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Recommended Citation
Kurata, Harley T.; Marton, Laurence J.; and Nichols, Colin G., "The polyamine binding site in inward rectifier K+ channels." Journal of General Physiology. 127,5. 467-480. (2006).
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The Polyamine Binding Site in Inward Rectifier K⁺ Channels

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The Journal of General Physiology
Published April 10, 2006
http://www.jgp.org/cgi/doi/10.1085/jgp.200509467

INTRODUCTION

The term rectification is used to describe the property of certain ion channels to preferentially allow currents to flow in one direction (either into or out of the cell). Rectification is a critical feature of many functional groups of channels, including K⁺ channels and glutamate receptors. Within the structural family of inwardly rectifying K⁺ (Kir, KCNJ) channels, there is a spectrum of rectification. Within the structural family of inwardly rectifying K⁺ (Kir, KCNJ) channels, there is a spectrum of rectification that depends in large part on the presence of a negatively charged amino acid residue, often termed the “rectification controller” in the pore-lining M2 helix (Lu and MacKinnon, 1994; Wible et al., 1994; Nichols and Lopatin, 1997; Lu, 2004). Under physiological conditions, weakly rectifying channels (e.g., Kir6.2) allow considerable outward currents at depolarized potentials, whereas strongly rectifying channels (e.g., Kir2.1, Kir6.2[N160D]) are able to nearly completely prevent ion permeation in the outward direction (Nichols and Lopatin, 1997; Lu, 2004). Variability in the strength of inward rectification is related to differences in channel sensitivity to polyamines, with strongly rectifying channels exhibiting a potent and strongly voltage-dependent block by intracellular polyamines (Lopatin et al., 1994; Ficker et al., 1994; Fakler et al., 1995).

To block Kir channels, polyamines enter and occlude the central K⁺-selective pore of the channel. The affinity and voltage dependence of block varies with the identity of the blocking polyamine, spermine generally being the most potent and voltage-dependent blocker and shorter polyamines (e.g., spermidine, cadaverine, and putrescine) exhibiting weaker affinity and voltage dependence (Lopatin et al., 1995; Nichols and Lopatin, 1997; Pearson and Nichols, 1998; Guo and Lu, 2003; Guo et al., 2003). The steep voltage dependence of polyamine blockade likely arises in part from interactions of the blocking molecule with permeating ions, as movement of the blocker through the channel pore forces occupant permeant ions to traverse the membrane electric field (Spassova and Lu, 1998; Pearson and Nichols, 1998; Lu, 2004).

A general concept underlying interpretation of the voltage dependence of channel blockade is that it should correlate with the depth of the blocking site in the pore; entry of polyamines into a deep blocking site in Kir channels should displace more K⁺ ions (or traverse a larger fraction of the transmembrane field) than polyamines binding to a shallower site. And although it is well known that channel block by intracellular polyamines is the underlying mechanism of inward rectification, the details of this process, and particularly the specific physical location of polyamine binding, remain incompletely resolved (Lopatin et al., 1995; Guo et al., 2003; Kurata et al., 2004; John et al., 2004; Lu, 2004). Some studies have suggested a model of “shallow” spermine block of Kir channels, with spermine binding between the “rectification controller” residue and several rings of negatively charged residues located in the cytoplasmic domain of the channel (Guo and Lu, 2003; Guo et al., 2003). These authors have argued that binding of spermine at a relatively shallow site in the pore can result in a large voltage dependence of block by displacing a column of at least five K⁺ ions along the Kir pore (Lu, 2004; Shin and Lu,

Abbreviation used in this paper: MTSEA, 2-aminoethyl methanethiosulfonate.
Others have proposed a “deep” model of spermine block, suggesting that spermine binds between the “rectification controller” residue and the selectivity filter (Chang et al., 2003; Kurata et al., 2004; John et al., 2004). In both the deep and shallow models, displacement of K⁺ ions by spermine is likely to account for a large fraction of the voltage dependence of block, but in the deep model, the blocker is proposed to reach a much deeper site in the pore, such that displacement of K⁺ ions from the selectivity filter is the logical source of the charge movement (Kurata et al., 2004; John et al., 2004). In this study, we address these contrasting models of polyamine blockade, using a novel variant of the “blocker protection” technique to determine the physical location of spermine binding in a Kir pore.

**MATERIALS AND METHODS**

**Kₐₑₑ Channel Constructs and Expression in COSm6 Cells**

General methods are described in detail in previous publications (Loussouarn et al., 2000). Point mutations were prepared by overlap extension at the junctions of relevant residues by sequential
PCR as described. All cysteine mutations employed in these experiments (L157C, L164C, and M169C) were constructed on the Kir6.2[N160D][C166S] background construct, for several reasons. An earlier study (Loussouarn et al., 2000) demonstrated Cd2+ accessibility of residue C166; however, currents in the C166S channel are insensitive to Cd2+ or modification by MTSEA. In addition, the C166S mutant channel exhibits considerably less rundown than WT Kir6.2, which is advantageous during long inside-out patch clamp recordings (Trapp et al., 1998). The N160D mutation is included to confer steeply voltage-dependent, high affinity binding of spermine and other polyamines. Earlier studies of Kir6.2 have demonstrated that the N160D mutation in the Kir6.2 pore (equivalent to residue D172 in Kir2.1/IRK1 channels) confers a high affinity for polyamines, and an effective valence of spermine block ($\delta z \sim 4.5$) essentially identical to that reported in Kir2.1 channels (Shyng et al., 1997; Guo et al., 2003; Kurata et al., 2004).

**Patch-clamp Recording**

COSm6 cells were transfected with pCMV6b-Kir6.2 (with mutations as described), pCEC-SUR1, and pGreenLantern (Invitrogen), as previously described (Loussouarn et al., 2000; Phillips et al., 2003). 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 Instruments, Inc.). The standard pipette (extracellular) and bath (cytoplasmic) solution used in these experiments had the following composition: 140 mM KCl, 1 mM EGTA, 1 mM K-EDTA, 4 mM K2HPO4, pH 7. (Guo and Lu, 2002). Spermine was purchased from FLUKA chemicals, putrescine was purchased from Sigma-Aldrich, and CGC-11179 was made available to us through CellGate Pharmaceuticals Inc. (Loussouarn et al., 2005). MTSEA (Toronto Research Chemicals) was dissolved in the standard recording solution on the day of experiments to make a 10 mM stock that was stored on ice. Further dilutions to 100 μM were prepared and used immediately for channel modification. Microsoft Solver was used to fit data by a least-squares algorithm.

**RESULTS**

**Blocking Properties of Spermine and CGC-11179**

We have adopted the technique of “blocker protection” (Del Camino et al., 2000) to investigate the location at which various polyamines bind stably to Kir channels at depolarized voltages. We began by characterizing the blocking properties of spermine and a longer synthetic polyamine analogue (CGC-11179) in strongly rectifying Kir6.2[N160D][C166S] channels (Fig. 1). Spermine exhibits a steeply voltage-dependent block ($\delta z \sim 4.5$) that is not significantly altered by the introduction of cysteine residues at pore-lining residues 157, 164, or 169 in the M2 helix of Kir6.2 (Fig. 1 C). The synthetic polycation CGC-11179 is a linear deca-amine, consisting of 10 amines separated by propyl linkers (for chemical structure, and size relative to spermine, see Fig. 8 or Loussouarn et al., 2005). In Kir6.2[N160D][C166S] and in cysteine-substituted channels (L157C, L164C, M169C), CGC-11179 exhibits slightly less potent block than spermine but with indistinguishable voltage dependence (Fig. 1 C).

**MTSEA Modification of the Kir6.2 Pore**

Proximity or overlap of a bound polyamine with introduced cysteines in the Kir6.2 pore should interfere with the rate of cysteine modification by methanethiosulfonate reagents. To examine this, we first determined the rate of MTSEA modification of the various substituted cysteine residues (Fig. 2). Cysteine modification by MTSEA
introduces a positively charged ethylamine adduct, and modification of cysteine residues substituted at pore-lining positions in Kir6.2 causes reduction of macroscopic current, reflecting a reduction of single channel current (Loussouarn et al., 2001; Phillips et al., 2003; Kurata et al., 2004). MTSEA application has no effect on ATP sensitivity (Phillips et al., 2003), and current is not rescued by PIP_2, indicating that changes in open probability or channel rundown do not substantially contribute to the overall current reduction in MTSEA.

The rate of MTSEA modification in various cysteine-substituted channels was determined as illustrated by the sample experiment in Fig. 2 A (the sample trace was collected from a patch expressing the M169C mutant channel). Immediately after MTSEA application, excised patches were pulsed to +50 mV with repeated brief repolarizations to −50 mV. This protocol was employed because MTSEA also blocks Kir6.2 in a voltage-dependent manner (Phillips et al., 2003), and brief repolarization to −50 mV is sufficient to relieve MTSEA block, allowing us to resolve the component of current reduction that is due to channel modification. The extent of current reduction depends upon the location of the substituted cysteine residue: modification of L164C or M169C channels reduces currents by 80–90%, whereas modification of L157C channels reduces currents by only ~50%, as a result of differing effects on single channel currents (Fig. 2 B; Loussouarn et al., 2001; Phillips et al., 2003). The overall time course of the reduction of macroscopic currents in each cysteine mutant is well approximated by a monoexponential fit (dashed blue lines in Fig. 2, L157C τ = 4.2 ± 0.7 s, n = 5; L164C τ = 3.9 ± 0.5 s, n = 4; M169C τ = 2.4 ± 0.3 s, n = 4).

Stable Voltage-dependent Binding of Spermine and CGC-11179 in the Kir6.2 Pore

The unique property of polyamine block that makes the present study possible is the remarkably slow unbinding rate of long polyamines such as spermine and CGC-11179 at depolarized voltages. When control or cysteine-substituted channels are blocked with either spermine or CGC-11179 at +50 mV, and then the blockers rapidly removed from the bathing solution (with the membrane voltage held at +50 mV throughout), a very slow release of the blocker from the pore is apparent (Fig. 3). The recovery of peak current at +50 mV (τ > 1 min) reflects the very slow unbinding of the blockers at this voltage. There is a very rapid relief of block and restoration of current upon repolarization to −50 mV, confirming that neither polyamine application nor prolonged clamping of the membrane at +50 mV causes significant rundown of currents.

Blocker Protection in Kir6.2 by Spermine and CGC-11179

The extremely slow off-rate of spermine and CGC-11179 at +50 mV allowed a unique experimental design in our blocker protection assays. The approach was to “preblock” channels with either spermine or CGC-11179 and then apply MTSEA, with the expectation that blocker occupancy should interfere with MTSEA modification of cysteines at locations that overlap the binding site, without interference from free blocker in solution. This preblocking approach eliminates many complications that may arise if MTSEA and the blocker of interest are applied simultaneously, where kinetic differences in access to a binding site could potentially confuse the interpretation of data (see DISCUSSION). Modification of a cysteine that is protected by a polyamine cannot occur until the polyamine has unbound from the channel pore, so the remarkably slow off-rate of either polyamine is the limiting factor in the assay. Importantly, the modification rates of our cysteine-substituted channels in 100 μM MTSEA (Fig. 2 B) were substantially faster than the off-rates of spermine or CGC-11179 at +50 mV (Fig. 3, A and B), and so changes in the rate of MTSEA modification after preblocking with a polyamine could be readily resolved.

Sample traces from typical blocker protection experiments in the L157C channel are illustrated in Fig. 4 (A and B). From a holding potential of −50 mV, patches were pulsed to +50 mV in the presence of spermine (A) or CGC-11179 (B) to completely block channels. The bathing solution was then changed to a polyamine-free solution and, where indicated by the downward arrow, the patch was exposed to polyamine-free solution containing 100 μM MTSEA. Due to the slow off-rate of either polyamine (Fig. 3), channels remain blocked in these steps. After a variable interval, patches were repolarized to −50 mV and immediately removed from the MTSEA-containing solution. Repolarization to −50 mV resulted in release of any blocking spermine, allowing measurement of the residual current after MTSEA exposure. Superimposed on the raw data is the monoexponential fit (dashed blue line) of the MTSEA modification rate in “unprotected” (i.e., unblocked) L157C channels (from Fig. 2). A considerably larger residual current remained when channels were modified after preblocking with either spermine (Fig. 4 A) or CGC-11179 (Fig. 4 B).

Experiments were performed on multiple patches with varied intervals in 100 μM MTSEA to determine the time course of MTSEA modification in channels preblocked with either spermine or CGC-11179 (Fig. 4 C). Importantly, we have previously shown that MTSEA modification of certain residues in the Kir6.2 pore can significantly affect the kinetics and affinity of spermine block (Kurata et al., 2004). Therefore, each patch can only be used once in these experiments, as any modification occurring in the first “run” can affect spermine occupancy and the apparent rate of modification in subsequent runs. Thus, each data point in Fig. 4 C is from a different patch, and not from cumulative MTSEA...
Overall, the rate of MTSEA modification of L157C channels was slowed ~10-fold after preblocking with either CGC-11179 (Fig. 4 C, red symbols; $\tau = 52 \pm 11$ s; unprotected $\tau = 4.2 \pm 0.7$ s) or spermine (Fig. 4 C, green symbols; $\tau = 44 \pm 7$ s). Thus, residue 157C (located between the “rectification controller” residue and the selectivity filter) is strongly protected against MTSEA modification by occupancy of the pore with either spermine or CGC-11179.

### Accessibility of Residue 164C in Spermine-blocked Channels

Similar protection experiments were conducted at several other sites in the Kir6.2 pore and, importantly, the profile of protection changes significantly with position. Most strikingly, at a slightly more shallow position in the inner cavity (L164C, one turn of the M2 helix below the rectification controller residue, toward the intracellular entrance of the channel), preblocking with spermine offers essentially no protection against MTSEA modification (Fig. 5, A and C; spermine $\tau = 5.4 \pm 0.5$ s; unprotected $\tau = 3.9 \pm 0.5$ s). In contrast, preblocking with the long polyamine analogue CGC-11179 still strongly protects against MTSEA modification at this position (Fig. 5, B and C; CGC-11179 $\tau = 35 \pm 8$ s). These results indicate that bound CGC-11179 overlaps with residue 164C, and occludes modification by MTSEA, while the shorter spermine fails to interfere with access of MTSEA to residue 164C.

We have previously demonstrated that the introduction of positive charges at position L164C dramatically reduces the channel affinity for spermine, with a pronounced acceleration of spermine off-rate (Kurata et al., 2004). This is most likely due to the close proximity of...
residues 164 and 160, such that introduction of positive charges at residue 164 counteracts the negatively charged rectification controller at residue N160D. An interesting consequence of this property of MTSEA-modified L164C channels becomes apparent in the spermine preblocking experiments (Fig. 5 A). Since spermine binding is essentially abolished in MTSEA-modified L164C channels, one would expect that MTSEA modification of a preblocked 164C channel would lead to exit of the blocking spermine ion shortly thereafter. This is indeed reflected in the experimental data; the application of MTSEA to L164C channels preblocked with spermine causes rapid relief of spermine block (Fig. 5 A), considerably faster than the intrinsic rate of spermine unbinding in the absence of MTSEA (compare with L164C in Fig. 3 A). In contrast, accelerated unblock is not apparent when MTSEA is applied to 164C channels preblocked with CGC-11179 (Fig. 5 B). Thus, 164C is protected in CGC-11179-blocked channels and not accessible to MTSEA. In spermine-blocked channels, the 164C residue is accessible to MTSEA, with modification leading to a decrease in spermine affinity and rapid exit of spermine from the pore. The result is channel unblock (with kinetics similar to the rate of 164C modification) to a current level corresponding to the fully MTSEA modified state of the channels (Fig. 5 A).

Blocker Protection Is Absent at Residue 169C

The profile of protection is different again at residue 169, which is located at the cytoplasmic end of the inner cavity. Preblocking with either spermine or CGC-11179 fails to substantially alter modification of M169C by MTSEA (Fig. 6, spermine $\tau = 2.7 \pm 0.2$ s; CGC-11179 $\tau = 3.3 \pm 0.3$ s; unprotected $\tau = 2.4 \pm 0.3$ s). We previously demonstrated that after MTSEA modification of residue 169C, entry and exit of spermine from the inner cavity is considerably slowed (Kurata et al., 2004); the preblocking protection experiments presented here reinforce this point. As shown in Fig. 6 C, the rate of M169C modification is not altered when channels have been preblocked. However, after the MTSEA modification step in preblocked channels, there is an obvious bi-exponential time course of current recovery upon repolarization, due to the appearance of a slow activation component ($\tau = 360 \pm 40$ ms; $n = 6$) that reflects the slow unbinding of spermine from MTSEA-modified channels at $-50$ mV. This phenomenon is apparent in the sample traces shown in Fig. 6 A, and has been expanded in Fig. 7 (A and B). To demonstrate this point further, we have compiled data from several 169C patches exposed to MTSEA for varying durations (Fig. 7 C). As the time of exposure is prolonged, the relative weight of the slow component is increased, as expected if the slow component is related to trapping of spermine by modification of the 169C residue. Thus, MTSEA modification of 169C is unaltered by the presence of spermine, and modification actually traps
the blocker in the inner cavity. The dramatic slowing of the spermine off-rate also illustrates that preblocked spermine molecules can remain within the Kir6.2 pore during and after the modification step at residue 169. There is no obvious slowing of CGC-11179 unblock after modification. However, the off-rate of CGC-11179 is considerably faster than that of spermine at −50 mV (Fig. 1, A and B), leaving us unable to resolve with certainty whether MTSEA modification of residue 169C can also trap the CGC-11179 compound in the pore.

**Protection Effects of Putrescine in the Kir Pore**

To characterize the localization of polyamines in the pore in more detail, we also determined the protection profile of putrescine in cysteine-substituted channels. These experiments require a slightly different experimental design than described earlier for spermine and CGC-11179, because putrescine does not exhibit the remarkably slow off-rate at +50 mV that is characteristic of the longer polyamines. Therefore, 1 mM putrescine was maintained.
in the bathing solution throughout the entire protocol, ensuring that a significant fraction of channels were blocked during the application of MTSEA. Apart from this detail, the design was identical to the experiments described in Figs. 4–6. Patches expressing L157C (Fig. 8A), L164C (Fig. 8B), or M169C (Fig. 8C) mutants were preblocked in putrescine at +50 mV, exposed to 100 μM MTSEA for a variable duration (with persistent exposure to putrescine), and repolarized to −50 mV to determine the extent of current reduction due to MTSEA exposure. Data from multiple patches of each mutant are compiled in Fig. 8 (righthand panels) together with the unprotected modification rates (from Fig. 2) at each position. At position L157C, putrescine occupancy resulted in significant protection ($\tau = 15.8 \pm 1.3$ s; unprotected $\tau = 4.2 \pm 0.7$ s), although the effects were more modest than the protection of this site by spermine and CGC-11179 (Fig. 4). The protective effects of putrescine are smaller at both 164C (Fig. 8B; $\tau = 7.1 \pm 0.5$ s; unprotected $\tau = 3.9 \pm 0.5$ s) and 169C (Fig. 8C; $\tau = 3.6 \pm 0.3$ s; unprotected $\tau = 2.4 \pm 0.3$ s). While the protective effects of putrescine appear to be more diffuse than for spermine or CGC-11179, which may reflect the technical limitations of the protocol (see DISCUSSION), residue 157C is clearly the most strongly protected of the three residues examined (Fig. 8).

The protection profile of each blocker is summarized in Fig. 9, where the mean unprotected and protected time constants of MTSEA modification are plotted at each residue examined. Data from multiple patches of each mutant are compiled in Fig. 8 (righthand panels) together with the unprotected modification rates (from Fig. 2) at each position. At position L157C, putrescine occupancy resulted in significant protection ($\tau = 15.8 \pm 1.3$ s; unprotected $\tau = 4.2 \pm 0.7$ s), although the effects were more modest than the protection of this site by spermine and CGC-11179 (Fig. 4). The protective effects of putrescine are smaller at both 164C (Fig. 8B; $\tau = 7.1 \pm 0.5$ s; unprotected $\tau = 3.9 \pm 0.5$ s) and 169C (Fig. 8C; $\tau = 3.6 \pm 0.3$ s; unprotected $\tau = 2.4 \pm 0.3$ s). While the protective effects of putrescine appear to be more diffuse than for spermine or CGC-11179, which may reflect the technical limitations of the protocol (see DISCUSSION), residue 157C is clearly the most strongly protected of the three residues examined (Fig. 8).

The protection profile of each blocker is summarized in Fig. 9, where the mean unprotected and protected time constants of MTSEA modification are plotted at each residue examined. The plot is lined up with a depiction of the KirBac1.1 M2 helix, with colors highlighting the equivalent residues examined in the present study (Kuo et al., 2003). Residue L157C is strongly protected by spermine and CGC-11157, and less so by putrescine. Modification of L164C is substantially slowed only in the presence of CGC-11179, and no blockers protected L169C channels from modification.

**DISCUSSION**

**Molecular Basis of Polyamine Block**

Steeply voltage-dependent block by polyamines accounts for the unique rectification properties of strong inwardly rectifying potassium channels (Lopatin et al.,
1994; Ficker et al., 1994; Fakler et al., 1995; Guo and Lu, 2002). However, the molecular details underlying this process have remained controversial, particularly with regard to the physical location of spermine binding (Guo and Lu, 2003; Kurata et al., 2004; John et al., 2004). Crystal structures have revealed that the pores of inwardly rectifying potassium channels are considerably longer than an individual spermine molecule and are lined by multiple rings of negative charges (Kuo et al., 2003). This has led to one proposed model in which spermine and other polyamines are bound stably between the negatively charged rectification controller residue in the inner cavity (D172 in Kir2.1, equivalent to N160D in Kir6.2 examined in the present study) and multiple negatively charged residues in the cytoplasmic domain of the channel (Fig. 10, Model A; Nishida and MacKinnon, 2002; Guo and Lu, 2003; Guo et al., 2003; Pegan et al., 2005). With relatively shallow spermine binding in the Kir pore, the voltage dependence of polyamine block must then arise entirely from the oblique displacement of a column of K⁺ ions as a polyamine molecule approaches its binding site (Lu, 2004; Shin and...
An alternative model is a deeper binding site for spermine in the inner cavity, between the rectification controller residue and the selectivity filter (Chang et al., 2003; Dibb et al., 2003; John et al., 2004; Kurata et al., 2004), with the head of spermine lying near or within the selectivity filter (Fig. 10A, Model B). In this case, charge movement can be the result both of significant polyamine movement through the membrane field and displacement of K\(^{+}\) ions from the inner cavity and the selectivity filter.

Several studies have now employed thermodynamic mutant cycle analysis to probe the location of spermine block. Varying conclusions have been drawn and support has been argued for each of the models above (Guo and Lu, 2003; Guo et al., 2003; Kurata et al., 2004). In all instances, the analysis has been hampered by the drawback that ∆∆G values have been derived from changes in apparent “overall” Kd values, and thus interpreted in the context of a single barrier binding equilibrium. However, it has long been known that at least two sequential equilibria are required to adequately describe the kinetic and steady-state properties of spermine block in Kir2.1, with a peripheral, only weakly voltage-dependent binding and a deeper voltage-dependent site responsible for steep rectification (Lopatin et al., 1995; Shin and Lu, 2005). In such a sequential model, mutations that alter an early equilibrium, but leave the deep spermine binding site unchanged, can affect the apparent Kd (see Eq. 1a in Shin and Lu, 2005). If interpreted in terms of a single barrier model, this will incorrectly imply disruption of the deep site responsible for steep voltage-dependent rectification. Given these significant potential pitfalls for interpretation of mutant cycle analyses, a blocker protection study potentially provides a far more direct approach for identifying the physical location of polyamine binding sites.
Blocker Protection Profile of Spermine and CGC-11179

The blocker protection properties we have described for spermine and CGC-11179 seem to exclude stable binding at more shallow sites in the Kir pore and clearly support a model in which the blockers bind at a deep site. In the residues examined here, spermine protected only residue 157C (between the rectification controller and selectivity filter) from modification. Modification of residues at more shallow sites in the pore (164C and 169C) was unaltered by the presence of preblocked spermine. Protection of residues at the more shallow 164C location required preblocking with the much longer synthetic polyamine CGC-11179, while at the most shallow location examined (169C), even CGC-11179 did not hinder modification by MTSEA. The protection profile of CGC-11179, when compared with the length of this compound (see Fig. 10, and later DISCUSSION), suggests that the head of this compound binds at a very deep site in the transmembrane region of the channel. The protection profile for spermine (together with its indistinguishable effective valence relative to CGC-11179, Fig. 1C) suggests a similarly deep binding site.

Many blocker protection studies have applied MTS reagents in the continuous presence of a blocker and have repetitively relieved block by voltage pulses to observe the extent of modification (Del Camino et al., 2000; Chang et al., 2003). In Kir channels, an important potential ambiguity arising from this approach results from modification reducing spermine affinity (Kurata et al., 2004). A conceivable situation is one in which rapid MTSEA entry into the inner cavity could precede blockade by spermine, allowing modification to take place before spermine reaches its binding site. If periodic voltage pulses are used to assess the extent of modification, this problem could be compounded with each repetitive pulse. At a location such as residue 157C, MTSEA modification does significantly reduce the potency and dwell time of spermine block (Kurata et al., 2004), and would thus reduce the ability of spermine to protect this site. Preblocking channels with either spermine or CGC-11179, and avoiding the use of repetitive voltage pulses, avoids the possibility that kinetic differences in access rates between spermine/CGC-11179 and MTSEA could mask or attenuate protection by a polyamine occupying the pore. The different protocol may account for some discrepancies in the results of our study compared with an earlier study in Kir2.1 (Chang et al., 2003), particularly the apparent absence of significant protection of Kir2.1 residue 169C (equivalent to 157C in our study) by spermine. It is reassuring, however, that both our study and a previous study (Chang et al., 2003) reported strong protection at deep sites in the Kir pore. While Chang et al. (2003) also reported some protection of Kir2.1 residue 176C (equivalent to 164C in our study), this effect was very modest compared with deeper sites in the pore, indicating that the model of deep spermine binding extends to the physiologically important strongly rectifying channel Kir2.1.

A second consideration in the interpretation of these data is the volume or capacity of the inner cavity. The data are significantly different from what one would predict based on a model of shallow polyamine binding (Guo and Lu, 2003; Guo et al., 2003), and before dismissing it, we have considered the possibility that MTSEA could bypass spermine and modify residues that in fact overlap or lie beyond the spermine binding site. It has been suggested, for example, that the relatively weak voltage dependence of block by divalent cations such as Ba2+ and Mg2+ might involve them bypassing K+ ions in the pore (and hence not requiring movement of K+ ions through the field), to reach a blocking site that is considerably deeper than has been proposed for spermine (Jiang and MacKinnon, 2000; Lu, 2004). While space-filling considerations suggest that this is improbable in the present case (given the substantially larger sizes of spermine and MTSEA relative to Ba2+ or K+), this issue provided a major impetus for examination of CGC-11179 (Loussouarn et al., 2005). Importantly, we observed a clear extension of the protected region of the inner cavity when occupied by CGC-11179 vs. spermine (Figs. 5 and 9), arguing against the possibility that MTS reagents are somehow bypassing the blocking polyamine to access substituted cysteine residues.

Models of Polyamine Binding in the Kir Pore

In Figs. 9 and 10, we have mapped the residues examined in the present study onto equivalent positions in the published crystal structure of the KirBac1.1 channel (Kuo et al., 2003). The mapped residues have been color coded, based on their protection profile. Residue L157 (red, equivalent to M135 in KirBac) is protected by both spermine and CGC-11179. Residue L164 (yellow, equivalent to T142 in KirBac) is protected by CGC-11179, but not spermine. The shallowest residue examined (M169, green, equivalent to A147 in KirBac) is not protected by either spermine or CGC-11179. Although the experimental design differed somewhat, and the protection effects were considerably smaller than for spermine and CGC-11179, putrescine only protected residue 157C, with little or no protection of residues 164C and 169C (Figs. 8 and 9). Adjacent to the full channel structure in Fig. 10, we have shown structures of fully extended spermine, CGC-11179, and putrescine, positioned in locations that are consistent with our data. The leading amines of both spermine and CGC-11179 are placed at a similar location, reflecting their indistinguishable effective valences (Fig. 1). Spermine is located between the rectification controller residue (160D) and the selectivity filter, accounting for its inability to protect against MTSEA modification of residues 164 and 169. The considerably longer CGC-11179 extends further toward the cytoplasmic vestibule of the channel,
where it is able to protect against MTSEA modification of residue 164. Importantly, even with its head placed in the entrance to the selectivity filter, a fully extended CGC-11179 molecule would still extend slightly beyond residue 169, suggesting that this extremely long and flexible polyamine may not remain in its fully extended conformation in the inner cavity. Although the boundaries of the protection effects of putrescine are not as clear as those observed for spermine and CGC-11179, partial protection of only 157C, located above the rectification controller residue, is entirely consistent with the proposal that it binds between rectification controller and the entrance of the selectivity filter (Fig. 10).

Previous characterization of Kir2.1 channels has demonstrated that the effective valence of block by diamines and polyamines increases up to a maximum of \( \sim 5 \) at an alkyl chain length of eight or nine (Pearson and Nichols, 1998; Guo et al., 2003). The shallow binding model proposed by Guo et al. located the trailing amine of diamines or of spermine between the rectification controller and the negatively charged residues in the cytoplasmic domain of the channel (Fig. 10, Model A). With longer polyamines/diamines, the leading amine was proposed to reach deeper into the pore toward the rectification controller residue, resulting in the displacement of more K\(^+\) ions, with a larger effective valence in consequence (Guo et al., 2003). One important potential problem with this model is that it seems to imply multiple K\(^+\) ions around the entrance to or in the inner cavity. That is, if the trailing amines of diamines/polyamines bind at essentially a fixed location, the difference in the position of the leading amines of putrescine and spermine, for example, would be only 10–12 Å. In the model of Lu and colleagues, this difference would need to account for a difference in valence of \( \sim 3 \), suggesting the displacement of three additional K\(^+\) ions through the membrane field. However, there is little or no evidence to suggest such close spacing of K\(^+\) ion binding sites at this shallow location in the pore.

The blocker protection data in the present study seems to rule out stable binding of polyamines at a shallow location in the pore, as residue 169C is not protected by any of the polyamines examined (Figs. 6 and 8). The protection profile is far more consistent with the alternative model of deep spermine binding, in which the trailing amine of diamines or polyamines binds near the rectification controller, with the leading amine approaching or entering the selectivity (Fig. 10 B). This model essentially places spermine and other diamine blockers between the rectification controller and the selectivity filter, and the displacement of additional (closely spaced) K\(^+\) ions from the selectivity filter by the longest polyamines can then logically account for their larger effective valence (for various descriptions of this model see Chang et al., 2003; Dibb et al., 2003; Phillips and Nichols, 2003; John et al., 2004; Kurata et al., 2004). Importantly, the displacement of ions from binding sites identified in KcsA (one K\(^+\) ion in the inner cavity, and two K\(^+\) ions in the selectivity filter) could generate a maximal effective valence of 3, clearly insufficient to make up the large valence associated with spermine block. To account for this discrepancy, one possibility is displacement of K\(^+\) ions from additional binding sites at more shallow locations in the pore. It has been suggested that there may be one or more K\(^+\) binding sites in the cytoplasmic domain of Kir2.1 (Nishida and MacKinnon, 2002; Shin et al., 2005), and the crystal structure of KsAP appears to contain two K\(^+\) ions in the inner cavity (rather than a single cavity ion, as in KcsA) (Jiang et al., 2003). A second possibility is that one or more amines of a blocking spermine ion traverse a segment of the transmembrane field, and thus directly contribute to the valence of block. This could potentially arise by partial entry of spermine into the selectivity filter (see below), or if the distribution of the transmembrane field in Kir channels differed from that predicted in MthK (where the field drops almost entirely across the selectivity filter) and extended partially into the inner cavity.

Selectivity Filter Entry of Polyamines

Although the present study unambiguously indicates a spermine binding site deep in the inner cavity, an issue that remains difficult to resolve is whether it is plausible that spermine block involves entry into the selectivity filter. This remains an important question in understanding the mechanism underlying strong voltage dependence of polyamine block. One recent study demonstrated that steeply voltage-dependent block is maintained in a polyamine analogue with expanded head groups (decane-bis-trimethylammonium). This study concluded that block occurs in or below the inner cavity (Shin and Lu, 2005), but this hinges on the assertion that the bis-trimethylammonium head cannot enter the filter. Other studies have presented evidence consistent with slow permeation of spermine and other polyamines through Kir channels, indicating that barriers for spermine entry into (and even permeation through) the selectivity filter are not insurmountable (Guo and Lu, 2000a,b; Dibb et al., 2003; Makary et al., 2005).

We have also suspected spermine binding near or within the selectivity filter based on comparisons with the well-characterized properties of quaternary ammonium ions. Although the physical location of the intracellular binding site for TEA and other quaternary ammonium ions in Kir channels is not completely understood, all structural evidence (Zhou et al., 2001; Lenaues et al., 2005) and blocker protection studies (Del Camino et al., 2000) suggest that these blockers occupy the cavity ion or dehydration transition site in the inner cavity of several other model K\(^+\) channels.
(i.e., Shaker and KcsA). In Kir channels, the effective valence of TEA blockade is normally <2 (Guo and Lu, 2001), while that of spermine is considerably larger. The possibility remains that the quaternary ammonium blocking site in Kirs differs significantly from other K+ channels (Shin et al., 2005). However, these observations collectively suggest that TEA may actually reach the cavity ion or dehydration transition site in Kir channels to achieve its effective valence. More careful determination of the quaternary ammonium binding site in Kir channels, by either structural studies or blocker protection studies, will likely provide significant new insights into this issue. The deep binding model can explain the even larger effective valence of spermine by entry into the selectivity filter and displacement of filter K+ ions (Kurata et al., 2004), a proposal that is entirely consistent with the results of our present study. Also consistent with this suggestion, and supporting the hypothesis that the selectivity filter comprises a final barrier to the exit of spermine to the extracellular solution, is the finding that disruption of the selectivity filter by mutagenesis of ion pairs in the P-loop of Kir2.1 and Kir3.1/3.4 can abolish spermine block, and instead allow spermine permeation (Yang et al., 1997; Dibb et al., 2003; Makary et al., 2005).

Conclusion

Pore occupancy by spermine can inhibit MTSEA modification of cysteine residues substituted at pore-lining positions in the pore of Kir6.2[N160D] channels. The pattern of protection is extended to more shallow pore-lining residues when channels are blocked with the extended polyamine analogue CGC-11179. The data unambiguously support a model of strong inward rectification in which spermine stably binds with its trailing amine near the rectification controller residue (D172 in Kir2.1, N160D in Kir6.2) and its leading amine located near or within the selectivity filter.

We are grateful to Gildas Loussouarn for many useful discussions early in this project.

This work was supported by National Institutes of Health grant HL54171 (to C.G. Nichols). H.T. Kurata is supported by a Canadian Institutes of Health Research Fellowship.

Lawrence G. Palmer served as editor.

Submitted: 7 December 2005
Accepted: 20 March 2006

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