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Intracellular polyamines mediate inward rectification of Ca^{2+}-permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors

(spermine/spermidine/excitatory amino acid receptor)

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ABSTRACT **α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors that lack the glutamate receptor GluR2 subunit are Ca^{2+}-permeable and exhibit inwardly rectifying current responses to kainate and AMPA. A proportion of cultured rat hippocampal neurons show similar Ca^{2+}-permeable inwardly rectifying AMPA receptor currents. Inward rectification in these neurons was lost with intracellular dialysis and was not present in excised outside-out patches but was maintained in perforated-patch whole-cell recordings, suggesting that a diffusible cytoplasmic factor may be responsible for rectification. Inclusion of the naturally occurring polyamines spermine and spermidine in the recording pipette prevented loss of rectification in both whole-cell and excised-patch recordings; Mg^{2+} and putrescine were without effect. Inward rectification of Ca^{2+}-permeable AMPA receptors may reflect voltage-dependent channel block by intracellular polyamines.**

Glutamate, acting at α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-selective receptors, is the principal neurotransmitter in the central nervous system responsible for fast synaptic excitation (1). Four AMPA receptor genes have been identified that encode AMPA-selective glutamate receptor (GluR) subunits (2, 3), each of which exists in alternatively spliced forms (4). AMPA receptors are multimeric proteins whose functional properties depend on their subunit composition. Homomeric or heteromeric receptors assembled from GluR1, GluR3, and Glu4 are permeable to Ca^{2+} and have inwardly rectifying current–voltage relationships, whereas AMPA receptors that contain GluR2 subunits are impermeable to Ca^{2+} and are outwardly rectifying (3, 5, 6). The Ca^{2+} permeability and rectification properties of the subunits are determined by the identity of the amino acid residue at a critical position (Q/R site) in the putative membrane segment 2 (M2) of each AMPA receptor subunit. The GluR1, GluR3, and GluR4 subunits contain a neutral glutamine residue at this site, whereas GluR2 usually contains a positively charged arginine that is introduced as a result of nuclear RNA editing (7–11). The mechanism responsible for the profound inward rectification of AMPA receptors that lack the GluR2 subunit is not well understood. It has been proposed that the absence of the positively charged arginine in GluR2 unmasks a binding site for an as yet unidentified intracellular blocking ion (7, 10) or, alternatively, that the GluR1, GluR3, and GluR4 subunits have an intrinsic voltage dependence that is canceled by the presence of GluR2 (6).

In the present study, we examined the mechanism underlying the inward rectification of Ca^{2+}-permeable AMPA receptors expressed in cultured rat hippocampal neurons. Native AMPA receptors are, for the most part, relatively impermeable to Ca^{2+}. Recently, however, a number of studies have demonstrated that there is variability in the Ca^{2+} permeability and rectification properties of native AMPA receptors expressed in cultured hippocampal neurons (12–15). Most cultured hippocampal neurons show outwardly rectifying, Ca^{2+}-impermeable responses to kainate and AMPA. These neurons, which typically are pyramidal in shape, have been designated type I. In contrast, a small proportion of cultured hippocampal neurons show inwardly rectifying AMPA-receptor responses and their AMPA receptors have high Ca^{2+} permeability (12–15). These so-called type II neurons have elliptical somata, fine neurites, and smaller overall size than type I neurons. Recently, it has been confirmed by using single-cell PCR that type II neurons fail to express mRNA for the GluR2 subunit (16). We used type II cultured rat hippocampal neurons to investigate the hypothesis that a soluble cytoplasmic factor accounts for the inward rectification of Ca^{2+}-permeable AMPA receptors. Our results support this hypothesis and, further, indicate that the polyamines spermine and spermidine are likely candidates for the cytoplasmic rectification factor.

METHODS

Cell Culture. Hippocampal neurons from 19-day Sprague–Dawley rat (Taconic Farms) embryos were grown in primary culture as described (17) and were used 5–8 days after plating.

Electrophysiology. Recordings were carried out at room temperature (23°C) in a control bathing solution containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM Hepes. The bathing solution also contained 1 μM tetrodotoxin to block voltage-gated sodium channels. The experiments were conducted in the absence of added glycine and in the presence of Mg^{2+} to suppress *N*-methyl-D-aspartate (NMDA) receptor currents. In some experiments, cells were perfused with bathing solution in which the NaCl was replaced with the impermeant cation *N*-methylglucamine, and $CaCl_2$ was raised to 10 mM. Whole-cell and excised outside-out patch voltage-clamp recordings were obtained with an Axopatch 200 amplifier (Axon Instruments, Burlingame, CA) by using patch electrodes (2–3 MΩ) filled with an intracellular solution containing 145 mM CsCl, 2 mM $MgCl_2$, 5 mM Hepes, 0.1 mM $CaCl_2$, and 1 mM EGTA. In some experiments, polyamines or Mg^{2+} were added to the intracellular solution. Currents were acquired in digital form and analyzed off-line by using the PCLAMP software package (Axon Instruments).

Perforated-patch recordings were obtained by using the antibiotic amphotericin B as described by Rae *et al.* (18). Intracellular solution was loaded into the pipette tip (to a

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GluR, glutamate receptor; NMDA, *N*-methyl-D-aspartate.
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distance of 200–300 μm) and then backfilled with the same solution containing amphotericin B (240 μg/ml). The increase in capacitative current response to 5-mV voltage steps was monitored to assess cell access resistance. Within minutes of obtaining the gigaohm seal, access resistance dropped to 7–10 MΩ. There was often a further gradual decrease during the recording period as observed by a gradual increase in the amplitude of agonist-evoked currents.

Drug Perfusion. Drugs were applied by using a multibarreled rapid perfusion system (see ref. 19) in which all barrels emptied by a common orifice (inside diameter, ≈600 μM) that was positioned within several hundred microns of the cell surface. One barrel contained bathing solution and the other barrels contained solutions of the agonists kainate or AMPA. The solution exchange time constant at the tip of a recording electrode was ≈2 msec. In whole-cell experiments, 1-sec agonist applications were separated by 5- to 6-sec wash periods, while in the patch experiments 100-msec agonist applications were applied at similar intervals.

Drugs. AMPA was obtained from Tocris Cookson (Essex, U.K.). All other drugs and chemicals were obtained from Sigma.

RESULTS

Fig. 1 provides a comparison of the kainate current–voltage relationships for representative type I and type II neurons. The type I neuron in Fig. 1*A* had a modestly outwardly rectifying current–voltage relationship in normal (Na^+ containing) extracellular medium. When the bathing medium was changed to a Na^+-free high Ca^{2+} solution, this cell passed no inward current. In contrast, the type II neuron in Fig. 1*B* had a strongly inwardly rectifying current–voltage relationship in normal bathing medium and passed inward current in the high Ca^{2+} medium. It has been observed that Ca^{2+}-permeable and Ca^{2+}-impermeable AMPA receptors expressed in *Xenopus* oocytes are differentially affected by extracellular application of the polyamine spermine (20). Thus, Ca^{2+}-permeable AMPA receptors lacking the GluR2 subunit (or containing an unedited version) were blocked by spermine, whereas Ca^{2+}-impermeable receptors containing the edited GluR2 subunit were not. We found similar differences in the effects of spermine on kainate currents in type I and type II neurons. While kainate responses were reduced in both cell types, the blocking potency in type II neurons was substantially greater than in type I neurons (Fig. 1 *A Right* and *B Right*). In experiments similar to those shown in Fig. 1, 300 μM spermine produced 61 ± 3% (n = 3) and 6 ± 2% (n = 4) block of 100 μM kainate in type II and type I neurons, respectively.

With ordinary (broken patch) whole-cell recording, the extent of inward rectification in type II neurons tended to decline progressively in the minutes after onset of the whole-cell recording mode. This is illustrated in the experiment of Fig. 2 *A* and *C* where there was strong inward rectification immediately after the whole-cell recording mode was established (t = 0 sec), but only minimal rectification 282 sec later; similar results were obtained in seven additional neurons (see Fig. 4). This observation suggested that rectification may depend upon an intracellular factor that is lost with intracellular dialysis. We reasoned that it may be possible to prevent the wash-out of rectification by using the amphotericin B-perforated-patch technique, which limits egress of cytoplasmic constituents larger in size than glucose (18, 21). This was confirmed in the experiment of Fig. 2 *B* and *C* where, by using the perforated-patch technique, rectification in a type II neuron was fully maintained for 282 sec. Comparable results were obtained in eight additional similar experiments (these data are summarized in Fig. 4). In three of the nine perforated-patch experiments, after acquiring sufficient measurements to demonstrate the maintenance of rectification, we broke the integrity of the patch. In these cells, there was subsequent loss of rectification during the 2-min period after patch breakage (see Fig. 4).

Several recent reports (22–24) have demonstrated that rectification of inwardly rectifying K^+ channels expressed in *Xenopus* oocytes is lost with patch excision and, furthermore, that this rectification can be restored by application of the polyamines spermine and spermidine to the intracellular face of inside-out patches, suggesting that cytoplasmic polyamines normally account for the inward rectification. It was therefore of interest to determine whether polyamines could play a similar role in mediating the rectifying properties of AMPA receptors in type II neurons. We first sought to determine whether inclusion of spermine in the recording pipette could prevent wash-out of rectification. As shown in Fig. 3 *A* and *C*, inward rectification in type II neurons was maintained when

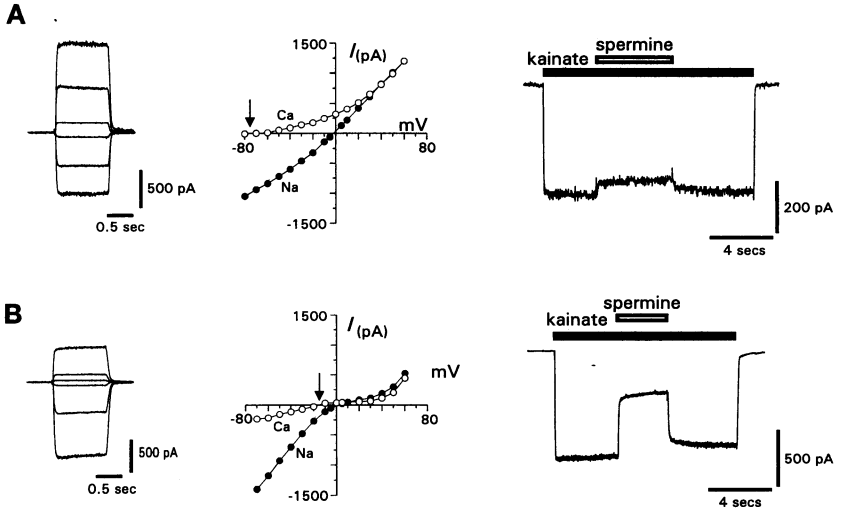

FIG. 1. Comparison of the rectification properties, Ca^{2+} permeability, and external spermine block of kainate-evoked currents (I) in a type I (*A*) and a type II (*B*) cultured hippocampal neurons. (*A* and *B*) Currents were evoked with 100 μM kainate at various holding potential levels between −80 to +60 mV in normal Na^+-containing buffer (●) and in Na^+-free buffer containing high (10 mM) Ca^{2+} and 140 mM *N*-methylgluconate (○). Sample traces are shown to the *Left* (holding potentials, ±10, ±30, and ±60 mV) and the current–voltage relationships are plotted in the *Middle*. The arrows indicate the null potential of the kainate-evoked current in high Ca^{2+} buffer. In the type I neuron, 300 μM spermine produced negligible block of the kainate current whereas there was a much larger block in the type II neuron (holding potential, −60 mV) (*Right*).

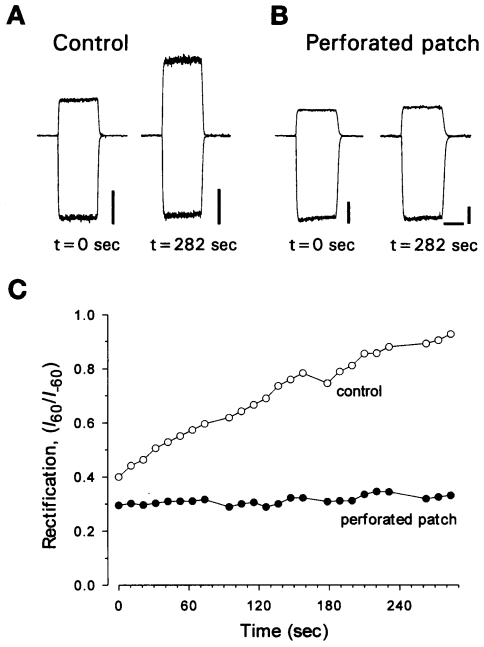

FIG. 2. Wash-out of rectification in type II neurons with conventional but not perforated-patch whole-cell recording. Kainate (100 μM)-evoked currents were recorded at +60 and −60 mV by using conventional whole-cell (*A*) and amphotericin B-perforated-patch (*B*) recording techniques. Calibration bars, 0.5 sec and 200 pA. In *C*, the rectification ratios for the cells shown in *A* and *B* are plotted as a function of the time after establishment of the whole-cell recording mode. There is a loss of inward rectification in the conventional whole-cell recording but not in the perforated-patch recording. The rectification ratio (I_{60}/I_{-60}) was calculated as the ratio of the current amplitude at +60 mV (I_{60}) to the current amplitude at −60 mV (I_{-60}). Zero time was taken to be the time at which rectification was first examined, within 10–20 sec after initiation of whole-cell recording or, in the perforated-patch recordings, at the time when access resistance stabilized.

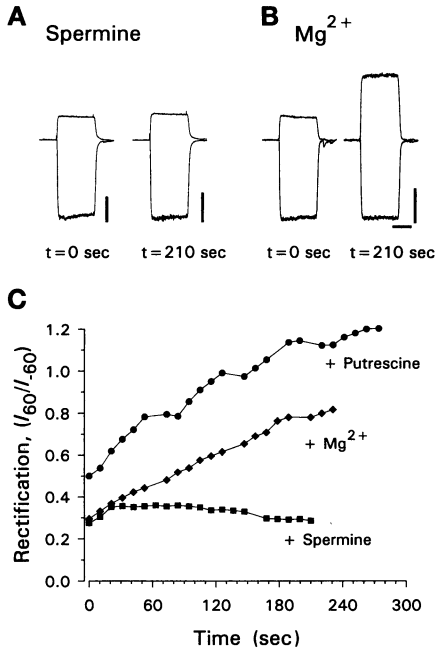

FIG. 3. Spermine, but not Mg^{2+} or putrescine, prevents wash-out of inward rectification in type II neurons. Kainate (100 μM)-evoked currents were recorded at +60 mV and −60 mV, by using conventional whole-cell electrodes filled with intracellular solution containing 100 μM spermine (*A*) and 10 mM Mg^{2+} (*B*). Calibration bars, 0.5 sec and 500 pA. The rectification ratios for the experiments of *A* and *B* as well as an additional experiment with 100 μM putrescine in the electrode solution are plotted in *C* as a function of the time after establishment of the whole-cell recording mode.

100 μM spermine was present in the intracellular recording solution but not with 100 μM putrescine or 10 mM Mg^{2+}. In similar experiments, we found that 10 μM spermine was insufficient to prevent wash-out of rectification and that 30 μM spermine had a partial effect. Spermidine was also able to prevent wash-out although it was of somewhat lower potency than spermine (Fig. 4).

The importance of intracellular spermine in determining the unique rectification properties of type II neurons was further evaluated in experiments in which the extent of rectification of AMPA currents was initially determined in the whole-cell recording configuration and then immediately examined in outside-out patches pulled from the same cells. An example of such an experiment is illustrated in Fig. 5*A*. In this type II neuron, the inward rectification of the rapidly desensitizing currents evoked by 1 mM AMPA was lost with patch excision. In fact, such patches demonstrated linear or slightly outwardly rectifying responses to AMPA (n = 4). In contrast, when 30 μM spermine was present in the recording pipette, inward rectification was maintained in the outside-out patch recordings as shown in the experiment of Fig. 5*B*, which is representative of data from four experiments (Fig. 5*E*). Spermine appeared to be slightly more potent than in the whole-cell-recording experiments (Figs. 3 and 4), possibly because cellular buffering mechanisms for spermine are lost in the patches. Type I neurons showed slightly outwardly rectifying AMPA responses in both whole-cell and patch recordings (n = 3; Fig. 5*C*). In experiments with four such cells, intracellular spermine failed to alter the rectification properties of the patch currents (Fig. 5 *D* and *E*).

DISCUSSION

A principal conclusion of the present work is that inward rectification of Ca^{2+}-permeable AMPA receptors in type II cultured hippocampal neurons is dependent upon a diffusible cytoplasmic factor. Inward rectification of kainate and AMPA receptor responses in such neurons was lost during the course of whole-cell recordings or immediately upon excision of

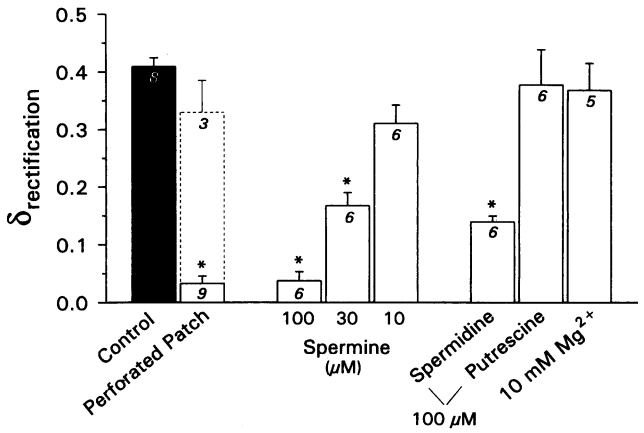

FIG. 4. Change in rectification of type II neurons during the course of whole-cell recording with ordinary broken-patch access (control), perforated-patch access, and broken-patch access with various additions to the pipette solution. Currents were activated by 100 μM kainate. The change in rectification ratio ($\delta_{rectification}$) was taken to be the difference in the rectification ratio at 2 min and time zero. Each bar represents the mean ± SEM; the number of cells tested in each group is indicated. The dashed bar indicates the change in rectification ratio in the 2-min period after rupture of the perforated patch. *, Significantly different from control at $P < 0.01$ (Newman–Keuls test).

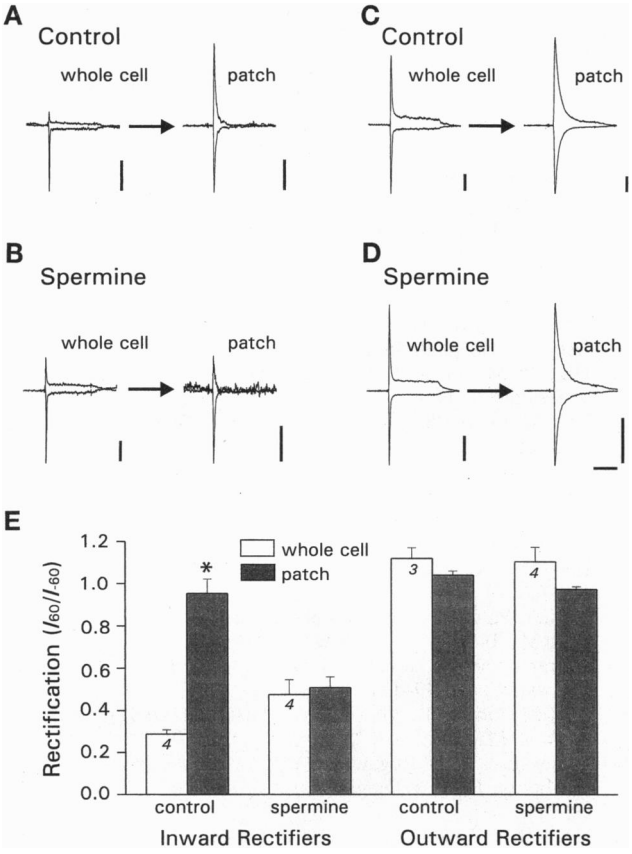

FIG. 5. Loss of rectification of AMPA-evoked currents in a type II neuron upon patch excision is prevented by intrapipette spermine (*A* and *B*), but spermine fails to affect the currents in a type I neuron (*C* and *D*). In *A–D*, the traces show 1 mM AMPA-evoked current responses at −60 and +60 mV. Currents obtained in the whole-cell recording configuration are shown to the left; currents shown to the right were obtained in excised outside-out patches pulled from the same cell. The duration of the AMPA application was 1 sec in the whole-cell recordings and 100 msec in the patch recordings. The patch currents illustrated represent the average of three to six responses (10–30 sec after patch excision). Recordings in *B* and *D* were carried out with 30 μM spermine in the pipette solution. Whole-cell and patch scale bars are 200 and 20 pA in *A* and *B*, respectively, and 400 and 50 pA, in *C* and *D*; time scales are 500 and 50 msec. (*E*) Rectification ratios (peak current) determined in a series of experiments similar to those illustrated in *A–D*. Each bar represents the mean ± SEM; the number of cells tested in each group is indicated. *, Significantly different from control at $P < 0.01$ (*t* test).

outside-out patches. The loss of rectification did not occur in amphotericin B-perforated-patch whole-cell recordings. It has recently been observed that Ca^{2+}-permeable AMPA receptors in patches from nonpyramidal interneurons of the rat neocortex (25) or from chicken cochlear neurons (26) have linear or outwardly rectifying current–voltage relationships. Thus, in these patch recordings, the outward rectification of AMPA responses may be due to a comparable loss of a diffusible rectification factor and not, as has been suggested (26), due to the presence of an as yet unidentified or modified AMPA receptor subunit with high Ca^{2+} permeability and outward rectification.

As is the case with inwardly rectifying K^+ channels (22–24), rectification of Ca^{2+}-permeable AMPA receptors could be maintained by the inclusion of spermine and spermidine in the intracellular solution. Since spermine and spermidine are present in the cytoplasm of mammalian cells (27), they are potential candidates for the endogenous diffusible rectification factor. The observation that rectification was maintained in amphotericin B-perforated-patch recordings provides an upper limit on the dimension of the diffusible rectification factor. The pore radius of amphotericin B pores is ≈4 Å, so that solutes larger than glucose are effectively excluded (21). Spermine and spermidine are of greater molecular weight than glucose and would not be expected to permeate amphotericin B pores. If spermine and spermidine are the cytoplasmic factors producing inward rectification of Ca^{2+}-permeable inwardly rectifying AMPA receptors, the following additional criteria should be fulfilled: (*i*) spermine and spermidine should only alter the rectification properties of type II neurons and have little effect on the current–voltage relationship of AMPA receptor responses in type I neurons, and (*ii*) the effects of spermine and spermidine on rectification should occur at concentrations that are within the range of those present in the cytoplasm. Both of these criteria were fulfilled. Thus, intracellular spermine and spermidine were selective for type II neurons, which show inwardly rectifying AMPA and kainate current responses; the polyamines had little effect on the outwardly rectifying kainate current responses of type I neurons. Moreover, spermine and spermidine effects occurred at relatively low concentrations that are within the free cytoplasmic levels believed to be present in mammalian cells (27). The effect on rectification was specific for spermine and spermidine; the cations putrescine and Mg^{2+} failed to prevent loss of rectification, indicating that charge alone does not account for the block. However, charge is likely to be a factor in the block, and indeed spermine, which has four positive charges at physiological pH, was a more potent blocker than spermidine, which has three positive charges.

Recombinant AMPA receptors composed of GluR1, GluR3, and GluR4 subunits show inwardly rectifying and Ca^{2+}-permeable AMPA responses similar to those of the type II neurons in the present study. Inward rectification of these AMPA-receptor-mediated responses is lost when the neutral glutamine present at the Q/R (arginine/glutamine) site of the M2 region is mutated to a positively charged arginine or when the AMPA receptor complex contains the GluR2 subunit (in which there is normally an arginine at the Q/R site). There has been considerable interest in the mechanism by which the Q/R site regulates the rectification properties of AMPA receptor subunits. It was initially proposed that the positively charged arginine shielded a binding site for an unknown cation (17). Subsequently, it was shown that rectification is also lost when a negatively charged aspartate 4 amino acids downstream from the Q/R site (position 616 in GluR3)—which has recently been demonstrated to lie at the intracellular mouth of the channel pore (28, 29)—is replaced by the neutral amino acid asparagine (10). This has led to the suggestion that in AMPA receptors composed of subunits in which an arginine is inserted at the Q/R site, the positively charged arginine forms a salt bridge with the downstream aspartate (10). In AMPA receptors formed from GluR1, GluR3, and GluR4 subunits that have a neutral glutamine at the Q/R site, the salt bridge does not form, thus uncovering an acceptor at this downstream site for a positively charged rectification factor. As rectification is also lost when the glutamine of the Q/R site is mutated to the shorter but still neutral asparagine (which would also not form a salt bridge), it would appear that this site in addition to the downstream site (i.e., position 616 in GluR3) is also important for binding of the rectification factor.

Our studies suggest that this rectification factor may be the polyamines spermine and spermidine. At physiological pH, these polyamines have multiple positively charged amine groups that could bind to the negatively charged aspartate at the intracellular mouth of the channel pore. Since there was a degree of structural specificity to the polyamine block (the related polyamine putrescine and the divalent cation Mg^{2+} failed to support rectification), additional interactions such as with the glutamine of the Q/R site must also be important in stabilizing polyamine binding in the channel. Interestingly,

Ca^{2+}-permeable AMPA receptors in type II neurons were not only selectively blocked by intracellular spermine but also by extracellular spermine (Fig. 1; see also ref. 20). The inhibitory effect of extracellular spermine was use-dependent (20) and voltage-dependent (unpublished observations), suggesting that it occurs by a channel-blocking mechanism, as does the block of NMDA receptors by extracellular spermine (30–32). However, the blocking potency of extracellular spermine at −60 mV was nearly 10-fold less than its potency as an internal blocker at +60 mV. The electrostatic repulsive effects of a positive charge at the Q/R site in Ca^{2+}-impermeable AMPA receptors would presumably destabilize polyamine binding, so that Ca^{2+}-permeable AMPA receptors lacking this positive charge would be more susceptible to block. However, in contrast to the situation for intracellular spermine, binding of external spermine in Ca^{2+}-permeable AMPA receptors would not be stabilized by the downstream aspartate in the internal channel mouth, thus possibly accounting for the lower blocking potency of external spermine.

For intracellular polyamines to exert a physiological role in regulating inward rectification of Ca^{2+}-permeable AMPA receptors, their free cytoplasmic levels must be in the range of concentrations where the block occurs. The effect of intracellular spermine and spermidine on AMPA receptors occurs at substantially higher concentrations than does their effect on inwardly rectifying K^+ channels (22–24). Nevertheless, polyamines are likely to be present free in the cytoplasm at micromolar concentrations (24, 27). These levels are within the appropriate range for block of AMPA receptors. Indeed the free concentrations are markedly higher than the affinity of certain (strongly) inwardly rectifying K^+ channels, so that the polyamine blocking site of these channels is likely to be saturated at all times. In contrast, polyamine levels are more likely to fluctuate within the range of concentrations relevant for block of AMPA receptors. Paradoxically, therefore, although polyamines have lower affinity for AMPA receptors, they are more likely to play a role in regulating AMPA-receptor function in response to physiological fluctuations in the intracellular spermine and spermidine levels of the cell. In this regard, it has been reported that synaptic stimulation (33) and seizure activity can stimulate brain polyamine metabolism (34, 35). Moreover, polyamine levels may be chronically altered in epileptic brain tissue (36–38). However, it remains to be determined whether alterations in polyamine levels induce changes in the activity of AMPA receptors that contribute to epileptic hyperexcitability.

Polyamines are now well recognized to have multiple actions on NMDA receptors (for review, see ref. 39), and it has been proposed (40) that changes in brain polyamine levels might regulate neuronal excitability by altering the activity of NMDA-receptor-mediated synaptic responses. Our present results indicate that changes in polyamine levels may also affect neuronal activity through their actions on Ca^{2+}-permeable AMPA receptors.

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