, a relatively weak rectification would tend to shorten action potentials, as well as reduce excitability. Glial cells are generally considered electrically inexcitable, but in these cells, weak rectification would tend to imply that relatively high K⁺ conductance will be present even at depolarized voltages, which may be relevant to K⁺ buffering in these cells.

Biophysical implications of variable and incomplete rectification. Various kinetic models have been proposed to account for the details of rectification.^{29–32} Our original model of 'long-pore plugging' proposed a shallow, weakly voltage-dependent site and a deep, strongly voltage-dependent binding site.³⁰ Recent models are still consistent with this general idea, and multiple mutagenesis studies have indicated that the shallow site is associated with the cytoplasmic vestibule of the Kir channel, whereas the deep site is in the inner

cavity or the entrance to the selectivity filter.^{21,22,29,31–34} In such a model, it is easy to see how shallow binding may not completely occlude the channel^{35,36} but it is difficult to imagine that significant K⁺ permeation could occur when the polyamine is bound at a deep site in the inner cavity. A small pedestal of non-blocked current at positive voltages and very low (< 1 μ M) spermine or spermidine concentrations has been reported previously for Kir2.1,³² leading to the proposal that there is a finite rate of polyamine permeation through the selectivity filter. In cyclic nucleotide-gated (CNG) channels, which are also blocked in a steeply voltage-dependent manner by polyamines,³⁷ this permeation seems to be significant, such that current-voltage relationships are biphasic, channels are blocked up to a certain point, but block is almost completely relieved at higher voltages.

So can permeation account for the much more significant plateau conductance that is evident in Kir4 channels with high polyamine concentrations and strong depolarizing voltages? Permeation is highly unlikely for philanthotoxin (Fig. 7), and, consistent with this notion, CNG channels show only monophasic block by PhTx.³⁷ In the present case, we have shown that PhTx can also induce strong inward rectification of Kir4.1, but there is no significant plateau of conductance. We thus suggest that the plateau of current in Kir4.1 channels is the result of a higher net permeation rate than in Kir2 channels.

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