

# Block of the Kir2.1 Channel Pore by Alkylamine Analogues of Endogenous Polyamines

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**ABSTRACT** Inward rectification induced by mono- and diaminoalkane application to inside-out membrane patches was studied in Kir2.1 (IRK1) channels expressed in *Xenopus* oocytes. Both monoamines and diamines block Kir2.1 channels, with potency increasing as the alkyl chain length increases (from 2 to 12 methylene groups), indicating a strong hydrophobic interaction with the blocking site. For diamines, but not monoamines, increasing the alkyl chain also increases the steepness of the voltage dependence, at any concentration, from a limiting minimal value of  $\sim 1.5$  ( $n = 2$  methylene groups) to  $\sim 4$  ( $n = 10$  methylene groups). These observations lead us to hypothesize that monoamines and diamines block inward rectifier  $K^+$  channels by entering deeply into a long, narrow pore, displacing  $K^+$  ions to the outside of the membrane, with this displacement of  $K^+$  ions contributing to "extra" charge movement. All monoamines are proposed to lie with the "head" amine at a fixed position in the pore, determined by electrostatic interaction, so that  $z\delta$  is independent of monoamine alkyl chain length. The head amine of diamines is proposed to lie progressively further into the pore as alkyl chain length increases, thus displacing more  $K^+$  ions to the outside, resulting in charge movement ( $z\delta$ ) increasing with the increase in alkyl chain length.

**KEY WORDS:** inward rectifier • polyamine • diamine • voltage dependence • potassium channel

## INTRODUCTION

The last five years have seen much progress in our understanding of inward rectifier  $K^+$  channel function. Beginning with the cloning of ROMK1 (Kir1.1; Ho et al., 1993) and IRK1 (Kir2.1; Kubo et al., 1993), several related families of inward rectifier  $K^+$  (Kir)<sup>1</sup> channel subunit genes have been cloned (see Nichols and Lopatin, 1997). These channel subunits differ in structure from the previously characterized voltage-gated (Kv) channel subunits in possessing only two, rather than six, transmembrane segments and lacking the highly positively charged S4 transmembrane segment, which serves as a voltage sensor in the Kv channels (Liman et al., 1991). Inward rectification is distinct from the voltage dependence of Kv channels in that conductance is not a function of the absolute membrane potential, but is instead related to the  $K^+$  reversal potential and is conferred by diffusible intracellular compounds, in particular the polyamines spermine and spermidine (and to a lesser extent putrescine and  $Mg^{++}$ ) (Matsuda et al., 1987; Vandenburg, 1987; Fakler et al., 1994; Ficker et al.,

1994; Lopatin et al., 1994, 1995). Strong rectification conferred by these compounds correlates with the presence of negatively charged residues in certain positions in the second (M2) transmembrane segment and in the COOH-terminal region (Tagliatela et al., 1995; Yang et al., 1995). Mutating these residues to uncharged residues decreases or abolishes polyamine-induced rectification (Yang et al., 1995). Likewise, mutation of corresponding neutral residues to negatively charged residues confers polyamine-induced rectification on channels that originally do not display strong rectification (Fakler et al., 1994, 1995; Lopatin et al., 1994; Glowatzki et al., 1995; Shyng et al., 1997). Experiments that reduce the levels of endogenous polyamines in situ reduce the rectification of Kir currents expressed in those cells (Bianchi et al., 1996; Shyng et al., 1996), confirming the physiological relevance of polyamine-induced rectification.

Much evidence is consistent with the concept that rectification results from positively charged ions binding to negatively charged residues in the channel. However, not all charged or polar molecules can block Kir channels. Lopatin et al. (1994) examined several related compounds and found that only the polyamines conferred strong rectification, while other bulkier, dipolar or nonlinear molecules (e.g., GABA, creatinine, lysine) failed to block at a concentration of 100  $\mu$ M. These results indicate that a molecule must possess both the correct structure and charge density or distribution to confer strong rectification. Because the most energetically favorable conformation of endogenous

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<sup>1</sup>Abbreviations used in this paper: DA, diamine; Kir, inward rectifier  $K^+$ ; MA, monoamine; PhEA, phenylethylamine; PhMA, phenyl methylamine; pPhDA, *p*-phenylenediamine.

polyamines in free solution is an extended linear chain (Romano et al., 1992), it seems likely that these molecules enter the long pore of the channel and lie in the pore to block it. In the present study, we have systematically measured the ability of a series of alkylamine analogues to block current flow through Kir channels to determine relevant structural features of polyamines in inducing inward rectification. We have examined monoaminoalkanes and diaminoalkanes with alkyl chains composed of 2–12 methylene groups, and find that changes in molecular length and charge have profound effects on channel block, with longer chain lengths increasing blocking affinity. Thus, the increased blocking affinity of spermine and spermidine, when compared with diamines, may not be attributed solely to increased blocker charge. Instead, we propose a model in which aminoalkanes act as “long pore plugs,” displacing K<sup>+</sup> ions to the outside as they enter the pore and binding both by electrostatic and hydrophobic interactions.

## METHODS

### *Expression of Channels*

Kir 2.1 was propagated in pBluescript SK(-). cRNA was prepared using Message Machine kits (Ambion Inc., Austin, TX). *Xenopus* oocytes were isolated using conventional techniques and pressure injected with (50–100 nl) cRNA (1–100 ng/ml). Oocytes were maintained at room temperature in ND96 solution (2 mM KCl, 96 mM NaCl, 1 mM MgCl<sub>2</sub>, 5 mM Na-HEPES) with 1.8 mM CaCl<sub>2</sub>, supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml). Channels were allowed to express for at least 18 h after injection. Before recording, each oocyte was incubated in a hypertonic solution (60 mM KCl, 10 mM EGTA, 40 mM HEPES, 250 mM sucrose, 8 mM MgCl<sub>2</sub>, pH 7.0) for ~10 min, and the vitelline membrane was removed manually.

### *Electrophysiology*

Oocytes were placed in a chamber continuously perfused with “KINT” solution (see below). Recording pipettes of ~20-µm tip diameter were pulled from soft glass (World Precision Instruments, Inc., Sarasota, FL), fire polished, and coated with a mixture of mineral oil and paraffin to reduce capacitive currents. Pipettes were filled with KINT solution and typically had series resistances from 0.2 to 0.9 MΩ. Recordings were made with an Axopatch 1D patch clamp amplifier (Axon Instruments, Foster City, CA). Data were filtered with the amplifier’s integral four-pole Bessel filter (typically at 5 kHz), digitized via a Digidata 1200 interface (typically at 10 kHz), and stored on the hard disk of a 486 microcomputer (Gateway 2000, North Sioux City, SD) for off-line analysis. Data were also displayed on a Gould chart recorder and stored on videotape using a Neurodata PCM digitizing interface. Data acquisition was controlled by pClamp 6 software (Axon Instruments).

After the pipettes were sealed to the oocytes, patches were excised and moved to a subchamber with a separate perfusion inlet, ensuring that endogenous polyamines released from the oocyte did not confer rectification on the channels in the excised patch. Patches were held at 0 mV and washed with fresh KINT solution until endogenous rectification was largely washed out. Patches expressing from 0.5 to 15 nA current at -50 mV were used in these experiments. Because several of the alkylamines tested ap-

peared to adhere to the tubing of the perfusion system, test solutions were applied by pipetting 100–200 µl of the solution directly into the subchamber containing the patch (perfusion of the subchamber with KINT was stopped during test solution application). When possible, several test solutions were applied to the same patch (either different concentrations of the same compounds or different compounds). Before a new compound was applied to the same patch, the previous compound was thoroughly washed from the patch (and currents were measured after washing to verify that wash was complete). Because inward rectifier current “ran down” after patch excision, and because intrinsic rectification continued to wash out of the patch for several minutes after excision, control measurements were made frequently throughout the course of an experiment.

To test the blocking affinity of a given compound, patches were typically held at 0 mV and briefly pulsed to a “conditioning potential” (e.g., -80 mV, 5 ms) to relieve voltage-dependent channel block, and then through a series of test potentials (e.g., up to 70 mV in 10-mV increments). In experiments where block kinetics were measured, additional voltage pulses of equal amplitude were made to 0 mV after the test voltage pulses. The currents recorded during these voltage steps were used for subtracting capacitive artifacts from the beginning of the test voltage step.

### *Solutions and Chemicals*

KINT solution was composed as follows (mM): 140 KCl, 10 K-HEPES, 1 K-EGTA, 1 K-EDTA, pH 7.35. KD98 solution was composed as follows (mM): 98 KCl, 5 K-HEPES, 1 MgCl<sub>2</sub>, pH 7.5. Alkylamine compounds were purchased from commercial sources (Sigma Chemical Co., St. Louis, MO, and Aldrich Chemical Co., Milwaukee, WI), or were obtained from Dr. Carl Romano (Washington University School of Medicine). All compounds tested in this study contained amine groups attached to terminal methylene groups of the molecule’s alkyl chain. To simplify the presentation, we refer to these compounds by using a nomenclature that indicates the number of amine groups present on the compound and the number of carbons in the alkyl chain. A given monoamine compound is referred to as MA<sub>n</sub>, and a given diamine compound is referred to as DA<sub>n</sub>, where *n* is the number of carbons in the alkyl chain. For example, the compound 1-amino-hexane is referred to as MA6, while the compound 1,5-diaminopentane is referred to as DA5. Other compounds examined in this study included the monoamines benzylamine (phenyl methylamine, PhMA), phenylethylamine (PhEA), and the diamine *p*-phenylenediamine (pPhDA). Compounds were prepared as 100 mM concentrated stock solutions in H<sub>2</sub>O (pH ~7.3), and then diluted into working stock solutions of 1 mM in KINT. Serial dilutions of the 1 mM stocks were used in these experiments.

### *Data Analysis*

To measure kinetics of channel block and unblock, data were fit to functions of the form  $I = K_{\text{ofs}} + K \cdot \exp(-t/\tau)$  (single exponential) or  $I = K_{\text{ofs}} + K_1 \cdot \exp(-t/\tau_1) + K_2 \cdot \exp(-t/\tau_2)$  (double exponential), where *I* is the current amplitude, *K*<sub>ofs</sub> the offset, *t* is time,  $\tau$  a time constant, and *K* (or *K*<sub>1</sub>, *K*<sub>2</sub>) a scaling factor. Offset coefficients were included to correct for incomplete subtraction of leak conductances. When appropriate, capacitance currents and other time-dependent currents not associated with channel block were subtracted from the data before fitting, either by subtracting residual currents after complete channel block or rundown, or by subtracting capacitance transients from equivalent voltage steps to 0 mV where no ionic current flows. Single exponential equations produced good fits to all relevant time-course data, except to unblock of the compound MA9, which followed a more markedly sigmoidal time course. The data were in this case fit by

assuming four independent blocking molecules [i.e.,  $I = [K_{\text{ofs}} + K^* \exp(-t/\tau)]^4$ ]. For a given concentration of a compound, relative blocking affinity was determined by fitting data to a Boltzmann equation of the form  $G_{\text{Relative}} = 1/[1 + \exp(V_m - V_{1/2})/(-K_{\text{Slope}}) + K_{\text{ofs}}(V_{1/2}, K_{\text{Slope}}, \text{ and } K_{\text{ofs}} \text{ left as free coefficients), which is equivalent to the Woodhull equation } G_{\text{Relative}} = 1/[1 + ([X]/K_{\text{d}(0\text{mV})}) \cdot \exp(V_m \delta Z F / RT)] + K_{\text{ofs}}$ , where  $\delta$  is the "electrical distance" into the transmembrane electrical field from the cytoplasmic face,  $Z$  is the charge of the blocking particle, and  $K_{\text{d}}$ ,  $R$ ,  $F$ , and  $T$  have their usual meanings. In these experiments, the effective valence ( $Z\delta$ ) was thus  $25.5 \text{ mV}/K_{\text{Slope}}$ , and  $K_{\text{d}(0\text{mV})} = [X] \cdot \exp(V_{1/2}/K_{\text{Slope}})$ , where  $[X]$  is the concentration of the test compound. These equations are most useful for describing channel block by a simple first order kinetic scheme. While these equations will not accurately describe channel block occurring by more complicated kinetic schemes, they provide useful empirical descriptions of channel block.

## RESULTS

### Block of Kir2.1 Channels by Endogenous Polyamines

Kir2.1 channels were expressed at high levels in *Xenopus* oocytes (whole cell current  $>20 \mu\text{A}$  at  $-30 \text{ mV}$  in KD98). Kir2.1 current was recorded using macropatch pipettes containing KINT solution ( $\sim 150 \text{ mM K}^+$ ). In

the cell-attached configuration, strongly inwardly rectifying currents were observed in response to voltage ramps from  $-50$  to  $+50 \text{ mV}$  ( $V_{\text{hold}} = 0 \text{ mV}$ , ramp duration =  $160 \text{ ms}$ ). Immediately after patch excision, current amplitude increased and rectification decreased, as described previously (Lopatin et al., 1994). As patches were moved from the vicinity of the oocyte into a fresh stream of KINT, rectification continued to decrease. The decrease of rectification was a continuous process, and in some patches the current measured in response to the voltage ramp became essentially ohmic (between  $-50$  and  $+50 \text{ mV}$ ). To measure channel block by applied compounds, patches were held at  $0 \text{ mV}$ , stepped briefly to a negative potential (typically  $-80 \text{ mV}$  for  $5 \text{ ms}$ ) to relieve channel block, and then stepped to test potentials between  $-80$  and  $+70 \text{ mV}$  in  $10\text{-mV}$  increments, repeating every  $0.5 \text{ s}$ . Currents typically showed some deactivation on stepping to potentials below  $-50 \text{ mV}$ . Such deactivation is frequently seen in inside-out patches. Since  $\text{K}^+$  is the only monovalent ion in the solutions, it does not appear to result from extracellular monovalent ion block (Harvey and Ten Eick, 1989; see also Nichols and Lopatin, 1997). Deactivation

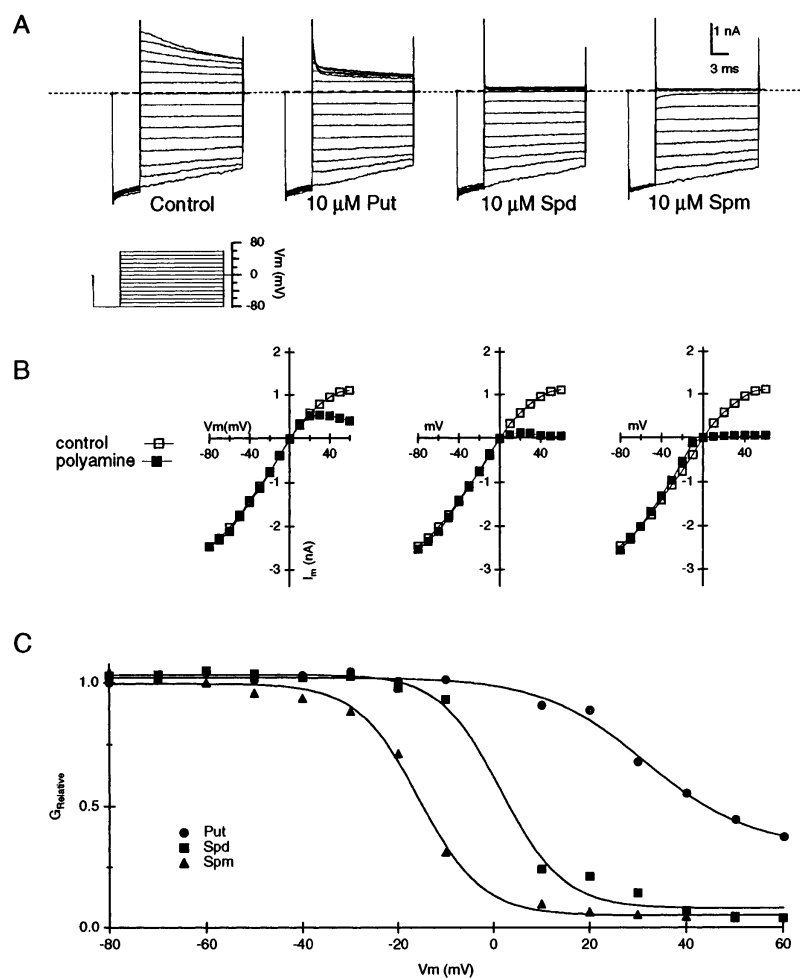


FIGURE 1. Endogenous polyamines cause inward rectification of Kir2.1 currents. (A) Kir2.1 currents recorded from an inside-out membrane patch in response to voltage steps to voltages between  $-80$  and  $+60 \text{ mV}$  from a prepulse to  $-80 \text{ mV}$  from a holding potential of  $0 \text{ mV}$  as indicated. The control recording was obtained  $\sim 3$  min after patch excision, and the patch was then sequentially exposed to polyamines as indicated. (B) Steady state current-voltage relationships plotted from the above current traces. (C) Relative conductance ( $G_{\text{Relative}}$ )-voltage relationships for the currents in A.  $G_{\text{Relative}}$  defined in the text.

occurred with a simple exponential time course, occurred more rapidly at more negative potentials, and was not obviously affected by the presence or absence of di- or monoamines. For test potentials at and above +20 mV, a variable, but very slow, current inactivation was observed that was also well fit by a single exponential decay (see Fig. 1). There remains some controversy as to whether this residual rectification results from an incomplete washout of endogenous polyamines (Lopatin et al., 1994, 1995) or from a gating process that is independent of polyamine block (Aleksandrov et al., 1996; Shieh et al., 1996). However, the residual process is well resolved from the exogenous polyamine block that we examine in the present experiments, and this question is not considered in this paper.

In addition to these voltage-dependent inactivation processes, channels also commonly displayed "run-down," which occurred with variable time course from patch to patch (as fast as 2 min for complete rundown of current, not present at all in some patches). As shown previously, different endogenous polyamines block Kir

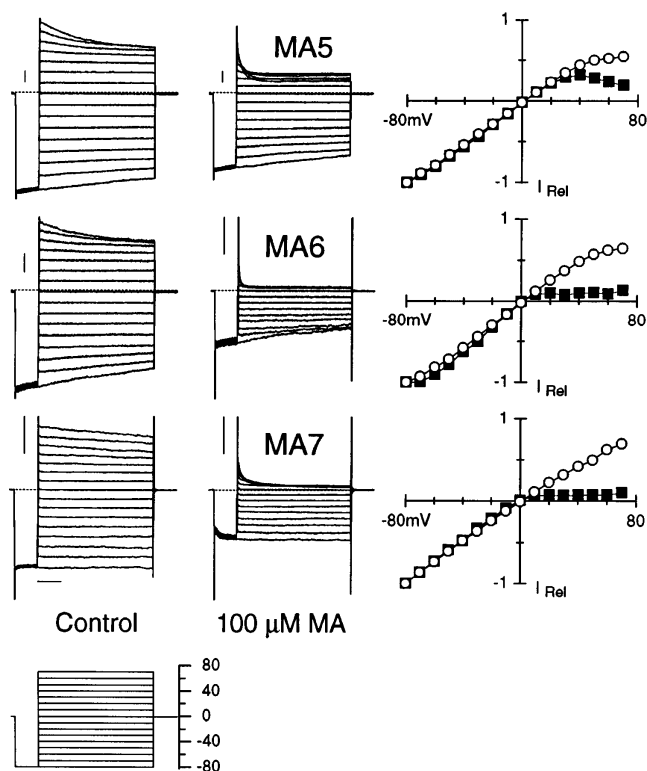


FIGURE 2. Monoamino alkanes cause weak inward rectification of Kir2.1 currents. (*left*) Kir2.1 currents recorded from an inside-out membrane patch in response to steps to voltages between -80 and +70 mV after a prepulse to -80 mV from a holding potential of 0 mV as indicated. In each case, the control recordings were obtained after removal of rectification after patch excision, and the patch was then exposed to 100  $\mu$ M monoamines as indicated. Scale bars: 2 nA and 5 ms. (*right*) Steady state current-voltage relationships plotted from the above current traces.

channels with different affinities and kinetics (Lopatin et al., 1995). Fig. 1 illustrates channel block by 10  $\mu$ M putrescine, spermidine, and spermine. Block by these compounds is highly voltage dependent. As considered in detail in Lopatin et al. (1995), there is both a shallow voltage dependence at very negative voltages and a steeper component at more depolarized potentials, which may indicate multiple binding sites; i.e., one at the inner entrance to the channel pore, and one deeper site within the pore. A single exponential function provides a reasonable fit to the steeper component and may provide a reasonable quantitative description of the deep block at a single site. As shown in the relative conductance ( $G_{\text{Relative}}$ )-voltage relations (Fig. 1 C), the steepness of block increases from putrescine ( $z = +2$ ) to spermidine ( $z = +3$ ) to spermine ( $z = +4$ ). The following experiments used a series of mono- and diaminoalkanes to examine the role of polyamine structure and charge in determining biophysical blocking properties.

#### Alkylmonoamine Block of Kir2.1 Channels

Cytoplasmic application of short chain monoamines (MA1-MA4) had no obvious effect on Kir2.1 currents at concentrations up to 1 mM (at test potentials as de-

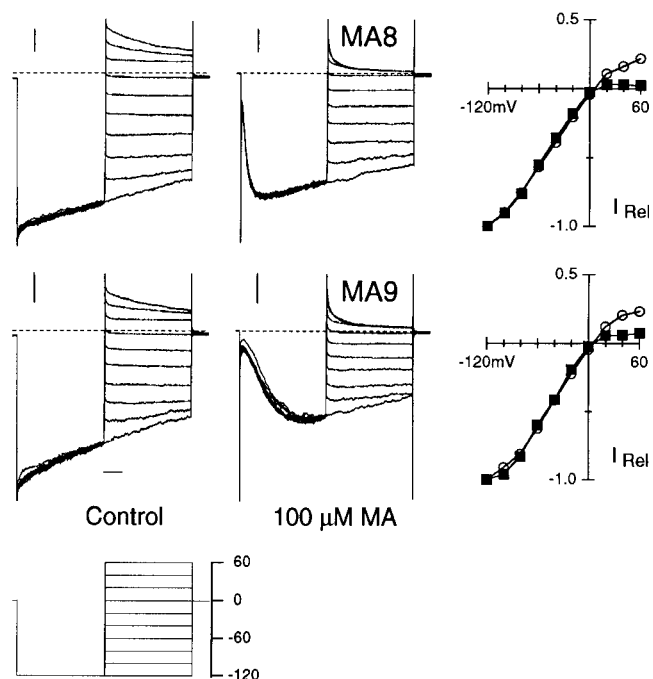


FIGURE 3. Longer monoamines cause inward rectification with very slow unblock rates. (*left*) Kir2.1 currents recorded from an inside-out membrane patch in response to steps to voltages between -120 and +60 mV after a prepulse to -120 mV from a holding potential of 0 mV as indicated. In each case, the control recordings were obtained after removal of rectification after patch excision, and the patch was then exposed to 100  $\mu$ M monoamines as indicated. Scale bars: 1 nA and 5 ms. (*right*) Steady state current-voltage relationships plotted from the above current traces.

polarized as 70 mV). Compounds with longer alkyl chains (5–12 methylene groups) produced voltage-dependent block of Kir2.1 current at concentrations below 100  $\mu$ M. Fig. 2 illustrates block of Kir2.1 currents by 100  $\mu$ M MA5, MA6, and MA7. At depolarized poten-

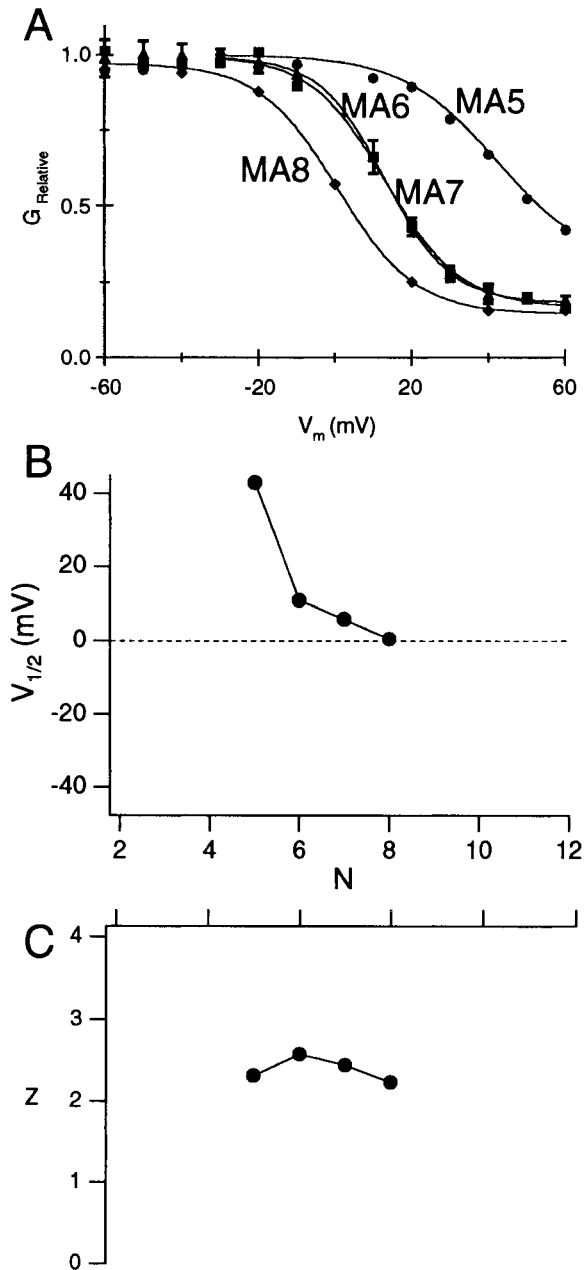


FIGURE 4. Monoamine blocking affinity increases with alkyl chain length. (A) Relative conductance ( $G_{\text{Relative}}$  see text)–voltage relationships obtained for block of Kir2.1 currents by different alkyl chain length monoamines at a concentration of 100  $\mu$ M (mean values from  $n = 2$ –6 patches,  $\pm$ SEM where  $n > 2$ ). Smooth lines are fits of a Boltzmann function (see text) to averaged data. (B)  $V_{1/2}$  of block as a function of alkyl chain length ( $N$ ) from fits to the data in A. (C) The effective valence of block ( $Z$ ) as a function of alkyl chain length ( $N$ ) from fits to the averaged data in A.

tials, the time dependence of block was clearly resolved. Longer monoamines (MA8, MA9, and MA10) also blocked Kir2.1 current in a voltage dependent fashion, although block was increasingly more potent with increasing alkyl chain length, and unblock at negative potentials occurred at a much slower rate. The slower kinetics of these compounds required that different voltage protocols be used to study their blocking properties (Fig. 3). By using longer duration conditioning pulses (20 ms) to a more negative potential ( $-120$  mV), complete channel unblock at negative potentials was achieved, allowing kinetic and steady state measurements. Single Boltzmann functions were fit to relative conductance ( $G_{\text{Relative}}$ )–voltage plots for 100  $\mu$ M monoamines (Fig. 4 A). The data demonstrate a very clear dependence of blocking affinity on chain length,  $V_{1/2}$  increasing by  $\sim -10$  mV per additional methylene group (Fig. 4 B), whereas the apparent effective valence (i.e., voltage dependence) of monoamine block ( $z\delta$ ) was relatively constant at a value  $\sim 2.2$  (Fig. 4 C). The marked increase in affinity with chain length indicates a very significant hydrophobic component in polyamine block.

The current decay from residual endogenous rectification was generally well fit by a single exponential function, and the resulting time constants were voltage dependent (but typically in excess of 10 ms at  $+70$  mV, not shown). Application of monoamines (longer than MA4) increased the rate of current decay at depolarized potentials. Blocking time constants derived from single exponential fits were voltage dependent (decreasing at depolarized potentials), and approached a minimum value at the most depolarized potentials (Fig. 5). Despite the differences in blocking affinity for the different compounds, there was little difference between the blocking rates, the block time constant at a given potential increasing slightly as the alkyl chain length increased (Fig. 5), and decreasing as blocker concentration increased (data not shown).

The increase in blocking potency, as alkyl chain length increases (Fig. 4), results primarily from a decreased off rate as the chain length is increased. To compare unblock kinetics, patches were stepped from a holding potential of 0 mV to a positive “blocking potential” ( $+50$  mV for 10 ms), and then back to a series of test potentials (0 to  $-140$  mV in 20-mV increments, Fig. 6 A). For the shortest monoamine (MA5), current activation (i.e., unblock) was almost instantaneous upon repolarization. For medium length monoamines (MA6, MA7, and MA8), time-dependent relaxations resulting from unblock were apparent between 0 and  $-40$  mV (Fig. 6 A). Because little or no endogenous current deactivation occurred at these potentials, single exponential functions could be fit directly to the current traces. At more negative potentials, however,

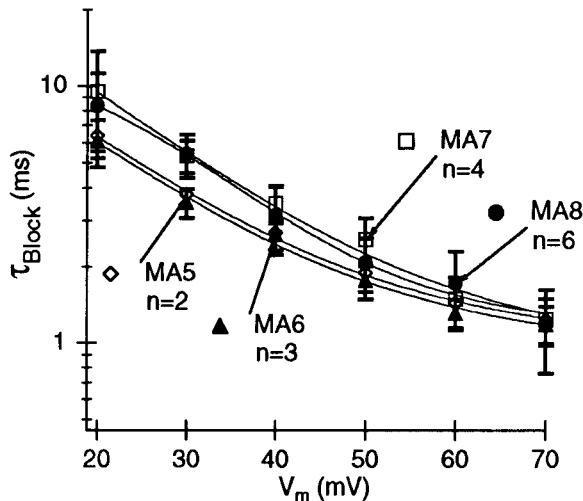


FIGURE 5. Monoamine block kinetics are voltage dependent. Time constant of block ( $\tau_{Block}$ ) obtained from single exponential fits to currents like those shown in Figs. 2 and 3 plotted versus membrane potential ( $V_m$ ).

current relaxations were superimposed on the endogenous voltage-dependent deactivation. Assuming that deactivation was independent of the unblock process, time constants for unblock were estimated after dividing the current measured in the presence of blocker by the current measured in the absence of blocker, and fitting a single exponential.<sup>2</sup> Unblock time constants decrease as an exponential function of voltage, as shown in Fig. 6 B. Increase in chain length is accompanied by increases in unblock time constants at a given potential. Addition of one methylene group to the alkyl chain increases the time constant (i.e., slows unblock) by a factor of  $\sim 10$ .

Block by these alkyl monoamine compounds was fully reversible, with observed decreases in current after MA application attributable to channel rundown. MA10 also caused voltage-dependent block of Kir2.1 channels, with relief of channel block at very negative potentials. In this case, however, recovery from block was incomplete ( $< 50\%$ ,  $n = 3$ ) after washing. MA11 and MA12 also potently inhibited Kir2.1 current, but the block appeared to be voltage independent, and the current amplitude did not recover after washing the compounds from the solution (not shown). It is not clear whether the irreversibility resulted from extremely slow unblock kinetics, or from current inhibition by a dis-

<sup>2</sup>Alternatively, the current in the presence of the blocker was fit as the product of two single exponential equations, one rising exponential attributable to the unblocking process, and one falling exponential attributable to deactivation. The unblock time constants determined by either method were very similar.

tinct mechanism. MA11 and MA12 are less soluble than the shorter monoamines, are very amphipathic in nature, and could conceivably be disrupting the activity of the channels by interaction with the lipid bilayer.

#### Diaminoalkane Block of Kir 2.1 Channels

All diamines tested (DA2–DA12) blocked Kir2.1 channels at  $100 \mu\text{M}$ . Representative currents, and current-voltage (I-V) relations in the absence and presence of shorter chain diamines (DA2–DA9) are shown in Fig. 7. As with monoamines, increasing the alkyl chain length increased the blocking affinity (Fig. 8, A and B). However, the kinetics of block and unblock were much faster, and this was particularly obvious for longer chain derivatives (compare DA8 in Fig. 7 with MA8 in Fig. 3). In contrast to the behavior of monoamines, the effective valence of diamine block increased steeply with increases in chain length (Fig. 8 C), the longest compound (DA12) having an effective  $z\delta > 4$ . This increase in effective  $z\delta$  was not obviously an artifact related to the shifted voltage dependence resulting from the accompanying increase in potency. As shown in Fig. 9 C, for a patch exposed to different [DA10], the fitted  $z\delta$  did not change for concentrations between 1 and  $100 \mu\text{M}$ . Since each diamine is divalent, the approximately monotonic increase of  $z\delta$  with alkyl chain length is striking, and suggests that increasing diamine length causes additional (i.e., non-amine) charges to move progressively further in the voltage field.

As shown in Fig. 9, unblock time constants were about two orders of magnitude faster for DA8 than for MA8, over a wide voltage range. At the same concentration, block time constants were also at least two orders of magnitude faster for DA8 than for MA8. Reducing DA8 concentration by 100-fold (from  $100$  to  $1 \mu\text{M}$ ) results in block time constants that are similar to the block time constants of  $100 \mu\text{M}$  MA8 (Fig. 9 A). Extrapolation of block time constants to zero applied voltage suggests that even in the absence of an applied field, and where the potencies of block are similar, the relaxation rate should be about two orders of magnitude higher for the diamine. For short to medium length diamines (DA2–DA7), channel block occurred almost instantaneously upon depolarization. As the alkyl chain length of diamines was increased, however, block did occur more slowly, as seen with monoamines (Fig. 9 B). Short and medium length diamines ( $< \text{DA7}$ ) unblocked very rapidly ( $< 500 \mu\text{s}$  for complete unblock) so that unblock kinetics were only measurable for DA8–DA12 (Fig. 10 A). While diamine unblock was faster than unblock of the corresponding monoamine, a similar relationship was seen between chain length and the rate of unblock, with longer diamines unblocking more slowly than shorter diamines (Fig. 10 B).

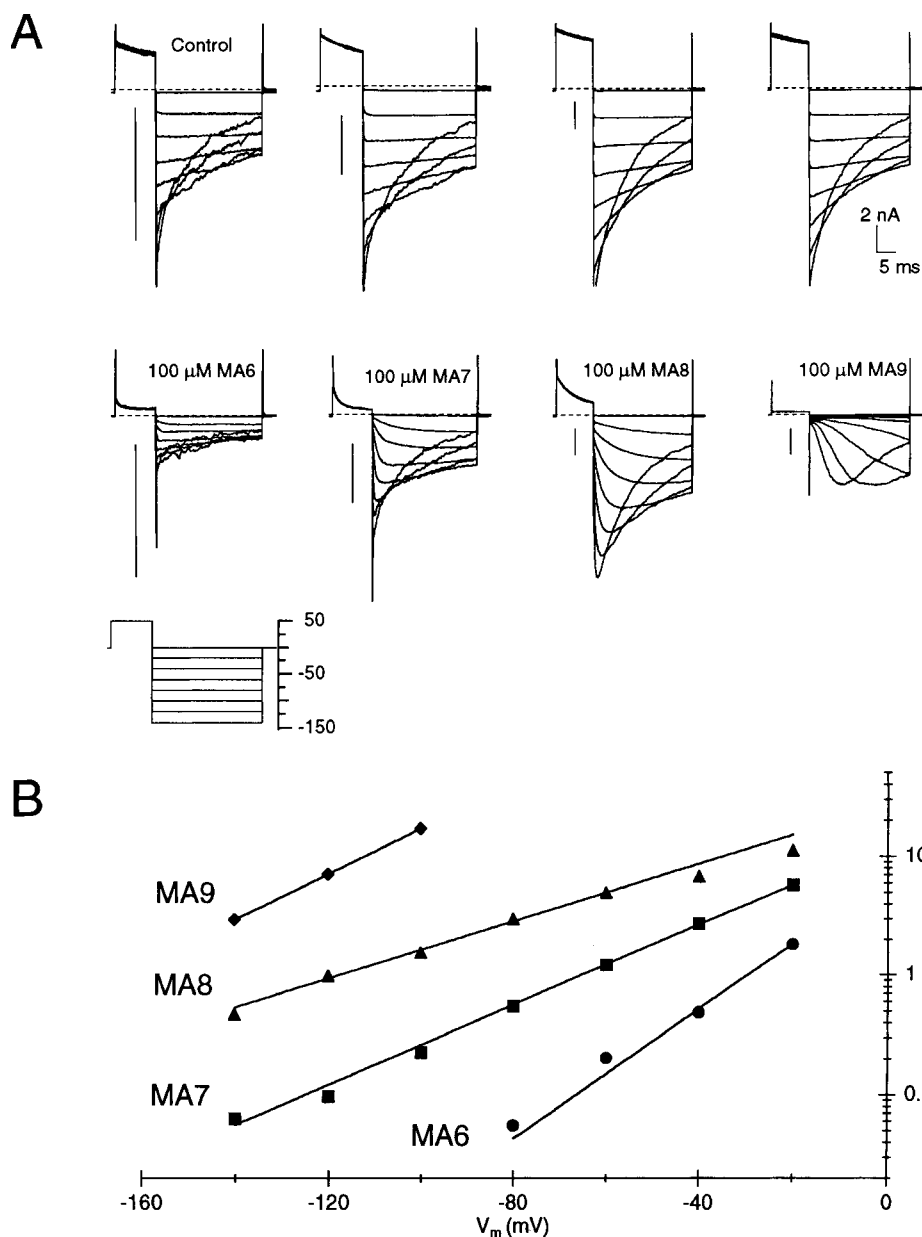


FIGURE 6. Monoamine unblock kinetics are strongly dependent on alkyl chain length. (A) Kir2.1 currents recorded from an inside-out membrane patch in response to voltage steps to potentials between 0 and  $-150$  mV after a prepulse to  $+50$  mV, from a holding potential of 0 mV as indicated. In each case, the control recordings were obtained after removal of rectification after patch excision, and the patch was then exposed to  $100 \mu\text{M}$  monoamines as indicated. Scale bars: 2 nA and 5 ms. (B) Time constant of unblock ( $\tau_{off}$ ) obtained from exponential fits (see text) to currents like those shown in A plotted versus membrane potential ( $V_m$ ).

*Block of Kir2.1 by Phenyl-substituted Mono- and Diamine Derivatives: The Effect of Bulky Hydrocarbon Groups*

While more than one polyamine molecule may be involved in channel block (Lopatin et al., 1995; Yang et al., 1995), we suggest that the steep voltage-dependent component results primarily from a single polyamine molecule entering deeply into the channel pore as an extended linear molecule. However, because the alkyl chains of mono- and diamino alkanes are not rotationally constrained, we cannot draw strong inferences about the physical dimensions of the pore from the effect of these compounds (since their conformations may be dramatically altered in the process of binding).

Arylamines and arylalkylamines contain aromatic groups that confer some structural rigidity to the molecules. PhMA ( $\text{NH}_3\text{-CH}_2\text{-C}_6\text{H}_5$ ) blocked Kir2.1 current with properties similar to the linear monoamines. Fig. 11 shows the voltage dependence of current block by  $100 \mu\text{M}$  PhMA. The block was reasonably well fit by  $V_{1/2} = +36$  mV. As with linear monoamines,  $100 \mu\text{M}$  PhEA was more potent ( $V_{1/2} = +28$  mV) without any obvious increase in steepness of voltage dependence ( $z\delta = 2.5$  and 2.3, respectively). In contrast, pPhDA was a very weak blocker of Kir2.1 channels. Fig. 11 shows original current records and  $G_{\text{Relative}}\text{-}V$  plots for currents in the presence of pPhDA.  $1 \text{ mM}$  pPhDA inhibited currents with  $z\delta = 1.2$  and  $V_{1/2} = +56$  mV.

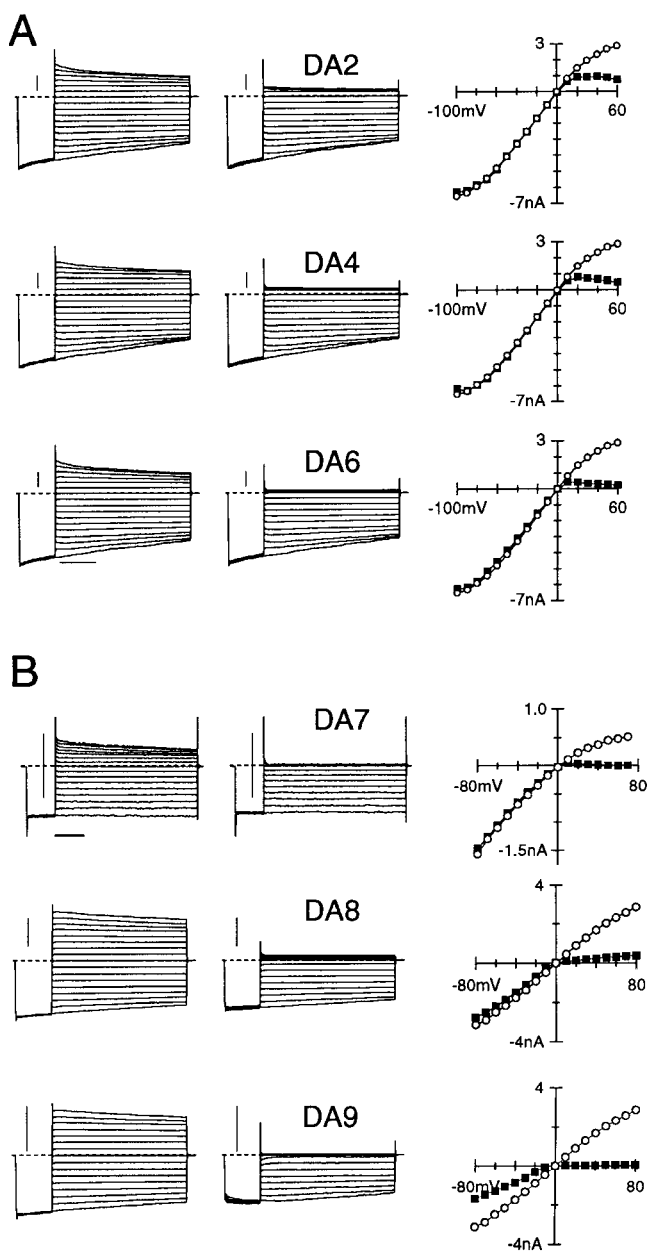


FIGURE 7. Diamino alkanes cause inward rectification of Kir2.1 with fast kinetics. (*left*) Kir2.1 currents recorded from inside-out membrane patches in response to steps to voltages between  $-100$  and  $+60$  mV (*A*), or between  $-80$  and  $+70$  or  $+80$  mV (*B*) after a prepulse to  $-100$  (*A*) or  $-80$  (*B*) mV from a holding potential of  $0$  mV. In each case, the control recordings were obtained after removal of rectification after patch excision, and the patch was then exposed to  $100 \mu\text{M}$  diamines as indicated. Scale bars:  $2$  nA and  $5$  ms. (*right*) Steady state current-voltage relationships plotted from the above current traces.

## DISCUSSION

### Alkylamine-induced Rectification of Kir Potassium Channels

Since the original descriptions of inward rectification of potassium conductance (Katz, 1949; Noble, 1965), it

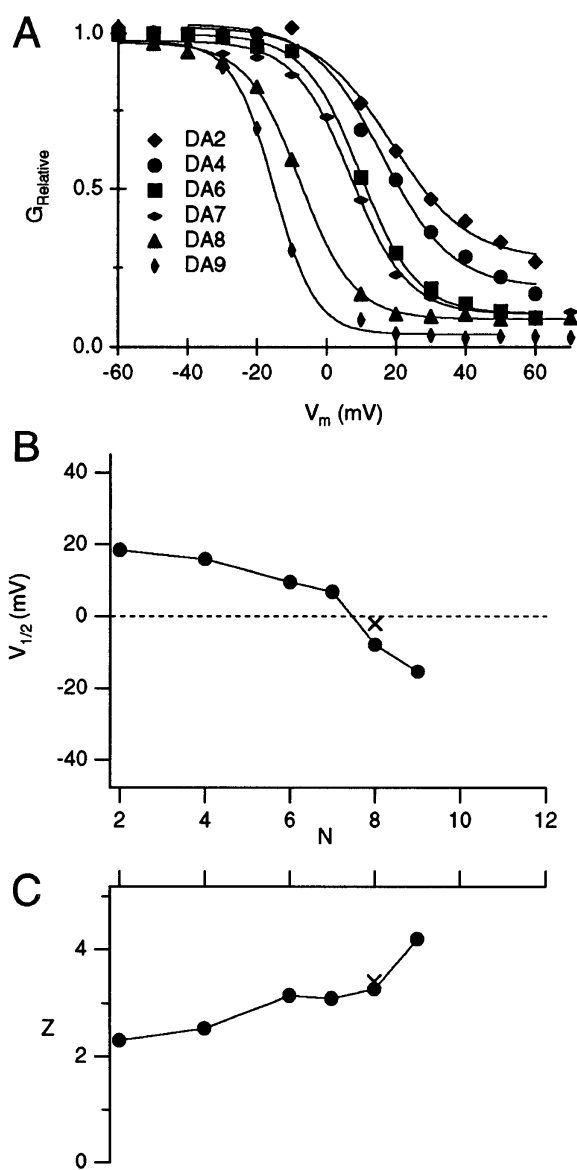


FIGURE 8. Diamino alkane blocking affinity and effective valency increase with chain length. (*A*) Relative conductance ( $G_{\text{Relative}}$ ; see text)-voltage relationships obtained for block of Kir2.1 currents by different alkyl chain length diamines at a concentration of  $100 \mu\text{M}$ . Smooth lines are best fits of a Boltzmann function (see text) to representative experiments in Fig. 7. (*B*)  $V_{1/2}$  of block as a function of alkyl chain length ( $N$ ) from fits to the data in *A*. (*C*) The effective valency of block ( $Z$ ) as a function of alkyl chain length ( $N$ ) from fits to the data in *A*. In *B* and *C*, X indicates the  $V_{1/2}$  and  $Z$  obtained for block by  $100 \mu\text{M}$  spermidine.

has been realized that rectification is a time-dependent process and that "activation" of the current at negative voltages can follow a multiexponential time course (Hagiwara et al., 1976; Stanfield et al., 1981; Kurachi, 1985; Ishihara et al., 1989; Oliva et al., 1990). Recent studies have shown that this intrinsic rectification is conferred by polyamines (spermine, spermidine, and putrescine), which block the channel from the intracel-



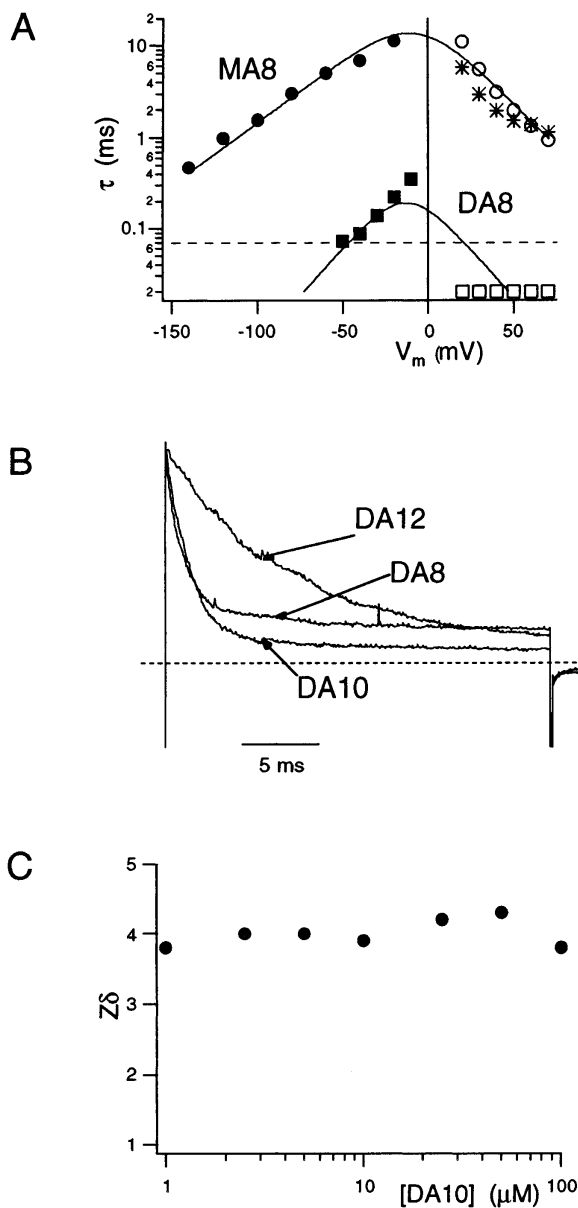


FIGURE 9. (A) Diamine block and unblock kinetics are much faster than monoamine kinetics. Time constants ( $\tau$ ) of block and unblock by 100 mM MA8 and DA8 obtained from experiments shown in Figs. 3, 6, 7, and 10. Plotted versus voltage. The line fitted to the MA8 data corresponds to a single site-blocking model with  $k_{on,0mV} = 5 \times 10^{-4} \text{ ms}^{-1} \cdot \mu\text{M}^{-1}$  and  $k_{off,0mV} = 0.03 \text{ ms}^{-1}$ ,  $Z_{on} = 2.2$ ,  $Z_{off} = 1.6$ . The line corresponding to DA8 is the same model with  $k_{on}$  and  $k_{off}$  100 $\times$  faster. The horizontal dashed line indicates the approximate limits of resolution for these experiments, so that block kinetics for 100  $\mu\text{M}$  DA8 were too rapid to measure (plotted below the line). Asterisks indicate  $\tau$  for block by 1  $\mu\text{M}$  DA8 (see text). (B) Representative current traces show that increasing the alkyl chain length slows channel block. Traces show block by 1  $\mu\text{M}$  DA8, DA10, and DA12 at 50 mV after a prepulse to  $-80$  mV. The capacitance transient has been digitally subtracted from the depolarizing pulses, and all traces have been scaled to the amplitude of current at  $-80$  mV. Dashed line indicates zero current level. (C) The valence of diamine block is independent of blocker concentration. Effective valence of block ( $Z$ ) by DA10 as a function of blocker concentration, obtained from a single patch subjected to a voltage protocol like that shown in Fig. 7.

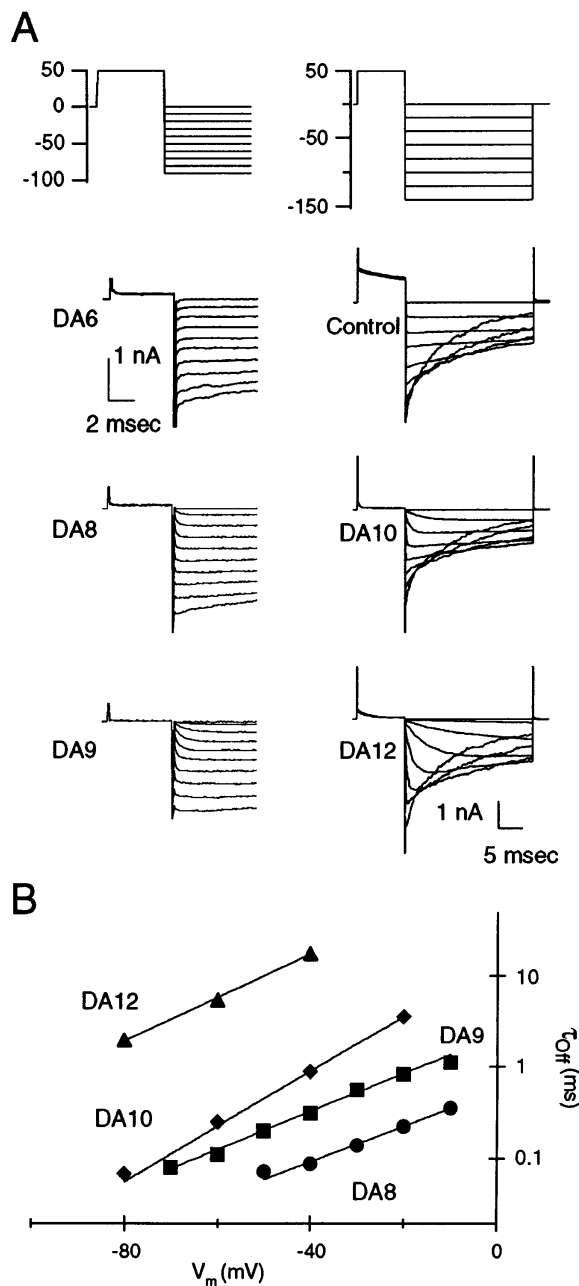


FIGURE 10. Diamine unblock kinetics are dependent on alkyl chain length. (A) Kir2.1 currents recorded from an inside-out membrane patch in response to voltage steps to voltages between 0 and  $-100$  or  $-150$  mV after a prepulse to  $+50$  mV, from a holding potential of 0 mV as indicated. The currents were obtained sequentially (DA6, Control, DA8, DA10, DA9, DA12) with exposure to 100  $\mu\text{M}$  diamines as indicated. Scale bars are 1 nA and 2 or 5 ms as indicated. (B) Time constant of unblock ( $\tau_{off}$ ) obtained from exponential fits (see text) to currents in A, plotted versus membrane potential ( $V_m$ ).

lular side of the membrane (Fakler et al., 1994, 1995; Ficker et al., 1994; Lopatin et al., 1994, 1995; Bianchi et al., 1996; Ishihara, 1996; Lopatin and Nichols, 1996; Shyng et al., 1996; Yamashita et al., 1996; Oliver et al., 1998). The strong rectification and time-dependent ac-

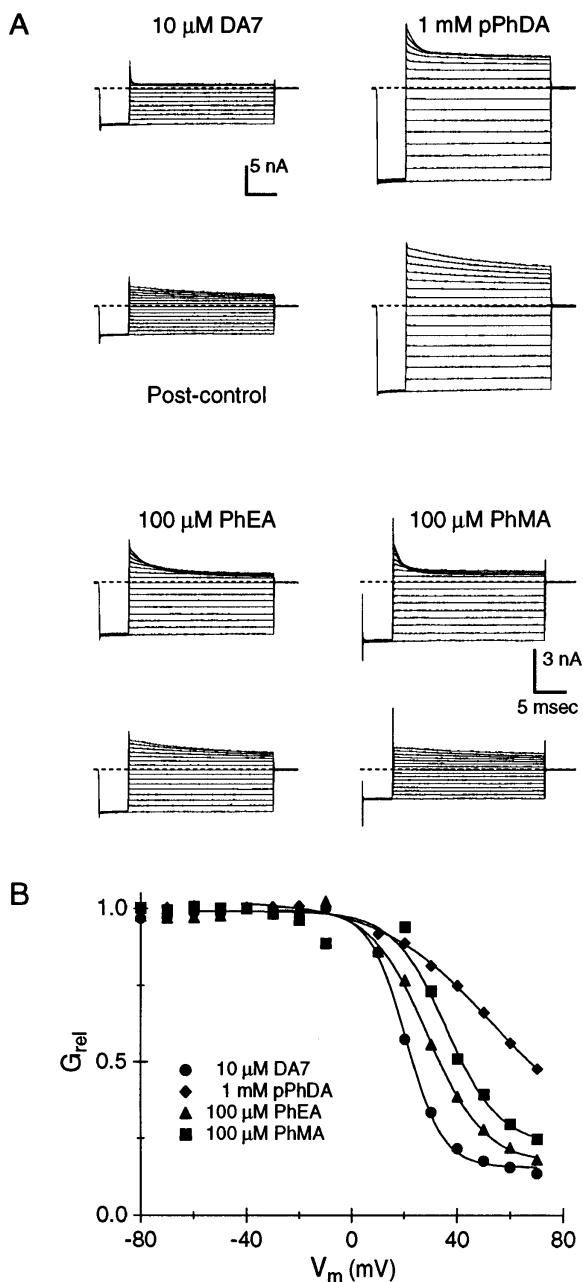


FIGURE 11. Block by phenyl amines is considerably weaker than block by alkylamines. (A) Kir2.1 currents recorded from an inside-out membrane patch in response to voltage steps to voltages between  $-80$  and  $+70$  mV after a prepulse to  $-80$  mV from a holding potential of  $0$  mV. Currents are shown in the presence of  $10 \mu\text{M}$  DA7,  $100 \mu\text{M}$  PhMA,  $100 \mu\text{M}$  PhEA, or  $1$  mM pPhDA. In each case, the post-control currents were obtained after washing out the test solution. Scale bars:  $5$  nA for pPhDA, PhEA, and DA7,  $2$  nA for PhMA, and  $5$  ms. (B) Steady state  $G_{\text{Relative}}$ -voltage relationships for the currents shown in A. The continuous lines are fits to the Boltzmann equation (see text).

tivation conferred by polyamines is relieved by increase of  $[\text{K}^+]$  on the outside of the membrane (Lopatin and Nichols, 1996; Oliver et al., 1998), and generally correlates with the presence of negatively charged residues

at a given position in the second (M2) transmembrane segments (Fakler et al., 1994; Ficker et al., 1994; Lu and MacKinnon, 1994; Lopatin et al., 1994; Shyng et al., 1997). While at least an additional residue in the COOH-terminal region is involved in polyamine binding (Tagliatela et al., 1995; Yang et al., 1995), mutating the critical M2 residues to a neutral glutamine generally decreases or abolishes polyamine-induced rectification. The potency and voltage dependence ( $z\delta$ ) of block increases from putrescine ( $z = +2$ ) to spermidine ( $z = +3$ ) to spermine ( $z = +4$ ), consistent with an electrostatic component to the blocking process. The first, and most consistent, result to come from the present study is that there is also a significant hydrophobic nature to the interaction. At  $0$  mV, the addition of one methyl group to either mono- or diaminoalkanes increases the blocking potency  $\sim 10$ -fold, corresponding to  $\sim 2.4$  kJ/mol. This is similar to the effect of increasing alkyl chain length on bis-quaternary amine block of Ca-activated and sarcoplasmic reticular  $\text{K}^+$  channels (e.g., French and Shoukimas, 1981; Miller, 1982). Studies in squid axon by Armstrong (1969) and on *Shaker* channels by Choi et al. (1993), using TEA analogues with one alkyl chain of variable length, also found that increasing the alkyl chain length increased the blocking affinity of the analogue, and that block was decreased by increasing the extracellular  $\text{K}^+$  concentration, indicating binding in the pore.

*The Mechanism of Polyamine-induced Inward Rectification: The Kir Channel Has a "Long Inner Pore" that May Accommodate Several  $\text{K}^+$  Ions, and Even the Longest Polyamines*

Biophysical and theoretical analyses of inward rectifier K channels have suggested that these channels consist of a long narrow pore (Hille and Schwartz, 1978; Hille, 1992), and this is now confirmed by the crystal structure of the bacterial KcsA  $\text{K}^+$  channel (Doyle et al., 1998). We previously suggested that "long pore plugging" by polyamines may account for rectification induced by the natural polyamines (Lopatin et al., 1995). The simple hypothesis was that, in addition to possible peripheral polyamine binding sites, a single polyamine should lay "vertically" inside the long narrow pore, binding through electrostatic interaction with a negatively charged binding site, and giving rise to the steeply voltage-dependent component of block. As we show here, both monoamines and diamines block Kir2.1 channels, with potency increasing as the alkyl chain length increases. This finding is inconsistent with the notion that block results from a purely electrostatic interaction with residues in the channel pore, and implies that some hydrophobic interaction of the alkyl chain stabilizes binding in the pore. Although somewhat of a surprise, the crystal structure of KcsA reveals

that the inner vestibule of the channel is actually very hydrophobic (Doyle et al., 1998), and this hydrophobic vestibule is indeed large enough to accommodate a molecule as large as spermine, in extended linear conformation (Nichols and Lopatin, 1997).

To account for the very high  $z\delta$  ( $\sim 5.4$ ) of spermine block, we previously suggested that two polyamine molecules may sequentially enter the channel pore to reach the fully blocked state. However, the strong interaction of polyamine block with external  $K^+$  ions (Lopatin and Nichols, 1996; Oliver et al., 1998) suggests an alternative probability, namely that the polyamine entry into, and binding in, the channel pore “sweeps”  $K^+$  ions outwards, contributing extra charge movement to the binding process (Ruppersberg et al., 1993). The present results indicate a large and gradual increase in  $z\delta$  (Fig. 8) of the steep part of the G-V curve, from a limiting minimal value of  $z\delta \sim 1.5$  ( $n = 2$  methylene groups) to  $z\delta \sim 4$  ( $n = 10$  methylene groups) as diamine alkyl chain length increases. It is not likely that the number of blocking particles changes (i.e., increases) as the blocking particles become larger, so the increase in  $z\delta$  cannot result from more charge being contributed by the blocking ion. Instead, the increase in charge associated with the blocking process may result from progressively more charge (i.e., permeating  $K^+$  ions) being swept out of the pore as the blocking particle increases in size (Ruppersberg et al., 1993).

Although the increase of potency of MA and DA compounds with increasing alkyl chain length is reminiscent of the block of delayed rectifier, Ca-activated, and sarcoplasmic reticulum K channels by substituted quaternary ammonium ions (e.g., Armstrong, 1969; French and Shoukimas, 1981; Miller, 1982), the significantly steeper voltage dependence of the block of the Kir channels (limiting  $z\delta = \sim 5.4$  for spermine, effective  $\delta \sim 1.3$ , compare  $\delta \sim 0.3$  for quaternary ammonium block of non-inward-rectifying K channels) may indicate significant mechanistic differences.<sup>3</sup> The results further contrast with those of Miller (1982), who found that for a series of bis-quaternary amines (similar structures to diamines, but with trimethyl amine groups replacing the amines),  $z\delta$  of block initially decreased as  $n$  increased from 2 to 5, and then increased again from 8 to 10 before saturating.

Miller’s (1982) results with bis-quaternary (bisQ) compounds could be accounted for by assuming that the “head” charge reaches a fixed binding site within the field, and that as the alkyl chain length increases, the

“tail” charge is pushed further and further out of the field until it is long enough and flexible enough to bend around and allow the tail charge to come back into the field. The much weaker voltage dependence of bisQ block of Maxi-K channels might suggest that bisQ compounds do not penetrate deeply into the Maxi-K channel pore. To account for the present results, we suggest that the relevant region of the pore of the Kir channel may be much narrower than in Ca-activated K channels, such that there is a longer physical distance of restricted ion flow, and over which the electrical field drops (Fig. 12). We hypothesize that this long pore allows even the longest DAs to remain in the field, and does not allow the “bend-over” phenomenon seen in the Maxi-K channel. In this case, in the absence of polyamines, the long pore may also accommodate more  $K^+$  ions than the short length of the pore that is bisQ accessible in the maxi-K channel. We further suggest that the DA charges may not occupy a single point in space or in the electric field, but may occupy a balanced position relative to the negative charges at the “rectification controller;” i.e., the two diamine-positive charges will be equidistant from the center of the ring of negative charges. Thus, as the alkyl chain length increases, a single DA will occupy more and more of the available space, with more and more  $K^+$  ions being displaced to the outside of the cell, moving more charge outwards and thereby increasing the net charge movement associated with channel block (Fig. 12). A similar suggestion could account for the results of Fakler et al. (1997) who observed that the apparent electrical distance for tetraalkylammonium block of Kir1.1 channels increased from 0.83 to 0.93 to 1.44 as the alkyl chain length increased from 2 to 3 to 4. In this case, the excess voltage dependence resulted from a slowed blocker off rate. The maximum diameter of the inner vestibule of the inward rectifier KcsA channel is in the order of 10 Å, and is accessed from the cytoplasm through a “tunnel” that is 18 Å long and  $\sim 5$  Å wide (Doyle et al., 1998). The impotency of pPhDA (Fig. 11) compared with hexyl-1,6-diamine (DA6, Figs. 7 and 8) may be in part due to the steric restrictions on access of the phenyl ring structure through the tunnel. The diameter of extended hexylamine is  $\sim 3$  Å, whereas the minimum diameter of the phenyl ring is  $\sim 5$  Å, similar to the dimensions of the access tunnel in the KcsA crystal structure (Doyle et al., 1998).

If the mechanism we propose is indeed causing increased steepness of voltage dependence with increase in diamine length, then how can we account for monamine block having a steepness of voltage dependence that is independent of alkyl chain length? We hypothesize that the major determinants of blocker depth within the inner pore are the charged groups on the blocker. Thus, for MAs, the single charge stabilizes at essentially

<sup>3</sup>Given that the blocking moiety in the polyamines is probably an unsubstituted amine (diameter  $\sim 2$  Å, similar to dehydrated  $K^+$ ), deeper access into the permeation pathway may be possible than for a tetramethylammonium group (diameter  $\sim 8$  Å, similar to hydrated  $K^+$ ).

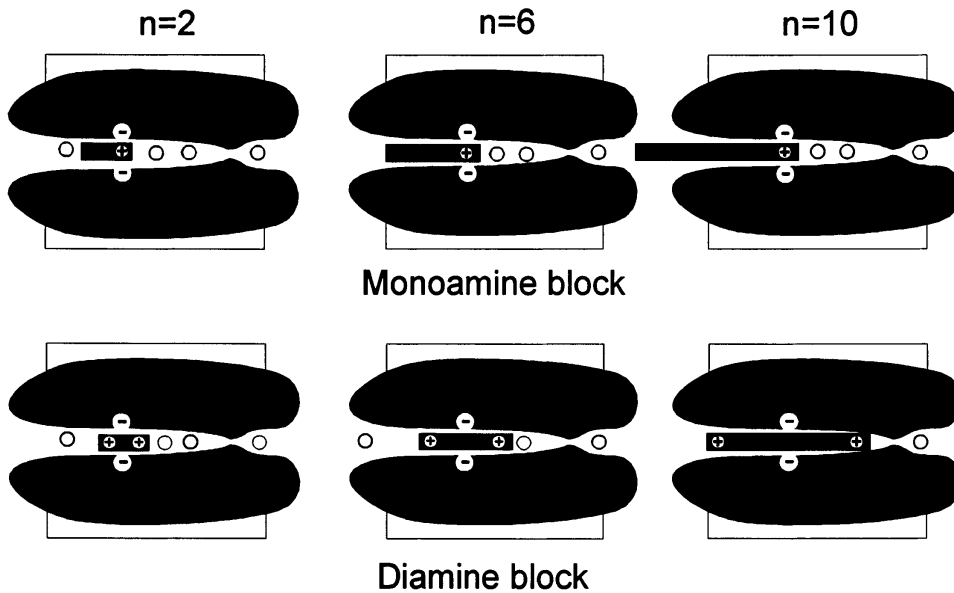


FIGURE 12. Accommodation of polyamines within the pore of Kir channels. Kir pores are hypothesized to have a long ( $>20$  Å) inner vestibule with strong electronegativity provided by the ring of negative charges at the “rectification controller” position (D172). While more peripheral negative charges (e.g., E224) may provide additional peripheral amine binding sites, a single diamine (*bottom*) can occupy the deep pore, the two amines being equidistant from the center of the ring of negativity at the rectification controller. As alkyl chain length increases, the head amine is pushed deeper into the pore, displacing  $K^+$  ions to the outside of the cell. Monoamines (*top*) can also occupy the pore, but the head amine remains in the ring of electronegativity provided by the rectification controller, the alkyl chain stretching back out of the pore.

the same depth (i.e., at the level of the “rectification controller” (the ring of four negative charges in the M2 segments)). The increasing alkyl chain then stretches further and further out of the pore, just as with monoQ block of the maxi-K channel, and the  $K^+$  ion displacing effect of increasing chain length observed with the DAs is absent (Fig. 12). Since the major determinant of the charge associated with block by long compounds is then due to sweeping out of  $K^+$  ions, so the present hypothesis predicts that block by triple or tetravalent species should differ from the divalent of the same length only by the additional  $\sim 0.5 z\delta$  resulting from moving the extra “middle” charge into the field. Indeed, we may compare the block produced by DA8 to the block of spermidine (which contains a total of seven methylene and one additional amine group). At the same con-

centration, the blocking potency and steepness are indeed comparable for both molecules (Fig. 8).

#### Conclusions

The present results, examining channel block by cytoplasmic alkylamines of different alkyl chain length, indicate that polyamine-induced steeply voltage-dependent rectification of inward rectifier channels has a significant hydrophobic component, in addition to electrostatic binding of the charged amines. Moreover, the length of the alkyl chain is an important determinant of the charge movement associated with channel block by *n*-alkyl-1,*n*-diamines. We propose a hypothesis to explain this phenomenon whereby the increasing alkyl chain displaces increasing numbers of  $K^+$  ions towards the outside of the membrane.

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