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Molecular Basis of Inward Rectification: Structural Features of the Blocker Defined by Extended Polyamine Analogs

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ABSTRACT

Polyamines cause inward rectification of Kir K^+ channels by blocking deep within the channel pore. We investigated structural constraints of polyamine block of strongly rectifying mutant K_{ATP} channels (Kir6.2[L164C,N160D,C166S] + SUR1). We studied three groups of polyamine analogs: 1) conformationally restricted linear tetra-amines with a cycloalkyl or alkene group between the second and third amines (CGC-11047, CGC-11093, CGC-11099, and CGC-11098), 2) conformationally restricted linear deca-amines with a cycloalkyl or alkene group between the fifth and sixth amines (CGC-11150, CGC-11179, and CGC-11241), and 3) cyclic tetra-amines (CGC-11174, CGC-11197, CGC-11199, and CGC-11254). All linear analogs cause a voltage-dependent block similar to that of spermine, but slightly weaker (at 1 μM, $V_{1/2}$ for spermine block = -10 ± 1 mV, $Z = 2.9 \pm 0.1$, $n = 19$; $V_{1/2}$ for polyamine analogs varies from -7 to $+10$ mV, $Z = 2.6$–3.9). These data indicate tolerance for conformational restriction and an upper limit to the voltage dependence of the blocking process. There was no voltage-dependent block by the cyclic compounds; instead, they induce irreversible rundown of the current. Structural models of Kir channels suggest that a narrow entry at the top of the cytoplasmic pore may exclude cyclic analogs from the inner cavity, thereby explaining the structure-activity relationship that we observe.

Inward rectification of Kir channels is distinct from the voltage dependence of Kv channels in that conductance is not a function of the absolute membrane potential. Instead, conductance is 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^{2+}) (Nichols and Lopatin, 1997).

There is much evidence consistent with the concept that rectification is conferred by positively charged ions binding to or near negatively charged residues in the channel, but details are lacking and there is no consensus as to where exactly these ions bind (Kubo and Murata, 2001; Xie et al., 2002, 2003; Guo and Lu, 2003; Guo et al., 2003; Kurata et al., 2004). Mutagenesis has indicated residues involved in the blocking process, and recent structural studies demonstrate the nature of the permeation path of Kir channels, within which the polyamines must bind (Nishida and MacKinnon, 2002; Kuo et al., 2003; Pegan et al., 2005). Further information may be obtained by examining the blocking abilities of a large collection of polyamine analogs that have been developed primarily in efforts to find anticancer agents (Seiler et al., 1998). We demonstrated previously that only the polyamines conferred strong rectification, whereas other bulkier, dipolar or nonlinear molecules (e.g., GABA, creatinine, lysine) fail to block these channels (Lopatin et al., 1994). These results indicate that a molecule must possess both the correct structure and charge density or distribution to confer strong rectification. Systematic examination of monoaminoalkanes and diaminoalkanes with alkyl chains composed of 2 to 12 methylene groups further indicates that changes in molecular length and charge have profound effects on channel block, with longer chain lengths increasing blocking affinity (Pear-

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; CGC-11047, (*Z*)-3,7,12,16-tetraazaoctadec-9-ene tetrahydrochloride; CGC-11093, 3,8,13,18-tetraaza-10,11-[(*E*)-1,2-cyclopropyl] eicosane tetrahydrochloride; CGC-11098, 3,8,13,18-tetraaza-10,11-[(*Z*)-1,2-cyclopropyl] eicosane tetrahydrochloride; CGC-11099, 3,8,13,18-tetraaza-10,11-[(*E*)-1,2-cyclobutyl] eicosane tetrahydrochloride; CGC-11150, 3,8,13,18,23,28,33,38,43,48-decaaza-(25*Z*)-pentacontene decahydrochloride; CGC-11174, 4-tridecyl-1,5,9,14-tetraazacycloheptadecan-2-one trihydrochloride; CGC-11179, 3,7,11,15,19,24,28,32,36,40-decaaza-(21*Z*)-dotetracontene decahydrochloride; CGC-11197, 4-tridecyl-1,5,9,13-tetraazacyclohexaadecan-2-one trihydrochloride; CGC-11199, 4-tridecyl-1,5,9,15-tetraazacyclooctadecan-2-one trihydrochloride; CGC-11241, 3,8,13,18,23,28,33,38,43,48-decaaza-[25,26(*E*)-1,2-cyclopropyl] pentacontane decahydrochloride; CGC-11254, 1,5,13-trimethyl-9-propyl-6-tridecyl-1,5,9,13 tetraaza-cycloheptadecane tetrahydrochloride.

son and Nichols, 1998; Guo and Lu, 2000). Thus, the increased blocking affinity of spermine and spermidine, compared with diamines, is attributable not solely to increased blocker charge but also to increasing size or hydrophobic interactions.

The determination of high resolution structures of bacterial K channels (Doyle et al., 1998; Zhou et al., 2001a,b; Jiang et al., 2002a,b) provides a template for comparative modeling of Kir channel pores. Such models predict that polyamines may experience no major energetic barriers in the pore and may actually enter the selectivity filter in causing strong inward rectification, with longer polyamines binding progressively deeper (Dibb et al., 2003; Rose and Nichols, 2003). Experimental evidence for the ability of negative charges throughout the inner cavity, including the entrance to the selectivity filter (Kurata et al., 2004), to confer voltage-dependent block provides further consistency with this picture of inward rectification.

High-resolution structural determination also shows that the Kir cytoplasmic domain includes a ~30-Å extension of the channel pore into the cytoplasm (Nishida and MacKinnon, 2002; Kuo et al., 2003; Pegan et al., 2005), but the consequences of this extension for the mechanism of rectification are unclear. In the present study, we have examined the blocking ability of further series of more complex polyamine analogs in three structural groups. The data indicate additional important structural features of the blocking moiety and argue for a role of the cytoplasmic pore in controlling polyamine-induced rectification.

Materials and Methods

Expression of K_{ATP} Channels in COSm6 Cells. COSm6 cells were transfected with pCMV6b-Kir6.2 (with mutations as described), pECE-SUR1, and pGreenLantern (Invitrogen, Carlsbad, CA), as described previously (Loussouarn et al., 2000). Point mutations were prepared by overlap extension at the junctions of relevant residues by sequential polymerase chain reaction as described previously (Loussouarn et al., 2000). The 'control' Kir6.2 construct had a deletion of 36 amino acids from the C-terminal end, as well as N160D and C166S mutations (Loussouarn et al., 2000). An additional mutation (L164C) in M2 renders the channels very insensitive to ATP and excised patch currents extremely stable (Loussouarn et al., 2001). 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 to 2 Hz; signals were digitized at 3 to 5 kHz and stored directly on computer hard drive using Clampex software (Axon Instruments, Union City, CA). The standard bath (intracellular) and pipette (extracellular) solution used in these experiments had the following composition: 140 mM KCl, 10 mM K-HEPES, and 1 mM K-EGTA, pH 7.3.

Data Analysis. Off-line analysis was performed using Fetchan, pSTAT (Axon Instruments), and Microsoft Excel (Microsoft, Redmond, WA). Wherever possible, data are presented as mean ± S.E.M. Microsoft Solver was used to fit data by least-square algorithm.

Chemicals. CGC- (previously SL) compounds were synthesized as described previously (Valasinas et al., 2001, 2003; Frydman et al., 2004). TB34, which was synthesized by the method of Igarashi et al. (1997), was kindly provided by Kazuei Igarashi (Chiba University, Chiba, Japan). Spermine was purchased from Fluka AG (Buchs, Switzerland).

Results

We examined the block of a strong inward rectifier channel generated by coexpression of Kir6.2[N160D, L164C, C166S, Δ36] (Kir6.2-rec) with SUR1 (Loussouarn et al., 2001) by internally applied CGC- compounds (Fig. 1), with the objective of comparing their blocking potency and voltage dependence with the prototypical spermine.

Alkyl Extended Linear Analogs Produce Spermine-Like Block. We first examined the blocking abilities of a series of conformationally restricted tetra-amine analogs with bis-ethyl extensions of the primary amines (Fig. 1, group 1). These compounds are flexible linear analogs (~22-Å extended length), but minimum width of the molecule is >2.2 Å, even at the central cyloalkyl groups. Two voltage protocols (Fig. 2, A and B) were used to determine both the steady -block and the blocking and unblocking kinetics. As is appar-

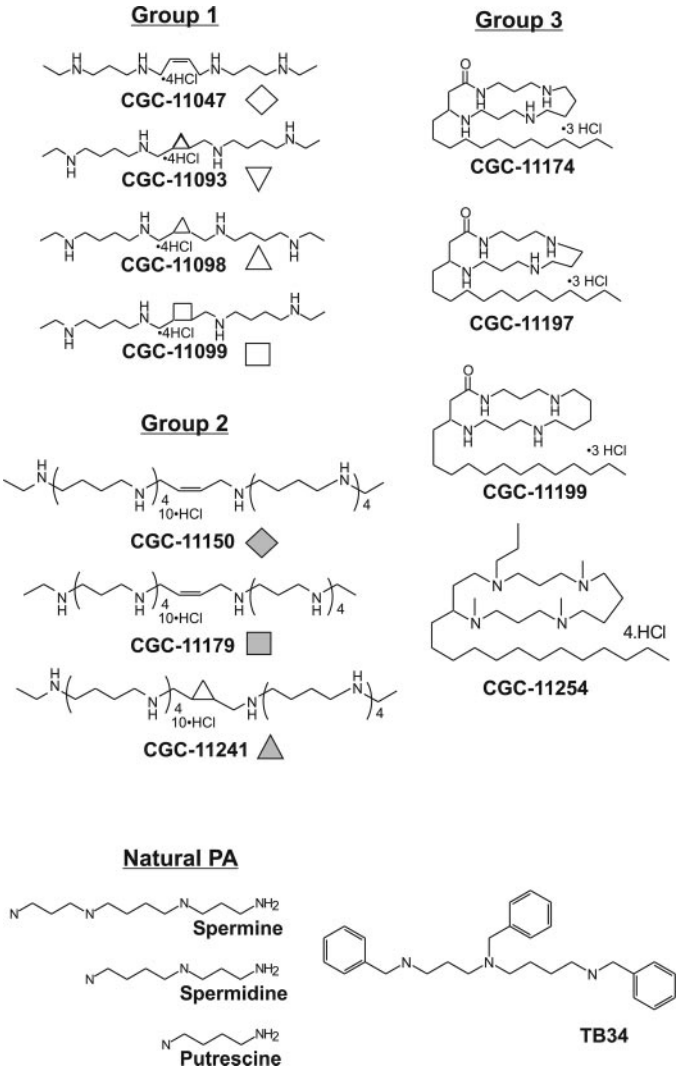

Fig. 1. Polyamine analogs examined in this study. Group 1, conformationally restricted linear tetra-amines with modified alkyl chains between the second and third amine. Group 2, conformationally restricted linear deca-amines with modified alkyl chains between the fifth and sixth amines. Group 3, cyclic tetra-amines. Natural polyamines are shown for comparison. TB34, a polyamine analog known to block NMDA channels in a voltage-dependent fashion has also been assayed. The symbols assigned to each of the group 1 and 2 molecules are used in subsequent figures, to aid the reader.

ent from the recordings in Fig. 2, the tetra-amine class 1 analogs, exemplified by CGC-11047 (Fig. 2, A and B) all caused voltage-dependent rectification of Kir6.2-rec with a voltage-dependence and potency that was similar to spermine (Fig. 3A). Using a single Boltzmann function to fit Grel-V curves, the estimated voltage-dependence of spermine block ($z\delta \sim 2.9$) is slightly lower than has previously been reported (Phillips and Nichols, 2003), which is probably the result of the use of a lower concentration of spermine (1 μM) than may be typically examined. Except for CGC-11047, all showed a slightly reduced potency compared with spermine that was accounted for by ~2-fold slowing of the on rate and increase of the off rate. An immediate conclusion from these experiments is that a free primary amine at the end of the blocking molecule is not a requirement for steep inward rectification (see *Discussion*). Likewise, a steric restriction in the center of the molecule does not significantly impede block. The relatively enhanced potency of CGC-11047 compared with the other group 1 compounds may be related to the spermine-like propyl groups between the primary and secondary amines.

Very Long Deca-Amines Indicate a Limiting Steepness to Rectification. Previous studies of rectification induced by diamine analogs indicate increasing voltage-dependence of block, as alkyl chain length is increased from 2 to 10 (i.e., up to the length of spermine) (Pearson and Nichols, 1998; Guo and Lu, 2003; Guo et al., 2003). The availability of a series of extended deca-amines (Fig. 1, class 2) affords the opportunity to test whether much longer, or more charged, polyamines can cause much steeper rectification. The extended length of these molecules is ~56 Å, but their minimal width is >2.2 Å. Figure 2, A and B, shows recordings from one example (CGC-11150) of these conformationally restricted deca-amines. Again, the blocking characteristics are similar to those of spermine, with a similar apparent gating charge but with faster on and off rates (see *Discussion*) underlying a slightly less potent block (Fig. 3). The similarity of apparent gating charge for CGC-11150, CGC-11179, and CGC-11241, to spermine, even though these compounds have 10 positive charges instead of 4, is striking and implies an upper limit to the charge movement associated with block is reached in these compounds (see *Discussion*). Equally strik-

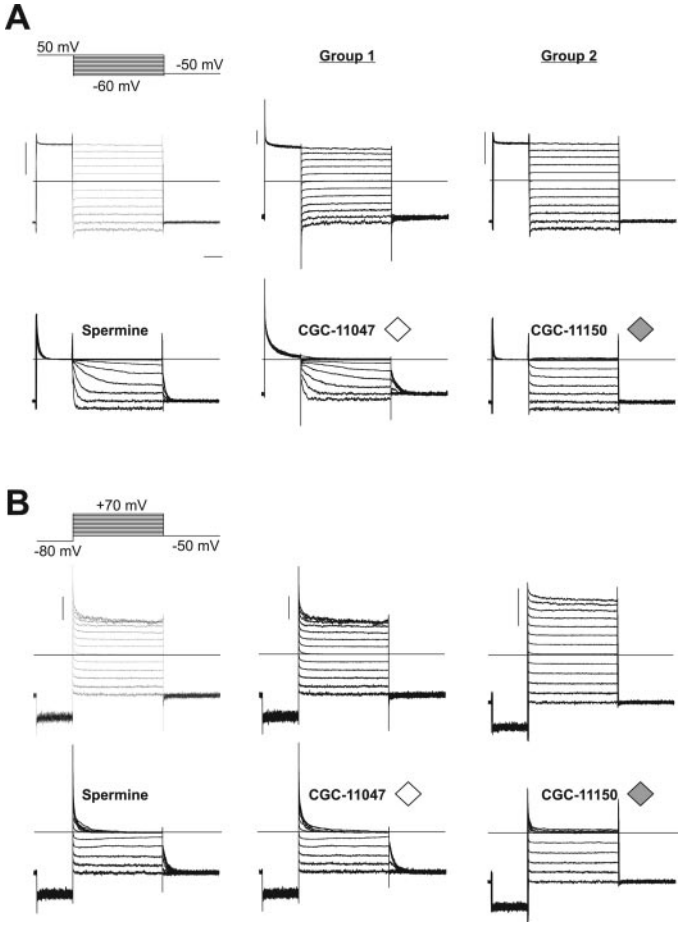

Fig. 2. Recordings of voltage-dependent block by spermine and by representative group 1 (CGC-11047) and group 2 (CGC-11050) polyamine analogs, using two distinct voltage step protocols. A, voltage steps to +50 mV causes polyamine block, then hyperpolarizing steps to voltages between +50 and −60 mV were used to examine the kinetics of unblock. B, voltage steps to −80 mV causes polyamine unblock, then depolarizing steps to voltages between −50 and +70 mV were used to examine the kinetics of block. In A and B, currents are shown in the absence (top) or presence (bottom) of 1 μM spermine or analog. Calibration bars, 250 pA and 100 ms.

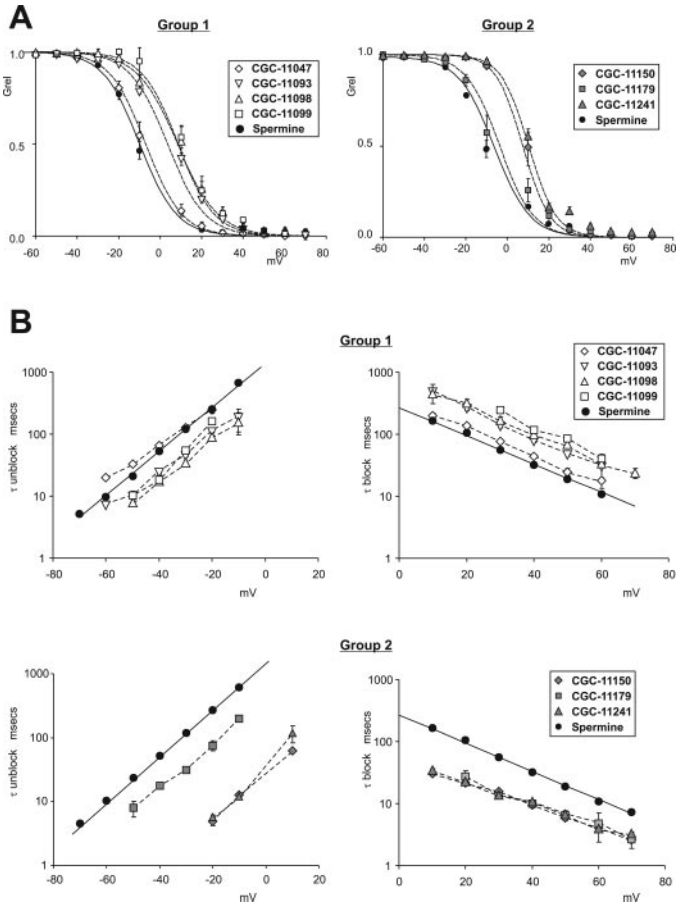

Fig. 3. Characteristics of voltage-dependent block by spermine and group 1 and 2 polyamine analogs. A, relative conductance (Grel, mean ± S.E.M., n = 5–10 for analogs, 16 for spermine) versus voltage relationships for spermine and group 1 (left) and group 2 (right) analogs, from records as in Fig. 2. Dashed and solid lines correspond to the averaged fits of Boltzmann functions to the Grel-V curves for individual patches. B, time constant of block (right) and unblock (left) obtained from single exponential fits to current relaxations, plotted versus membrane potential (mean values from n = 2–5 patches, ± S.E.M.). The solid line corresponds to exponential fit for mean spermine block/unblock rates versus membrane potential (n = 16) ($z\delta_{on}$ = 1.3 and $z\delta_{off}$ = 2.0).

ing is the considerable difference in the off rates of two compounds (>10-fold; Fig. 3), which differ only in the number of carbon atoms spacing the amines (three versus four) and in the *cis* (CGC-11179) versus *trans* (CGC-11150) arrangement of the penta-amine extensions. It is interesting that CGC-11241, which has the same structure as CGC-11150 but contains a cyclopropyl group in place of the *cis*-ethenyl group, has almost identical kinetics and potency to CGC-11150 (Fig. 3).

Cyclic Analogs Do Not Block the Channel. In sharp contrast to the group 1 and 2 analogs, CGC-11174, CGC-11197, and CGC-11199 induce no voltage-dependent block of the channel at a concentration of 1 μM (Fig. 4), suggesting that they do not enter the channel pore. At this concentration, however, they do provoke a rundown of the channel [such as polylysine (Shyng and Nichols, 1998)], possibly by screening the phospholipid charges that keep the channel open. An action of these compounds on the membrane lipids is suggested by a separate series of experiments, in which we applied each of the CGC- compounds to *Xenopus laevis* oocytes. At a concentration of 100 μM, none of the group 1 or 2 molecules had any noticeable effect, but each of the group 3 molecules caused a breakdown of the membrane and death of the oocytes within 2 h (data not shown).

Each of the above group 3 compounds contains a carbonyl group, which could be the cause of the lack of rectification induction. However, another related compound CGC-11254 (Fig. 1), lacking the carbonyl group, also failed to induce rectification at 1 μM (Fig. 5A) and caused rapid breakdown of the membrane patch at higher concentrations.

These results suggest that the cyclized polyamine analogs, with a minimal diameter across the cyclic group of ~5.5 Å, may not access the necessary site for induction of rectification, perhaps because they do not enter the inner cavity. Another interesting molecule in this regard is TB34, a derivative of spermidine, with a benzyl group attached to each

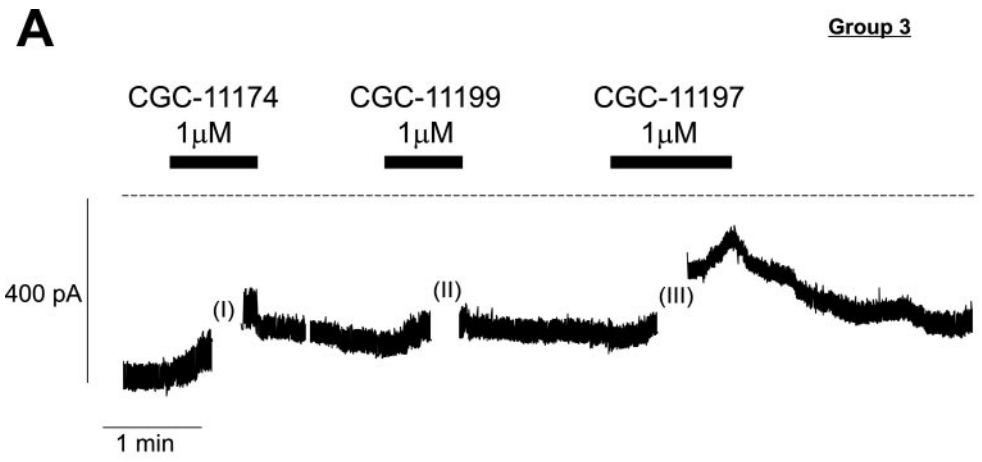

Fig. 4. Group 3 polyamine analogs (cyclic tetra-amines) induce no voltage-dependent block. A, recording of a patch expressing Kir6.2-rec with sequential application of group 3 polyamine analogs. Inward currents recorded at −50 mV are shown, and zero current is indicated by a dotted line. B, normalized current from ramp protocols (−100 mV to +50 mV in 4 s) in the absence or presence of polyamines analogs shows no voltage-dependent block.

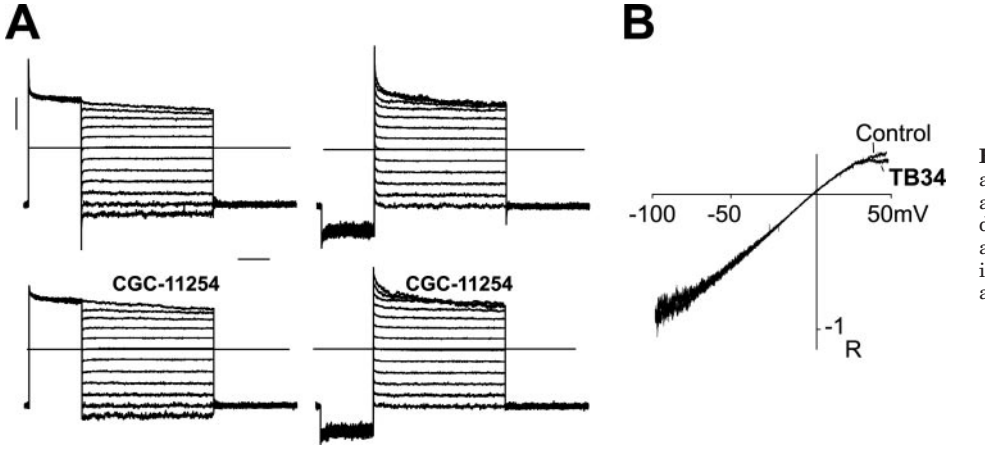

Fig. 5. A, recordings of currents in absence and presence of 1 μM CGC-11254 using voltage protocols as in Fig. 2, show no voltage-dependent block. Calibration bars are 1 nA and 100 ms. B, ramp protocols as in Fig. 4B, in the absence and presence of 10 μM TB34, also show no voltage-dependent block.

nitrogen. This molecule causes potent and steeply voltage-dependent block of NMDA receptor channels (Igarashi et al., 1997). However, with a minimal diameter of ~2.8 Å across the benzyl ring, it also failed to induce any rectification of Kir6.2-rec channels (Fig. 5B).

Discussion

Similarity of Rectification Induced by Extended Polyamines. Strong inward rectification of Kir channels is conferred by the naturally occurring polyamines (spermine, spermidine, and putrescine), which block the channel from the intracellular side of the membrane (Nichols and Lopatin, 1997). Molecular modeling, based on the crystal structures of KcsA and MthK (Doyle et al., 1998; Zhou et al., 2001; Jiang et al., 2002), has led us to propose that the voltage dependence of polyamine block arises primarily from entry of the compounds into the ion selectivity filter, thereby displacing charge-carrying K^+ ions from the filter, to the outside of the membrane (Rose and Nichols, 2003). This view has been challenged, however. At first, Nishida and MacKinnon (2002) suggested that polyamines should bind in the extended cytoplasmic pore of the channel and that the voltage dependence of block results from the obligate movement of a column of K^+ ions that extends from this region, through the inner cavity and selectivity filter. This view has been modified and further argued, in light of energetic coupling experiments, by Lu and colleagues (Guo and Lu, 2003; Guo et al., 2003).

The present experiments add more insight to the potential location and structural basis of polyamine block. Previous studies have indicated a requirement for a linear polyamine structure for steep voltage dependence of block (Lopatin et al., 1995; Pearson and Nichols, 1998). An untested possibility is that a primary 'head' amine must enter the blocking site first. This is clearly not required, because tetra-alkylamines with bis-ethyl extensions (group 1) can cause rectification that is as steep, and almost as potent, as spermine (Fig. 3). The voltage dependence of on and off rates for each of these compounds is remarkably consistent and similar to that of spermine (Fig. 3B). Further patterns emerge in the details: 1) the central restriction in group 1 molecules, whether cycloalkyl or an unsaturated double bond, seems not to limit the blocking ability. This may be expected if only the first alkyl segment (i.e., between N1 and N2) is required to enter the very restricted space of the selectivity filter, with the remainder of the molecule accommodated in the much wider inner

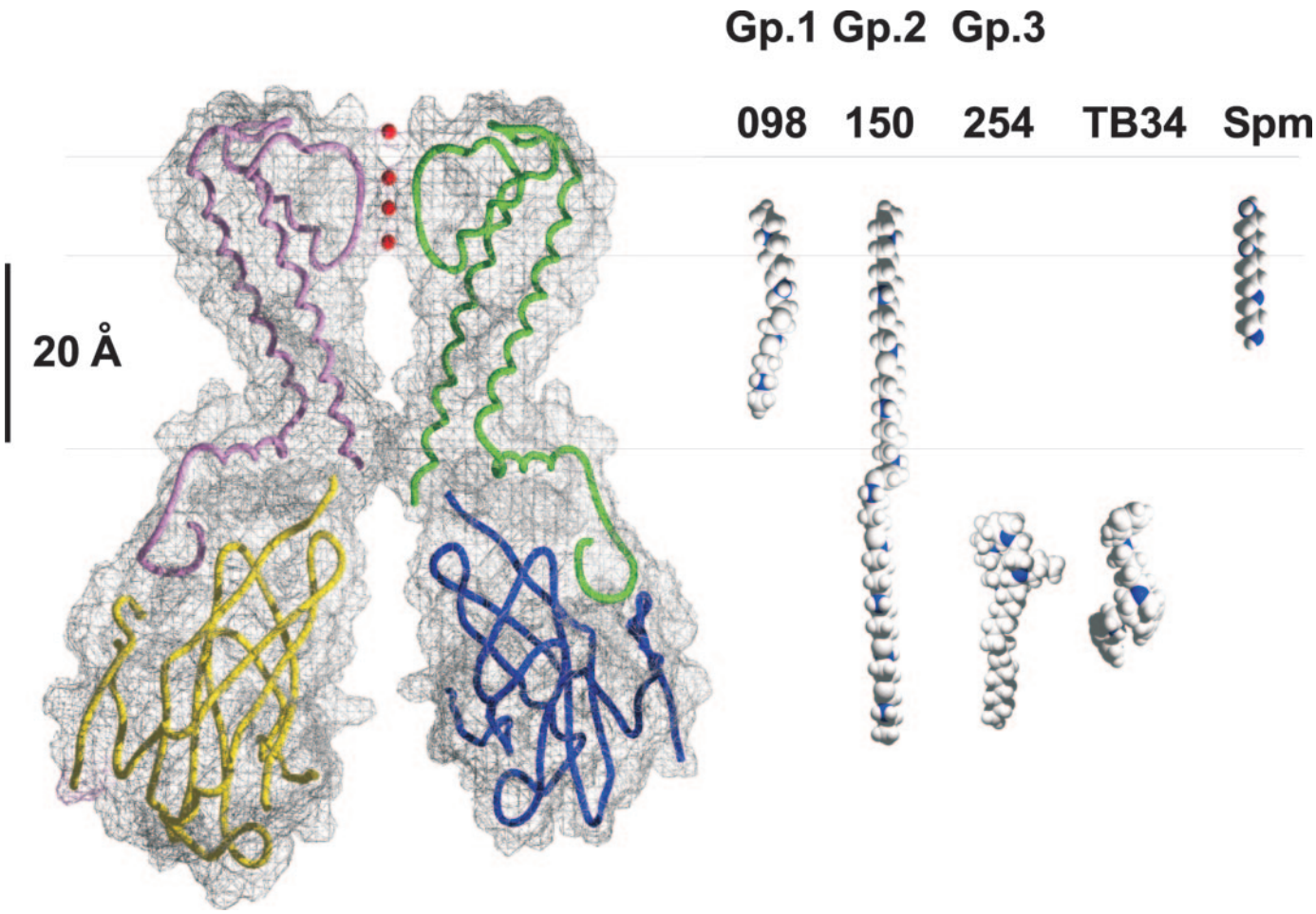

Fig. 6. Potential structural basis of polyamine block. Left, three-dimensional structure of the bacterial KirBac1.1 channel (Kuo et al., 2003) shown for two opposing subunits in ribbon and space-filling representation, together with representative polyamine analogs and spermine. It is envisioned that only the group 1 (CGC-11098) and 2 (CGC-11150) analogs as well as spermine can access the inner cavity and the deep binding site at the entrance to or in the selectivity filter (Kurata et al., 2004). Group 3 (CGC-11254) and TB34 analogs may be excluded from the inner cavity by the restriction at the top of the cytoplasmic pore (Pegan et al., 2005).

vestibule. 2) There is a systematic consequence of the first alkyl chain being a propyl (C_3) or butyl (C_4) group; the C_3 compounds (CGC-11047, spermine) are systematically more potent (approximately 5- to 10-fold slower off rate) than C_4 compounds (CGC-11093, CGC-11098, and CGC-11099). The same difference in potency is observed for C_3 (CGC-11179) versus C_4 (CGC-11150 and CGC-11241) deca-amines. It is tempting to speculate that this difference results from the more optimal N-N spacing with C3 linkers for accommodation in the K^+ binding sites in the filter (Fig. 6).

A Limiting Voltage Dependence of Rectification. Previous studies with diaminoalkanes indicate that the charge movement associated with block increases essentially monotonically as chain length increases, up to at least $Z = 4$ or 5 for C_{12} or spermine (Pearson and Nichols, 1998; Guo and Lu, 2003; Guo et al., 2003; Rose and Nichols, 2003; Kurata et al., 2004). The possibility that longer diamines might move yet more charge is not readily testable, because solubility becomes a problem, and there may be significant partition into the membrane, making experiment and interpretation difficult. However, the deca-amines studied here (group 2), with C_4 or C_3 alkyl spacers are readily soluble, and allow some examination of these questions. The blocking potency and voltage-dependence are strikingly similar to those of the tetra-alkylamines.

However, both on and off rates are speeded up ~4- to 10-fold compared with the tetra-amines. The extreme length of deca-amines makes it necessary that even if the head group can reach the selectivity filter, the tail will still be in the cytoplasmic pore (Nishida and MacKinnon, 2002). There is clearly a role for this region in controlling on and off rates of polyamines from the deep binding sites (Yang et al., 1995; Kubo and Murata, 2001; Xie et al., 2002; Kurata et al., 2004), and the possibility exists that prepositioning of the long deca-amine in the cytoplasmic pore is responsible for speeding both on and off rates. In this regard, it is important to note that in Kir2 family members, residues within the cytoplasmic pore [Glu224 and Glu299 (Yang et al., 1995), and the recently identified Asp255 and Asp259 (Pegan et al., 2005)] seem to control the potency of polyamine block, a result that may be interpreted to imply a role of these residues in forming the PA binding site (Guo et al., 2003). However, it is also conceivable that these residues instead form a 'predocking' site for polyamines (Xie et al., 2003; Kurata et al., 2004), thereby controlling the apparent affinity of a deeper site. Differential interactions of polyamines and CGC- analogs with cytoplasmic cavity residues could affect the estimated affinities of the deeper blocking site.

Sizing the Channel Entry. Figure 6 shows the structure of KirBac1.1 (Kuo et al., 2003), a model for eukaryotic Kir channels, together with space-filling models of representative polyamine analogs. All of the structures are quite flexible, with the exception of the cycolalkyl ring segments (in group 1 and 2), which are rigid planes, and the cyclic (group 3) compounds. The cycloalkyl rings cause a 'kink' of 10 to 20° in what is otherwise the straightest conformations (as can be seen for CGC-11098 versus spermine in Fig. 6), but they do not limit the overall flexibility of the molecule; all of the group 1 and 2 compounds are very flexible and can adopt very twisted as well as relatively straight conformations. The cyclized group 3 compounds maintain a planar cyclized segment, when analyzed dynamically, that is ~9.5 Å long by ~5.5 Å at the widest. All group 1 and 2 compounds have a maximum diameter of less than ~2.2 Å, even at the cyclobutyl group of CGC-11098. TB34 is also a flexible structure, except for the benzyl rings which have a minimum diameter of ~2.8 Å (C-C) across the ring.

The cytoplasmic extension of the Kir pore that is generated by the cytoplasmic domain (Nishida and MacKinnon, 2002; Kuo et al., 2003; Pegan et al., 2005) meets the inner cavity at what may be the region of the channel gate (Phillips and Nichols, 2003; Phillips et al., 2003). Relatively large, symmetric quaternary ammonium ions, at least up to tetrapentylammonium, can block Kir channels (Oliver et al., 1998; Guo and Lu, 2001) and in doing so must at least enter the cytoplasmic pore and block either at the top of the cytoplasmic pore (Guo and Lu, 2001) or in the inner cavity (Zhou et al., 2001). Because 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide-propyl triethylammonium can modify cysteine residues inside the inner cavity, it seems most likely to us that these molecules can cross into the inner cavity; presumably, however, there is a limit to the size of molecules that can gain entry. This seems to be exceeded by the cyclized group 3 compounds. None of these cause any voltage-dependent block, despite their similar chemical composition compared with the linear tetra-alkylamines (group 1) and considerably smaller size compared with the group 2 deca-amines (Figs. 4 and 5). The inability of TB34 to induce block, although it is a potent and steeply voltage-dependent blocker of NMDA receptor channels (Igarashi et al., 1997), suggests that the lower limit may be reached at the benzyl group, with a minimum diameter of ~2.8 Å.

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