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Inward Rectification of Both AMPA and Kainate Subtype Glutamate Receptors Generated by Polyamine-Mediated Ion Channel Block

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Summary

Ca^{2+}-permeable glutamate receptors assembled from subunits containing a GLN residue at the RNA editing site in membrane domain 2 show strong inward rectification. In HEK 293 cells transfected with the kainate receptor subunit GluR6(Q), inward rectification is lost in outside-out patches, suggesting a role for diffusible, cytoplasmic factors. Inclusion of different polyamines in the internal solution restored inward rectification, whereas Mg^{2+} (1 mM) was inactive. Spermine (K_d[0 mV] = 5.5 μM) was of higher affinity than spermidine (K_d[0 mV] = 25.4 μM) or putrescine (K_d[0 mV] = 1.2 mM). AMPA receptors assembled from $GluRA_{flip}$ showed even higher affinity for spermine (K_d[0 mV] = 1.5 μM). Analysis of the voltage dependence of whole-cell responses predicted intracellular free spermine and spermidine concentrations of 51 and 153 μM, respectively.

Introduction

Transcripts of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) and kainate receptor subunits, which contain an RNA editing site in an exon encoding part of the ion channel pore, have recently been shown to undergo oxidative deamination of adenosine to inosine at the editing site (Sommer et al., 1991; Higuchi et al., 1993; Melcher et al., 1995; Rueter et al., 1995; Yang et al., 1995). The resulting codon change, which causes substitution of an ARG for GLN residue in the GluR-B, GluR5, and GluR6 subunits (Sommer et al., 1991; Higuchi et al., 1993), has profound functional consequences for ion flow because the ARG residue encoded at the editing site regulates AMPA and kainate receptor channel rectification and Ca^{2+} permeability (Hollmann et al., 1991; Hume et al., 1991; Verdoorn et al., 1991; Burnashev et al., 1992b; Dingledine et al., 1992; Sommer et al., 1992; Egebjerg and Heinemann, 1993; Köhler et al., 1993; Jonas et al., 1994). The discovery that, as reported previously for the kainate receptor subunit GluR5 (Paschen et al., 1994), the extent of editing of GluR-B mRNA is also regulated in a tissue and developmentally specific pattern (Nutt and Kamboj, 1994) suggests that in the developing CNS the expression of Ca^{2+}-permeable AMPA and kainate receptors may be more widespread than generally recognized.

Although functional studies on native AMPA receptors typically show outward rectification with low permeability to Ca^{2+}, strong inwardly rectifying AMPA receptor responses with high Ca^{2+} permeability have recently been observed in subpopulations of hippocampal interneurons (Iino et al., 1990; Ozawa and Iino, 1993; McBain and Dingledine, 1993) and in Bergmann glial cells (Burnashev et al., 1992a; Muller et al., 1992). Native kainate receptors are less well characterized, but strong inwardly rectifying responses characteristic of the unedited form of GluR6 have recently been described in embryonic hippocampal neurons and in glial progenitor cells (Lerma et al., 1993; Puchalski et al., 1994). The mechanisms underlying inward rectification of glutamate receptor responses have not been established. Hume et al. (1991) proposed that the pore region of AMPA receptors contains a binding site for divalent cations, which becomes masked by the positively charged ARG residue introduced into edited subunits. Consistent with this, there is data suggesting that channel block by intracellular Mg^{2+} contributes to inward rectification for certain families of K^+ channels and for neuronal nicotinic acetylcholine receptors (Matsuda et al., 1987; Vandenberg, 1987; Mathie et al., 1990; Ifune and Steinbach, 1991). However, in sympathetic neurons and PC12 cells, nicotinic receptor inward rectification results for the most part from an unidentified but strongly voltage-dependent mechanism, which functions in combination with a modest voltage dependence of ion channel gating with relatively weak ion channel block by intracellular Mg^{2+} (Ifune and Steinbach, 1990; Mathie et al., 1990; Ifune and Steinbach, 1991; Mathie et al., 1991; Ifune and Steinbach, 1992; Sands and Barish, 1992).

Intracellular dialysis with Mg^{2+}-free solutions containing ATP, but not ADP, substantially reduces inward rectification for neuronal nicotinic receptors expressed in PC12 cells (Sands and Barish, 1992; Ifune and Steinbach, 1993). The interpretation of the latter result has remained ambiguous; one possibility is that rectification is regulated by phosphorylation of acetylcholine receptors or by an interaction of nicotinic receptors with other proteins sensitive to ATP; alternatively, ATP could chelate an unidentified intracellular cation distinct from Mg^{2+}, which produces inward rectification by ion channel block. Recent studies have revealed channel block by intracellular polyamines to be a major determinant of voltage-dependent ion flux in a family of inwardly rectifying K^+ channels, with block by Mg^{2+} playing a secondary role (Fakler et al., 1994; Ficker et al., 1994; Lopatin et al., 1994). Of particular relevance to prior work on nicotinic receptors was the demonstration that ATP chelates spermine (Watanabe et al., 1991), and that in the case of K^+ channels this is sufficient to account for the reduction of inward rectification by ATP (Fakler et al., 1995). The loss of inward rectification observed when the cytoplasmic face of outside-out patches from Xenopus oocytes expressing AMPA receptors was bathed in a simple salt solution with a low concentration of divalent cations (Burrill et al., 1993., Soc. Neurosci., abstract) raised the possibility that either Mg^{2+} or a diffusible cytoplasmic factor

might block current flow-through channels assembled from the unedited forms of glutamate receptor subunits. To address this, we have analyzed the sensitivity of recombinant AMPA and kainate receptor subunits to polyamines and to Mg^{2+}. Our results show that at concentrations known to be present in mammalian cells, the polyamines spermine and spermidine can completely account for the complex rectification properties of AMPA and kainate receptors assembled from subunits for which the GLN/ARG site in the second membrane domain is in its unedited form. In contrast to results obtained for inwardly rectifying K^+ channels, glutamate receptors show essentially no block by 1 mM $[Mg^{2+}]_i$.

Results and Discussion

A Diffusible Cytoplasmic Factor Accounts for Inward Rectification

Following transfection with the kainate receptor subunit GluR6(Q), HEK 293 cell responses to kainate and glutamate were rapidly and strongly desensitizing. In contrast, responses to domoate showed only moderate desensitization (28.4% ± 2.6%, mean ± SD; n = 7), such that at −60 mV, large amplitude currents were evoked in the continued presence of agonist (Figure 1A, left). Voltage ramps applied during the plateau response to domoate in whole-cell recording conditions revealed strongly rectifying responses similar to those described previously (Köhler et al., 1993). Leak subtracted current–voltage (I–V) relationships for whole-cell responses to domoate (Figure 1B) showed three distinct regions. First, at membrane potentials more negative than −50 mV, the I–V relationship was close to linear, but with an extrapolated reversal potential about 20 mV more negative than measured experimentally (the reversal potential for whole-cell responses to domoate was −2.4 ± 1.6 mV, mean ± SEM; n = 7); second, between +10 and +40 mV, the slope of the I–V relationship was close to zero, even though there were small, clearly detectable outward currents; third, above +50 mV, the amplitude of outward currents increased progressively on depolarization to +100 mV. To allow comparison of the complex behavior of the I–V relationship in experiments using different recording conditions, chord conductance ratios (G_r) at −50, +50, and +100 mV were calculated with respect to the chord conductance at −100 mV (Table 1).

In contrast to results obtained for Mg^{2+}-sensitive inward rectifier K^+ channels (Fakler et al., 1994, 1995; Ficker et al., 1994; Lopatin et al., 1994), the strong inward rectification of whole-cell responses to domoate was lost following the excision of outside-out membrane patches with 1 mM Mg^{2+} present in the internal solution (Figure 1A, right), suggesting that a cytoplasmic diffusible factor other than Mg^{2+} is responsible for inward rectification of glutamate receptor responses. Consistent with this hypothesis, and similar to studies on K^+ channels, the inclusion of 100 μM spermine in the patch-pipette solution restored strong inward rectification to domoate responses in a manner qualitatively similar to responses obtained with whole-cell recording (Table 1). In outside-out patches, the I–V relationship for responses to 50 μM domoate in the absence of spermine was close to linear at potentials between −100 to +50 mV, with weak outward rectification at membrane potentials between +50 and +100 mV (Figure 1C). Chord conductance ratios at −50, +50, and +100 mV had values

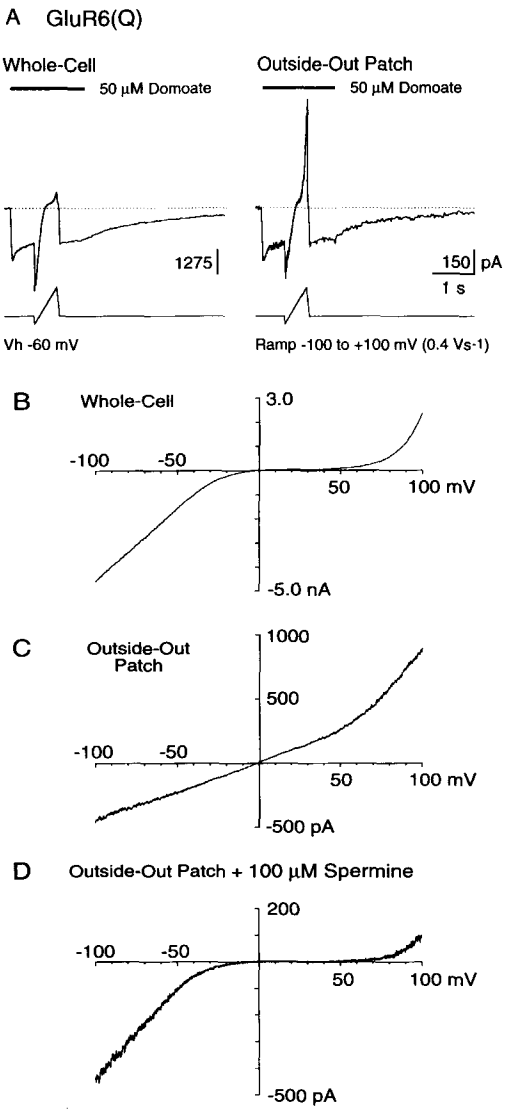

Figure 1. Inward Rectification for GluR6(Q) Is Lost Following Formation of Outside-Out Patches

(A) Whole-cell (left) and outside-out patch responses (right) to 50 μM domoate recorded from a single HEK 293 cell. To analyze the rectification of kainate receptor responses, the membrane potential was ramped from −100 to +100 mV; the change in noise preceding and following the ramp result from alterations in the sample clock frequency to allow capture of the slowly decaying response to domoate following agonist removal. Note that large outward currents were recorded for outside-out patches but not for whole-cell responses even though both recordings were obtained from the same cell.

(B) A typical I–V plot for whole-cell responses to 50 μM domoate recorded from another cell.

(C and D) Responses recorded in outside-out patches from additional cells. Control responses show outward rectification (C); inward rectification similar to that recorded under whole-cell conditions was obtained when 100 μM spermine was added to the internal solution (D).

Table 1. Rectification Ratios Calculated from Chord Conductance Measurements at −100, −50, +50, and +100 mV Using Various Recording Conditions and Spermine Concentrations

	G_{-50}/G_{-100}	G_{+50}/G_{-100}	G_{+100}/G_{-100}
GluR6(Q) whole cell	0.65 ± 0.08	0.02 ± 0.03	0.41 ± 0.16
GluR6(Q) outside-out patch	1.00 ± 0.01	0.99 ± 0.10	1.78 ± 0.33
GluR6(Q) patch + 100 μM spermine	0.51 ± 0.11	0.02 ± 0.03	0.26 ± 0.02
GluR6(Q) patch + 60 μM spermine	0.66 ± 0.03	0.02 ± 0.03	0.39 ± 0.04
$GluRA_{flip}$ patch + 60 μM spermine	0.44 ± 0.06	0.03 ± 0.04	0.10 ± 0.05

Values are mean ± SD of 4–9 observations.

of 1.00 ± 0.01, 0.99 ± 0.10, and 1.78 ± 0.33 (mean ± SD; n = 8), respectively. In contrast, with 100 μM spermine added to the internal solution, there was pronounced inward rectification (Figure 1D). However, although at +50 mV the block of domoate activated conductance with 100 μM intracellular spermine was greater than 95% for both outside-out patches and whole-cell recording, the degree of rectification at both −50 and +100 mV was significantly different ($p < .01$) for the two sets of experiments (Table 1). Nevertheless, the effect of 100 μM spermine was specific for GluR6(Q), since kainate receptors assembled from GluR6(R) with transmembrane domain 2 (TM2) in the edited form did not show inward rectification of responses to domoate either during whole-cell recording or when the pipette solution for outside-out patches contained 100 μM spermine (Figure 2). Previous work has shown that the rectification properties of GluR6 do not vary with RNA editing of sites within TM1 (Köhler et al., 1993); therefore, all of our experiments were performed using GluR6 cDNAs encoding TM1 in its fully edited state.

Voltage-Dependent Block Generated by Intracellular Polyamines

The failure of 100 μM spermine in outside-out patches to account fully for the rectification properties of kainate receptor responses analyzed during whole-cell recording suggested that either the concentration of spermine in the pipette solution did not match the free cytoplasmic concentration of spermine present in the intact cell, and/or that a different substance, possibly another endogenous polyamine, was responsible for rectification during whole-cell recording. To discriminate between these possibilities, we first analyzed data obtained in outside-out patches over a wide range of spermine concentrations using the Woodhull (1973) model of voltage-dependent block. We then used this information, together with that for additional polyamines, for comparison with whole-cell responses.

In outside-out patches, I–V relationships of responses to 50 μM domoate were determined with 1–100 μM spermine added to the pipette solution. Calculation of conductance–voltage (G–V) plots (Figure 3A; see Experimental Procedures) revealed a concentration- and voltage-dependent reduction in conductance by spermine, with maximum block at approximately +40 mV and relief of block on further depolarization. With increase in concentration of spermine, the onset of block of domoate-activated responses developed at progressively more negative membrane potentials (Figure 3A). Control responses to domoate recorded when no spermine was added to the internal solution showed moderate outward rectification over the range +50 to +100 mV (see Figure 1C). In the presence of spermine there was relief from block over the same membrane potential range. This effect was most pronounced at low (1–3 μM) concentrations of spermine, such that the conductance at +100 mV was approximately 1.5 times greater than at −100 mV, only slightly less than observed in the absence of spermine (Table 1). The underlying mechanism was not addressed in the present experiments, but is possibly due to permeation of spermine at strong membrane electric field strengths, as occurs for channel block by divalent cations at NMDA receptors and L-type Ca^{2+} channels (Mayer and Westbrook, 1987; Lansman et al., 1986).

The essentially parallel shift of G–V plots observed with increase in concentration of spermine over the range 1–100 μM suggests that, at membrane potentials below +20 mV, a simple model of voltage-dependent ion channel block should adequately describe the action of spermine over a wide range of membrane potential and spermine concentration. Consistent with this, fits of the Woodhull model of voltage-dependent block to G–V plots obtained

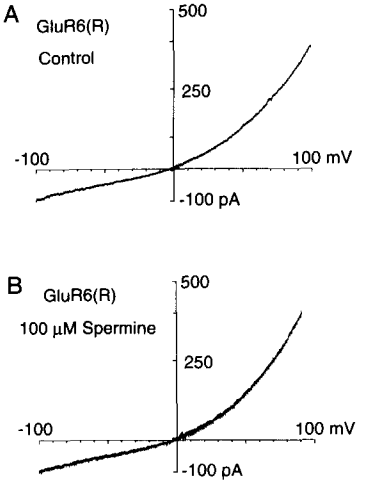

Figure 2. Editing of the Q/R Site Controls Sensitivity to Polyamines

Leak subtracted I–V plots for responses to 50 μM domoate recorded from outside-out membrane patches isolated from HEK 293 cells transfected with GluR6(R) and recorded with protocols identical to those shown in Figure 1; responses in (A) and (B) show mean values, each recorded from 3 patches, normalized to the response at −100 mV. Control responses (A) show weak outward rectification, similar to that observed for GluR6(Q). However, different from GluR6(Q), the addition of 100 μM spermine to the internal solution (B) failed to produce inward rectification.

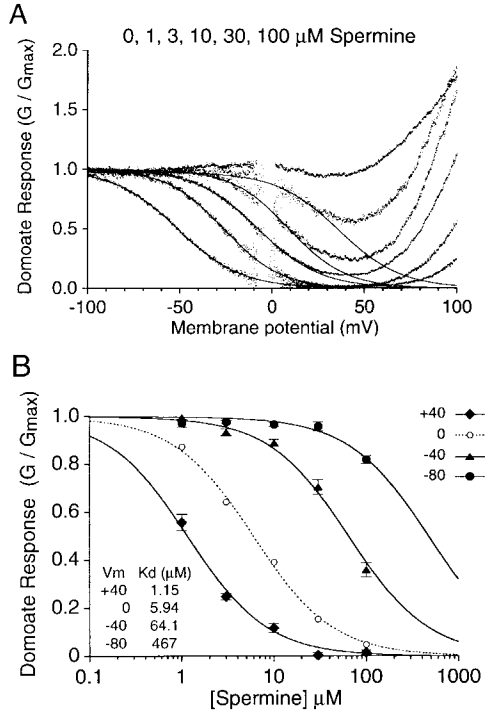

Figure 3. Concentration and Voltage-Dependent Block of Kainate Receptor Responses by Intracellular Spermine

(A) Leak subtracted G–V plots for responses to 50 μM domoate recorded from outside-out membrane patches isolated from HEK 293 cells transfected with GluR6(Q) and recorded with protocols identical to those shown in Figure 1. Control responses showed modest outward rectification. When spermine (1, 3, 10, 30, or 100 μM) was added to the internal solution, the domoate-activated conductance decreased with depolarization over the range −100 to +50 mV; at more positive membrane potentials, the blocking action of spermine was reduced. With increase in concentration of spermine, the onset of block developed at progressively more hyperpolarized membrane potentials. Block at potentials more positive than +50 mV also increased with concentration of spermine. Data points show mean values from 3–9 patches per concentration, normalized to the maximum conductance estimated from fits of the Woodhull equation to data from individual patches; lines drawn through the data points are least squares fits of the Woodhull equation to pooled responses over the range −100 to +20 mV and are extrapolated to +100 mV.

(B) Dose–response analysis for block of domoate responses by spermine at +40, −40, and −80 mV fit with a single binding site isotherm; data points show mean ± SEM for responses recorded from 3–9 patches per concentration of spermine; the dashed line and open symbols show responses at 0 mV estimated from fits of the Woodhull equation as shown in (A).

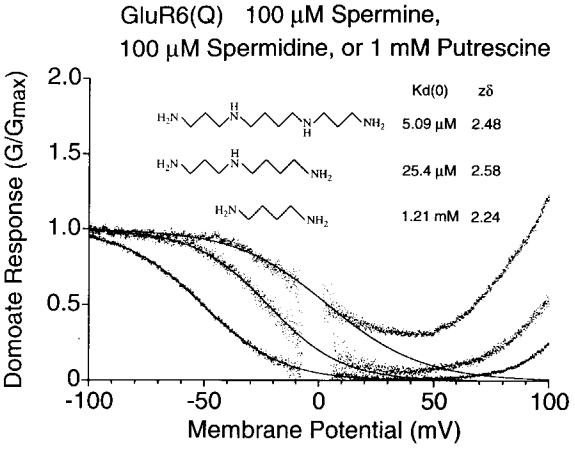

Figure 4. Structure–Activity Analysis for Block of Kainate Receptor Responses by Polyamines

Leak subtracted G–V plots for responses of GluR6(Q) to 50 μM domoate were recorded from outside-out membrane patches as described in Figure 2 with spermine (100 μM), spermidine (100 μM), or putrescine (1 mM) added to the internal solution. The plot shows mean values for 4–9 responses for each polyamine, normalized to the maximum conductance estimated from fits of the Woodhull equation to data from individual patches; lines drawn through the data points are least squares fits of the Woodhull equation to the pooled responses over the range −100 to +20 mV and are extrapolated to +100 mV. The inset shows the structure of the polyamines analyzed and estimates for K_d(0 mV) and zδ obtained from analysis of the pooled responses; block by spermine (left trace) was of greater potency than that produced by spermidine (middle trace) or putrescine (right trace).

with 1–100 μM intracellular spermine gave highly consistent values for the equilibrium dissociation for spermine at 0 mV membrane potential (K_d[0 mV]) and for the voltage dependence of block by spermine as determined by the product of δ, the fraction of the membrane electric field at the binding site for spermine, and the valence of spermine, which at physiological pH is estimated to be 3.8 (Palmer and Powell, 1974). Such analysis gave estimates of 5.53 ± 0.40 μM for K_d(0 mV) and 2.52 ± 0.04 for zδ, with G_{max} (4.28 ± 0.48 nS, n = 38; range, 0.81–13.02 nS) varying as a function of the number of channels in each patch.

To analyze further the effect of membrane potential on receptor affinity for spermine, the K_d was determined by dose–response analysis at membrane potentials of −80, −40, and +40 mV, using values obtained from normalized G–V plots. At any given membrane potential, concentration-dependent block by spermine was well fit by a single binding site isotherm (Figure 3B), with K_d values decreasing progressively with depolarization, from 467 μM at −80 mV to 1.15 μM at +40 mV. Comparison with data calculated for K^+ channels at +50 mV reveals the affinity of spermine for GluR6(Q) to be only 10-fold lower than for the IRK1 and BIR10 inwardly rectifying K^+ channels (Fakler et al., 1994, 1995).

When either of two other endogenous polyamines, spermidine and putrescine, were added to the internal solution, we observed inward rectification of domoate-activated responses in outside-out patches similar to that produced by spermine, with the exception that spermidine, and particularly putrescine, were of lower potency. Figure 4 shows normalized G–V plots for responses to domoate determined with 100 μM spermidine (n = 6), 1 mM putrescine (n = 4), or 100 μM spermine (n = 8) and fit with the Woodhull model of voltage-dependent block. The K_d(0 mV) values obtained for spermidine, 26.31 ± 3.72 μM, and putrescine, 1243 ± 150 μM, reveal these polyamines to be 4.8- and 225-fold less potent than spermine. Similar to results obtained for inwardly rectifying K^+ channels (Lopatin et al., 1994), the zδ value determined for spermidine (2.58 ± 0.08) was similar to that for spermine (2.52 ±

0.04); surprisingly, in view of the charge difference for putrescine, the zδ value for this compound (2.28 ± 0.06) was only slightly lower than that for the other polyamines.

Estimates for Free Intracellular Polyamine Concentrations

K_d(0 mV) and zδ values for spermine, spermidine, and putrescine, determined using outside-out patches as described above, were used to estimate the cytoplasmic concentration of these polyamines that could account for the rectification of whole-cell responses for GluR6(Q). This was initially achieved by assuming only a single polyamine to be present in the cytoplasm, with K_d(0 mV) and zδ constrained to their mean values for spermine, spermidine, or putrescine. Fits of the Woodhull model using K_d(0 mV) and zδ values for spermine or spermidine accurately described the voltage dependence of whole-cell responses to domoate (8 cells), whereas the lower value of zδ for putrescine gave rectification of weaker voltage dependence than observed experimentally. The responses for this analysis were recorded 112 ± 20 s after breakthrough and gave estimates of 64 μM, 322 μM, and 10.8 mM for the cytoplasmic concentrations of spermine, spermidine, and putrescine that would be required to produce rectification like that observed during whole-cell recording, assuming only a single polyamine to be present in the cytoplasm. Based on biochemical analysis of polyamine distribution in bovine lymphocytes and rat liver cells, which gave mean values of 46 μm and 138 μM for the free concentrations of spermine and spermidine, respectively (Watanabe et al., 1991), we reanalyzed whole-cell responses for domoate assuming both of these polyamines to be present in the cytoplasm at a concentration ratio of 1:3. Fits were indistinguishable from those for single polyamines and gave estimates for spermine and spermidine concentrations of 40 and 120 μM, respectively, in excellent agreement with biochemical estimates, suggesting that both spermine and spermidine are likely to contribute to rectification of glutamate receptor responses in neurons.

In 5 of 8 of the above experiments, the internal solution contained 20 mM Na_2ATP to test whether chelation of intracellular polyamines would reduce rectification. Following correction for the effects of series resistance and cell size on the dialysis exchange time constant (Pusch and Neher, 1988; Ifune and Steinbach, 1993), fits to multiple G–V plots recorded from single cells revealed the estimated polyamine concentration to decrease exponentially, at an average rate of 0.21 ± 0.05-fold per exchange time constant (mean ± SEM; 3 cells with 20 mM Na_2ATP held for between 2 and 8 exchange time constants; see Experimental Procedures). The slow wash out of rectification, similar to that found for inwardly rectifying nicotinic acetylcholine receptor ion channels in PC12 cells (Ifune and Steinbach, 1993), suggests that the free intracellular concentrations of polyamines are highly buffered. This is consistent with the results of biochemical analysis, which revealed that in mammalian cells, RNA and DNA generate binding sites with high capacity and low affinity for spermine and spermidine such that their free concentrations are typically <10% of total (Watanabe et al., 1991). In 6 cells (4 with 20 mM Na_2ATP) held for a sufficient period following the start of whole-cell recording to characterize the rate of decrease of polyamine concentration, extrapolation to time zero of exponential fits to the rate of decay of polyamine concentrations versus duration of whole-cell recording, assuming both spermine and spermidine to be present at a ratio of 1:3, gave an estimate for the free spermine and spermidine concentrations in intact cells of 51 ± 6.8 μM and 153 ± 20 μM, respectively.

Relief of Block with Strong Depolarization

The Woodhull model accurately described block by polyamines over the membrane potential range −100 to +20 mV, but did not account for the doubly rectifying whole-cell responses typical of GluR6(Q). To determine whether the complex rectification properties of whole-cell responses over the membrane potential range −100 to +100 mV could be fully restored by polyamines, we compared whole-cell responses, recorded on average 113 ± 20 s (mean ± SEM; n = 8) after breakthrough, with responses in outside-out patches recorded with 60 μM spermine added to the internal solution (n = 6). Figure 5A shows pooled I–V plots from these experiments superimposed for comparison and indicates that 60 μM spermine closely mimicked rectification of whole-cell responses, including the relief of block observed from +50 to +100 mV. Figure 5B shows the same data, transformed to G–V plots, with the data for whole-cell responses fit using the Woodhull model of block, with values for K_d(0 mV) and zδ constrained to the means determined for spermine in outside-out patches. Data points for responses in outside-out patches with 60 μM spermine almost exactly overlay the fit to the data for whole-cell responses, and chord conductance ratios at −50, +50, and +100 mV for whole-cell responses were essentially identical to those for outside-out patches with 60 μM spermine added to the internal solution (Table 1). Similar results were obtained for mixtures of 40 μM spermine and 120 μM spermidine, the concentrations of polyamines estimated to be present in mammalian cells (Watanabe et al., 1991). The relief of block recorded with strong depolarization most likely results from permeation of spermine and spermidine at high membrane electric field strengths, an effect which is not observed for inward rectifier K^+ channels (Fakler et al., 1995), as would be expected if there are substantial differences in the pore size of glutamate receptors versus K^+ channels.

AMPA Receptor Rectification Mediated by Intracellular Spermine

Although kainate receptor subunits show on average only 40% amino acid sequence homology with AMPA receptor subunits (Hollmann and Heinemann, 1994), their rectification properties are similar and are regulated by RNA editing of a site in TM2 for both receptor families (Sommer et al., 1991). This suggests that, similar to kainate receptors, intracellular polyamines are likely to be responsible for the inward rectification of AMPA receptors. In HEK 293 cells transfected with GluR-A_{flip}, strong inward rectification was observed for whole-cell responses when voltage ramps were applied during the steady-state response evoked by

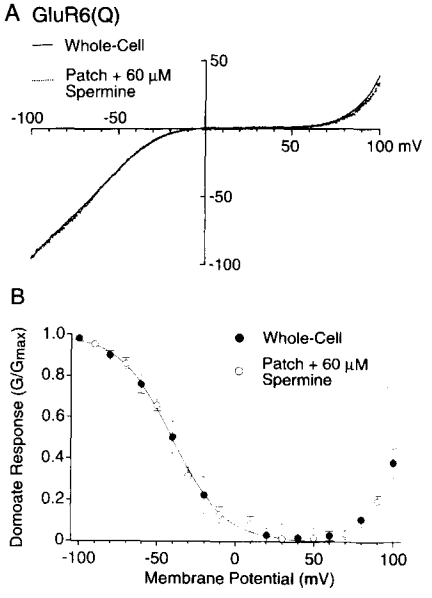

Figure 5. Analysis of Whole-Cell Responses for GluR6(Q)

(A) Superimposed I–V plots for whole-cell and outside-out patch responses to 50 μM domoate recorded as described in Figure 1; data points show mean values for 6–8 observations per recording condition, normalized to the current amplitude recorded at −100 mV. Whole-cell responses were recorded on average 113 ± 20 s (mean ± SEM; n = 8 cells) after breakthrough, using a polyamine-free internal solution. Data for outside-out patches was recorded using 60 μM spermine in the internal solution; this concentration closely matched the complex I–V relationship of whole-cell responses over the range −100 to +100 mV and was chosen based on analysis of G–V plots for whole-cell responses.

(B) G–V plots for pooled data from whole-cell responses recorded from 8 cells (mean ± SD) fit by the Woodhull equation, with K_d(0 mV) and zδ constrained to their mean values estimated from analysis of the action of spermine on outside-out patches; the free spermine concentration was estimated as 62.1 μM. Responses recorded from outside-out patches with 60 μM spermine added to the internal solution (mean ± SD, 6 observations) overlay the fit to whole-cell responses and show a similar relief of block at membrane potentials depolarized to +50 mV; similar results were obtained with a mixture of 40 μM spermine and 120 μM spermidine.

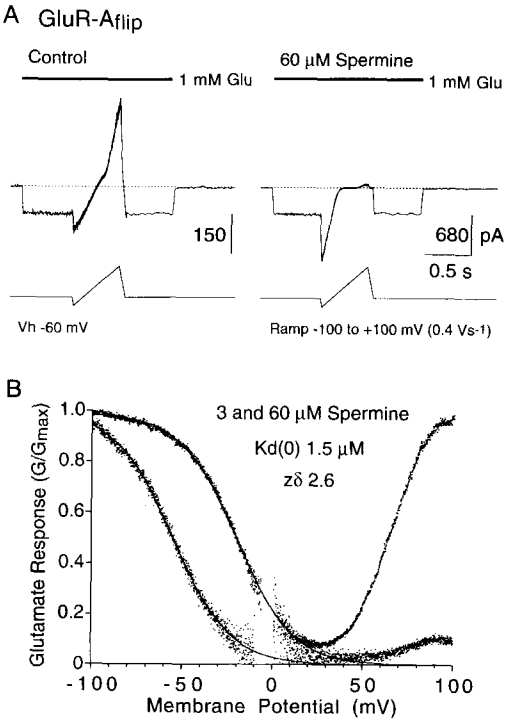

Figure 6. Inward Rectification of AMPA Receptors Generated by Intracellular Spermine

(A) Responses to 1 mM glutamate applied to outside-out patches isolated from HEK 293 cells transfected with GluR-A_{flip}; 100 μM cyclothiazide was added to the extracellular solution to block desensitization. During application of glutamate, the membrane potential was ramped from −100 to +100 mV; the changes in noise preceding and following the ramp result from alterations in the sample clock frequency. Note that large outward currents were recorded with control internal solution but not when 60 μM spermine was added to the internal solution.

(B) Analysis of G–V plots for responses to glutamate with 3 or 60 μM intracellular spermine; note the strong relief of block at membrane potentials depolarized to +30 mV for 3 μM but not 60 μM spermine. Data points show mean values recorded from 4 or 5 outside-out patches per concentration of spermine, normalized with respect to G_{max} estimated from fits of the Woodhull equation to data from individual patches; lines drawn through the data points are least squares fits of the Woodhull equation to the mean values over the range -100 to +20 mV and are extrapolated to +100 mV. Pooled analysis of such results shows the affinity of spermine for AMPA receptors assembled from GluR-A_{flip} to be 3.6 times higher affinity than for kainate receptors assembled from GluR6(Q).

1 mM L-glutamate in the presence of 100 μM cyclothiazide (data not shown). Inward rectification, however, was eliminated in outside-out membrane patches (Figure 6A, left) suggesting that, as for kainate receptors, a diffusible substance in the cytoplasm confers inward rectification. The inclusion of spermine in the pipette solution restored inward rectification (Figure 6A, right). As for kainate receptors, the Woodhull model of voltage-dependent block gave good fits to G–V plots for responses to glutamate determined with different intracellular concentrations of spermine (Figure 6B). For measurements on 9 patches made with 3 or 60 μM spermine, estimates for K_d(0 mV) (1.53 ± 0.36 μM) indicated a slightly greater than 3-fold higher affinity of spermine for AMPA versus kainate receptors ($P < .001$; analysis of variance with Fisher's PLSD) with no obvious change in zδ (2.65 ± 0.10). As would be expected from the different affinity of AMPA and kainate receptors for glutamate and domoate (Stern-Bach et al., 1994), comparison of traces in Figure 1 and Figure 6 reveals the rate of deactivation of AMPA receptor responses following removal of glutamate (τ_{off} = 3.5 ± 0.5 ms) to be much faster than the deactivation of responses to domoate for GluR6, for which a slowly decaying component gave a prolonged tail current (τ_{off2} = 4.3 ± 0.8 s; amplitude, 43.2% ± 5.9%).

Rectification of Fast Desensitizing Responses to Glutamate

Native AMPA and kainate receptors that possess inwardly rectifying I–V relationships also exhibit profound desensitization (Burnashev et al., 1992a; Lerma et al., 1993). In principle, the nonstationary kinetics of neurotransmitter responses at synaptic sites could regulate the extent of polyamine block of glutamate receptors if the rate of onset

of block is slower than the duration of the agonist response or polyamines interfere with receptor desensitization. The experiments described above examined spermine block when the agonist response was at equilibrium and, for AMPA receptors, were performed with desensitization abolished by 100 μM cyclothiazide (Partin et al., 1993). To address these issues, we measured the kinetic properties of kainate receptor responses activated by 1 mM L-glutamate in outside-out membrane patches isolated from HEK 293 cells transfected with GluR6(Q), both in the presence and absence of 60 μM spermine. We then tested whether the amount of block by spermine was similar to previous estimates made for equilibrium responses to domoate. These experiments were performed using GluR6(Q) because, for many patches taken from cells transfected with GluR-A_{flip}, responses to 1 mM L-glutamate were too small for reliable analysis in the absence of cyclothiazide.

In outside-out membrane patches taken from cells transfected with GluR6(Q), concentration jump applications of 1 mM L-glutamate at −60 mV elicited large amplitude (control, 1.33 ± 0.50 nA; 60 μM spermine, 0.56 ± 0.21 nA), rapidly desensitizing membrane currents. Figure 7A shows typical responses at various holding potentials (range, −100 to +100 mV; 20 mV increments) in the absence (left) or presence (right) of 60 μM spermine. I–V relationships constructed from peak responses revealed strong inward rectification in the presence of 60 μM spermine and weak outward rectification in the absence of spermine, suggesting that the rate of onset of block by 60 μM spermine was sufficiently rapid to give qualitatively similar results to those obtained for equilibrium responses to domoate (Figure 7B). Consistent with block by spermine of the peak response to glutamate, in preliminary experiments with 60 μM internal spermine we observed exponential relaxations with submillisecond kinetics evoked by voltage jumps applied during equilibrium responses to domoate, confirming rapid onset of and recovery from block. G–V plots determined for peak responses to glutamate with 60 μM internal spermine (n = 3) were well fit by the Woodhull model of block (Figure 7C) and gave essentially identical estimates for K_d(0 mV) (6.77 ± 1.67) and zδ (2.44 ± 0.20; mean ± SD; n = 3) to values obtained under equilibrium conditions. In addition, similar to responses obtained for domoate, rapidly desensitizing responses to L-glutamate also showed relief from block by 60 μM spermine at membrane potentials above +50 mV, with the chord conductance ratio at +100 mV (0.50 ± 0.10) similar to that for responses to domoate (Table 1).

The possibility that spermine may promote inward rectification of kainate receptors by altering receptor desensitization was investigated by analysis of the rate of onset and extent of desensitization evoked by 1 mM L-glutamate at different membrane potentials in the presence and absence of spermine. The onset of desensitization was well fit by the sum of two exponentials with similar time constants in the presence and absence of spermine (values at −60 mV were as follows: control, τ_{des1} = 3.23 ± 0.20 ms, τ_{des2} = 71.3 ± 23.9 ms, A_1 = 97.7% ± 0.56% [n = 4]; 60 μM spermine, τ_{des1} = 3.30 ± 0.36 ms, τ_{des2} = 50.5 ± 5.6 ms, A_1 = 97.6% ± 0.49% [n = 3]). The extent of desensitization was similarly unaffected by spermine (values at −60 mV were as follows: control, 99.2% ± 0.20%; 60 μM spermine, 99.0% ± 0.53%). Although in the presence of spermine the glutamate-activated conductance was strongly voltage dependent (Figure 7C), the kinetics of onset and extent of desensitization were essentially independent of membrane potential (Figures 7D and

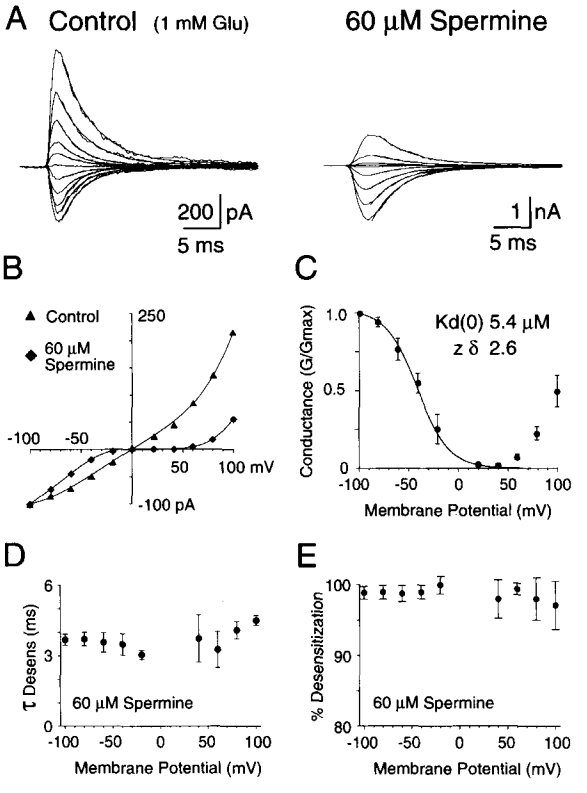

Figure 7. Inward Rectification of Rapidly Desensitizing Responses to Glutamate Requires Intracellular Spermine

(A) Concentration jump responses to 1 mM glutamate applied to outside-out patches isolated from HEK 293 cells transfected with GluR6(Q) and recorded with control internal solution (left) or with 60 μM intracellular spermine (right). Responses were recorded at 20 mV intervals over the range −100 to +100 mV. Lines drawn through the data points are double exponential functions fit to the desensitizing phase of the response to glutamate.

(B) I–V plots for similar responses, normalized to have an amplitude of −100 pA at -100 mV (scale factors: control, 0.31; 60 μM spermine, 0.043).

(C) Analysis of G–V plots for pooled responses to 1 mM glutamate recorded with 60 μM intracellular spermine and fit to the Woodhull equation over the range −100 to +40 mV. At membrane potentials depolarized to +60 mV, there is pronounced relief of block of outward current flow by spermine; data points show mean ± SD (n = 3). Estimates for K_d(0 mV) and zδ were similar to those estimated for equilibrium responses to 50 μM domoate.

(D and E) Analysis of the voltage dependence of the kinetics of onset and extent of desensitization recorded with 60 μM spermine; data points show mean ± SD (3 patches). Despite strong inward rectification over the range −100 to +40 mV and relief of block at more depolarized potentials, there is no apparent change in either the rate of onset of desensitization (D) or the extent of desensitization at equilibrium (E). Values for control responses, recorded in the absence of spermine, were similar to those shown in (D) and (E).

7E) and were similar at all potentials for control responses and for 60 μM intracellular spermine.

Functional Significance of Glutamate Receptor Ion Channel Block by Polyamines

Our experiments were performed on recombinant AMPA and kainate receptors, but similar results have been recently obtained for native AMPA receptors in cultured hippocampal neurons (Donevan and Rogawski, 1995). Together, these results help to explain a paradoxical observation concerning the high Ca^{2+} permeability but outward rectifying properties of AMPA receptor responses in patches removed from cortical interneurons (Jonas et al., 1994). Based on the properties of recombinant AMPA receptors, which link Ca^{2+} permeability to inward rectification (Hollmann et al., 1991; Hume et al., 1991; Verdoorn et al., 1991; Burnashev et al., 1992b; Dingledine et al., 1992), it would have been predicted that native AMPA receptor responses with high Ca^{2+} permeability would show strong inward rectification. That this was not seen in outside-out patches removed from cortical interneurons (Jonas et al., 1994) can be explained by loss of the cytoplasmic gating mechanism generated by polyamine-mediated ion channel block. In contrast, the extent of inward rectification of glutamate receptor responses observed during whole-cell recording for hippocampal neurons expressing native kainate receptors (Lerma et al., 1993) and for Bergmann glial cells, which lack the GluR-B subunit (Burnashev et al., 1992a), agrees well with that expected for intracellular polyamine concentrations in the range 50–200 μM. Although prior analysis of I–V plots for inwardly rectifying glutamate receptor responses in these cells emphasizes the block of outward current flow at strongly depolarized membrane potentials, our analysis of G–V plots reveals significant attenuation of inward current flow close to the resting potential. Calculations based on our estimate of the free spermine and spermidine concentrations in HEK 293 cells reveal that at a resting membrane potential of −70 mV there will be 36% block of the inward current through homomeric AMPA receptors generated from GluR-A and 18% block for kainate receptors generated from GluR6(Q); during depolarization to −50 mV, the threshold for action potential initiation, the degree of block increases to 67% for AMPA receptors and 42% for kainate receptors. One physiological consequence of ion channel block by polyamines for inwardly rectifying glutamate receptors responses will be voltage-dependent modulation of Ca^{2+} influx. The reduction in inward current with depolarization, and essentially complete block of outward current within the physiological range of membrane potentials encountered during action potential firing, will also allow depolarizing responses mediated by inwardly rectifying glutamate receptors to initiate the activity of other voltage-dependent ion channels, without introducing a shunt at more depolarized membrane potentials. It is also possible that polyamine block of Ca^{2+} influx at the resting potential helps to prevent cell damage resulting from excessive Ca^{2+} influx during activation of glutamate receptors.

Common Mechanisms Regulate Rectification of Voltage-Dependent and Ligand-Gated Ion Channels

Rapid progress in the analysis of ion channel structure and function has given rise to the concept that structural domains are shared among ion channels from diverse families (Jan and Jan, 1994; Wo and Oswald, 1995a). Recent proposals for a revised transmembrane topology for glutamate receptor ion channels, with TM2 forming a reentrant loop, raised the possibility that the ion channel pore formed by TM2 has structural features comparable to that formed by the H5 or P region of K^+ channels (Hollmann et al., 1994; Stern-Bach et al., 1994; Wo and Oswald, 1994; Bennett and Dingledine, 1995; Wo and Oswald, 1995b). The discovery that polyamines confer inward rectification to both glutamate receptor ion channels and to inwardly rectifying K^+ channels strengthens this concept and suggests that polyamines have been conserved as the cytoplasmic gates for a diverse range of ion channel proteins. In addition to K^+ channels and to glutamate receptors, two other families of ligand-gated ion channels show pronounced inward rectification: neuronal nicotinic receptors and ATP receptors (Brake et al., 1994; Valera et al., 1994; Couturier et al., 1990; Charnet et al., 1992). The washout of inward rectification for nicotinic receptors following perfusion of PC12 cells with solutions containing ATP (Sands and Barish, 1992; Ifune and Steinbach, 1993) suggests that polyamines are reasonable candidates for the intrinsic rectification mechanism in neuronal nicotinic receptors, and perhaps also for purinergic receptor ion channels. The latter are believed to have a novel structure, different from that for other ligand-gated ion channels, with only two hydrophobic membrane regions surrounding an H5 or P region–like sequence, comparable to the structure for inwardly rectifying K^+ channels (Brake et al., 1994; Valera et al., 1994; Jan and Jan, 1994). However, the structure of the pore region for Torpedo nicotinic receptors, and presumably for other members of the superfamily of 50 kDa ligand-gated ion channel subunits, including neuronal nicotinic receptors, is currently thought to be generated by an alpha helical segment distinct from that present in K^+ channels (Unwin, 1993). Clearly, further work is required to identify the molecular structures responsible for polyamine block in individual ion channel subunits.

Experimental Procedures

Expression of Recombinant Receptors in HEK 293 Cells

HEK 293 cells (ATCC CRL 1573) were maintained in MEM with Earle's salts, 2 mM L-glutamine, and 10% fetal bovine serum (5% CO_2; 37°C). Cultures were split upon reaching 70%–80% confluency and for transfection were plated at a density of 20,000 cells/ml onto the center of 35 mm plastic dishes (NUNC). Twenty-four hours later, vectors carrying cDNAs for GluR-A_{flip}, GluR6(Q), or GluR6(R) were cotransfected with a vector for the cDNA of green fluorescent protein using the calcium/phosphate technique described by Chen and Okayama (1987). In each case, cDNA for GFP was present at a ratio of 1:4 of the total DNA (20–40 μg/ml).

Recording Conditions

Whole-cell and outside-out patch recordings were performed on isolated HEK 293 cells 40–72 hr after transfection using an Axopatch-200A amplifier (Axon Instruments). Current records were filtered (8-

pole Bessel filter) at 0.5–2 kHz, digitized at 0.04–5 kHz as required, and stored on a MacIntosh IIfx computer using an ITC-16 interface under control of the program Synapse (available by anonymous FTP from Zippy.NIMH.NIH.GOV in the directory \PUB\SYNAPSE). During whole-cell recording, the series resistance (3.5–10 MΩ) was compensated by 95%. For outside-out patch recordings, in which high expression levels of AMPA or kainate receptors were desired, the observation of intense GFP fluorescence (Chalfie et al., 1994) was used as a marker of transfection efficiency (excitation 450–490 nm; 510 nm dichroic; 520 nm barrier). Recordings were made with thin-wall borosilicate glass pipettes (resistance, 2–5 MΩ). Because preliminary experiments showed that in outside-out patches inward rectification was lost even when the internal solution contained 1 mM Mg^{2+}, experiments were performed using the following solution composition: 140 mM CsCl, 10 mM CsF, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 5 mM CsBAPTA (pH 7.2; osmolarity, 295 mOsm) to which polyamines were added as required. Internal solution containing 20 mM Na_2ATP was made using the above composition but with 110 mM CsCl. The extracellular recording solution contained 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, and 0.01 mg/ml phenol red (pH 7.3; osmolarity, 295 mOsm). Agonists were dissolved in standard extracellular solution and applied using a stepper motor-based fast perfusion system (Vyklicky et al., 1990). Cyclothiazide was dissolved in DMSO at 20 mM before dilution with extracellular solution (final DMSO concentration after dilution was 0.5%). Experiments were performed at room temperature.

Data Analysis

I–V relationships for agonist responses were leak subtracted from an average of 2–3 control responses and fit with a ninth order polynomial to estimate the reversal potential (V_{rev}). G–V plots during a ramp from −100 to +100 mV (0.4 Vs^{-1}) were calculated from the equation $G = I_m/(V_m - V_{rev})$ using the program Kaleidagraph (Synergy Software); data points surrounding the reversal potential and depolarized to +20 mV were masked during subsequent fits to the Woodhull equation:

$$G_{Vm} = G_{max}\left(\frac{1}{1 + \frac{[P]}{K_d}}\right)$$

where

$$K_d = K_d(0\ mV)\exp\left(\frac{V_m z (1 - \delta) F}{RT}\right)$$

and G_{max} is the agonist-activated conductance at a membrane potential sufficiently hyperpolarized to relieve block (typically −100 mV); [P], the internal concentration of polyamine; K_d, the dissociation constant at a membrane potential V_m; $K_d(0\ mV)$, the dissociation constant at 0 mV membrane potential; z, the valence of the polyamine; and δ, the fraction of the membrane electric field at the polyamine binding site; with F, R, and T having their standard values. To analyze voltage-dependent block of whole-cell responses assuming both spermine and spermidine to be present simultaneously, the above definition of K_d was used in the equation

$$G_{Vm} = G_{max}\left(\frac{1}{1 + \frac{[Spm]}{K_{d1}} + \frac{[Spd]}{K_{d2}}}\right)$$

where K_{d1} and K_{d2} are the voltage-dependent dissociation constants for spermine and spermidine.

The exchange time constant during whole-cell recording was calculated according to Pusch and Neher (1988) using the equation $\tau_{exchange} = 0.6(Cm / 5.91)^{3/2} \times Rs(M)^{1/3}$, where Cm is the HEK 293 cell capacitance (15.1 ± 3.0 pF), Rs, the series resistance (5.7 ± 0.8 MΩ, mean ± SEM; n = 9) and M, the molecular weight (202 for spermine). Values for $\tau_{exchange}$ varied from 30.4 to 288 s (85.6 ± 29.1; mean ± SEM). Exponential analysis of the time constants for desensitization and deactivation was performed using Synapse. Tests for statistical significance were performed using analysis of variance.

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