Concentration-Jump Analysis of Voltage-Dependent Conductances Activated by Glutamate and Kainate in Neurons of the Avian Cochlear Nucleus

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ABSTRACT We have examined the mechanisms underlying the voltage sensitivity of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptors in voltage-clamped outside-out patches and whole cells taken from the nucleus magnocellularis of the chick. Responses to either glutamate or kainate had outwardly rectifying current-voltage relations. The rate and extent of desensitization during prolonged exposure to agonist, and the rate of deactivation after brief exposure to agonist, decreased at positive potentials, suggesting that a kinetic transition was sensitive to membrane potential. Voltage dependence of the peak conductance and of the deactivation kinetics persisted when desensitization was reduced with aniracetam or blocked with cyclothiazide. Furthermore, the rate of recovery from desensitization to glutamate was not voltage dependent. Upon reduction of extracellular divalent cation concentration, kainate-evoked currents increased but preserved rectifying current-voltage relations. Rectification was strongest at lower kainate concentrations. Surprisingly, nonstationary variance analysis of desensitizing responses to glutamate or of the current deactivation after kainate removal revealed an increase in the mean single-channel conductance with more positive membrane potentials. These data indicate that the rectification of the peak response to a high agonist concentration reflects an increase in channel conductance, whereas rectification of steady-state current is dominated by voltage-sensitive channel kinetics.

INTRODUCTION

Voltage-dependent conductances have been described for various ligand-gated ion channels such as the *N*-methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) subtypes of glutamate receptors and nicotinic acetylcholine receptors. The voltage dependence of the conductance of the *N*-methyl-D-aspartate receptor is thought to serve a critical function in regulating the activity of the receptor by requiring coincident ligand binding and depolarization for channel opening (reviewed by McBain and Mayer, 1994). In other receptors, the voltage sensitivities of gating and channel block have a less dramatic effect on channel function; nevertheless, studies of voltage dependence have allowed inferences to be made about the dynamics of transmitter-receptor interactions (Magleby and Stevens, 1972; Andersen and Stevens, 1973; Sheridan and Lester, 1977; Zhang and Trussell, 1994).

The strong inward rectification of AMPA receptors in some cultured hippocampal neurons (Iino et al., 1990) and in Bergmann glia (Müller et al., 1992; Burnashev et al., 1992) is indicative of high permeability to Ca^{2+}; these cell types express AMPA receptors lacking the GluR2 subunit (Burnashev et al., 1992; Bochet et al., 1994). Similarly, when heteromeric AMPA receptors lacking GluR2 are expressed in nonneuronal cells, the channels are highly permeable to Ca^{2+} and have inwardly rectifying current-voltage (I-V) relations (Hollmann et al., 1991; Verdoorn et al., 1991). The presence of GluR2 abolishes inward rectification and reduces Ca^{2+} permeability (Hollmann et al., 1991; Verdoorn et al., 1991). Consistent with widespread expression of GluR2, studies of AMPA receptors generally have demonstrated low Ca^{2+} permeability and either linear or outwardly rectifying I-V relations. However, some studies have identified native AMPA receptors exhibiting both outwardly rectifying I-V relations and substantial Ca^{2+} permeability (Gilbertson et al., 1991, Jonas et al., 1994, Otis et al., 1995). It has recently been suggested that inward rectification results from diffusible, cytoplasmic channel blockers (Bowie and Mayer, 1995). Thus, high Ca^{2+} permeability and outward rectification in responses obtained from excised patches might result from the loss of such a blocking agent. However, these properties can also be observed in whole cell recordings. For example, in the nucleus magnocellularis (nMAG) of the chick brain stem, synaptic current I-V relations are strongly outwardly rectifying, and the synaptic AMPA receptors have a high Ca^{2+} permeability (Otis et al., 1995).

The biophysical origin of outward rectification of AMPA receptors has been controversial. Studies of hippocampal neurons provide evidence that outward rectification of currents at AMPA receptors could result from the voltage sensitivity of the rate of entry into a desensitized state (Patneau et al., 1993). By contrast, data from spinal trigeminal neurons suggest that rectification arises from open

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channel block by Ca^{2+} (Gu and Huang, 1991). We have studied the basis for the outward rectification of ligand-gated currents at the AMPA receptors of voltage-clamped neurons of the nMAG. Rectification of current responses does not seem to be due solely to a voltage-dependent transition into the desensitized state nor to channel block by divalents. Instead, the open time, or burst duration, of the receptors seems to increase with membrane potential. Additionally, the single-channel conductance increases at positive holding potentials. Some of these data have been presented in abstract form (Raman and Trussell, 1992b; Otis et al., 1994).

MATERIALS AND METHODS

Neurons were enzymatically isolated from the nMAG of late-stage embryonic chicks (E18-E21), as described in Raman and Trussell (1992a). Responses to glutamate-receptor agonists solely reflect activation of AMPA receptors due to enzymatic cleavage of the *N*-methyl-D-aspartate receptor (Raman and Trussell, 1992a). The bath solution in the recording chamber contained 140 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 20 mM glucose, 1 mM pyruvate, and 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Tocris Neuramin, Bristol, UK), buffered to pH 7.3 with NaOH. Solutions were applied to outside-out patches (see below) with either the flow-pipe system (Raman and Trussell, 1992a) for voltage ramp responses or the piezoelectric/theta tube system (Raman et al., 1994) for experiments requiring extremely rapid application of agonist. Control solution had the same composition as bathing solution without pyruvate or CNQX, except as indicated in the low-divalent experiments. Drug solutions consisted of agonist dissolved in the corresponding control solutions as described in Raman et al. (1994). For the experiments using cyclothiazide, a 100-mM stock solution of cyclothiazide dissolved in dimethyl sulfoxide was diluted to the desired concentration in both the drug and the control solutions. All drugs were obtained from Sigma (St. Louis, MO) except kainic acid (Cambridge Research Biochemicals, Natick, MA), CNQX (Tocris Neuramin), aniracetam (gift of Hoffman-La Roche, Nutley, NJ), and cyclothiazide (gift of Eli Lilly, Indianapolis, IN).

Electrodes were pulled from borosilicate glass (Raman and Trussell, 1992a) and filled with a solution containing 70 mM Cs_2SO_4, 85 mM sucrose, 4 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, and 5 mM BAPTA, buffered to pH 7.3 with CsOH. Voltage-clamp recordings were made in either the whole-cell or outside-out patch mode (Hamill et al., 1981) with an Axopatch 1D patch clamp amplifier (Axon Instruments, Foster City, CA). Analysis was accomplished with pCLAMP software. Nonstationary variance analyses were performed as described previously (Sigworth, 1980; Hestrin, 1992; Raman and Trussell, 1995). The I-V data in Fig. 1 *D* were obtained by applying voltage jumps from −110 to +90 mV in the presence of 1 mM glutamate and subtracting jump responses in the absence of glutamate. All measurements of holding potential are corrected for junction potentials. Data are given as mean ± SD, and statistical significance was taken to be $p < 0.05$ (Student's *t*-test) unless otherwise stated. In some of the figures, electrical artifacts from the piezoelectric device have been removed for clarity.

RESULTS

Voltage sensitivity of agonist-evoked currents in patches

Responses to 30-ms applications of glutamate were measured at holding potentials from −100 to +100 mV in 20-mV steps. Current traces such as those shown in Fig. 1 *A* exhibit outward rectification of the peak current amplitude, as well as an increase in the time course of desensitization with more positive holding potential. Furthermore, as illustrated in the inset to Fig. 1 *A*, the amplitude of the steady-state current rectifies strongly, such that the extent of desensitization is reduced at positive potentials. The 10–90% rise time of the currents in response to 10 mM glutamate was generally between 0.13 and 0.18 ms at all potentials and was not voltage dependent. However, at this concentration, the rise time is probably limited by the time course of solution exchange over the patch (Raman and Trussell, 1995), and so we cannot determine whether the actual rise time of the response to high agonist concentrations is voltage sensitive.

The I-V for the peak currents evoked by 10 mM glutamate in five patches is shown in Fig. 1 *B*. Although the I-V is fairly linear at negative potentials, rectification develops at potentials positive to the reversal potential near 0 mV. Averages of measurements made at positive and negative potentials as well as statistical differences are given in Table 1. The I-V for the steady-state currents was more difficult to obtain, as the steady-state current in patches was often undetectable. However, for a set of five patches in which nonzero steady-state current was observed, there was a clear upward turn in the steady-state I-V relation, as shown in Fig. 1 *C*. Indeed, the rectification of the steady-state current seemed greater than that of the peak current. Rectification was not an artifact of patch excision, as I-Vs also turned upward for glutamate-activated currents in whole cells (Fig. 1 *D*). From the data shown in Fig. 1 *C*, we examined how membrane potential affects the extent of desensitization. The ratio of steady-state to peak current expressed as a percentage was determined for five patches and ranged between 0.15 and 3.5 at −80 to −100 mV and between 0.5 and 6.9 at +80 to +100 mV. On average, the fraction of current remaining at steady-state increased 3.43 times per patch over this potential range.

In Fig. 2 *A*, the traces at +100 mV and −100 mV shown in Fig. 1 *A* are scaled to illustrate the apparent slowing of desensitization at positive potentials. Fig. 2 *B* shows the voltage dependence of the time constant of desensitization (τ_{des}) obtained by a single-exponential fit to the desensitizing phase of the currents. At negative potentials, the time course of desensitization is fairly constant, but at positive to 0 mV, τ_{des} lengthens. However, the time course of desensitization to high concentrations of glutamate is better fit with the sum of two exponentials (Raman and Trussell, 1992a). We therefore used double-exponential curve fits to assess whether the briefer component ($\tau_{fast,\ des}$) or the longer component ($\tau_{slow,\ des}$) rectified preferentially, or whether the relative contribution of the slow component increased. In order to make the best estimates of the time constants, data were generally used only if at least 14 records were averaged; consequently only two extreme potential ranges were examined from −60 to −80 mV and from +60 to +80 mV. Plots of $\tau_{fast,\ des}$ and $\tau_{slow,\ des}$ are shown in Fig. 2 *C* for six patches at negative and five patches at positive holding potentials. $\tau_{fast,\ des}$ increased slightly at positive potentials

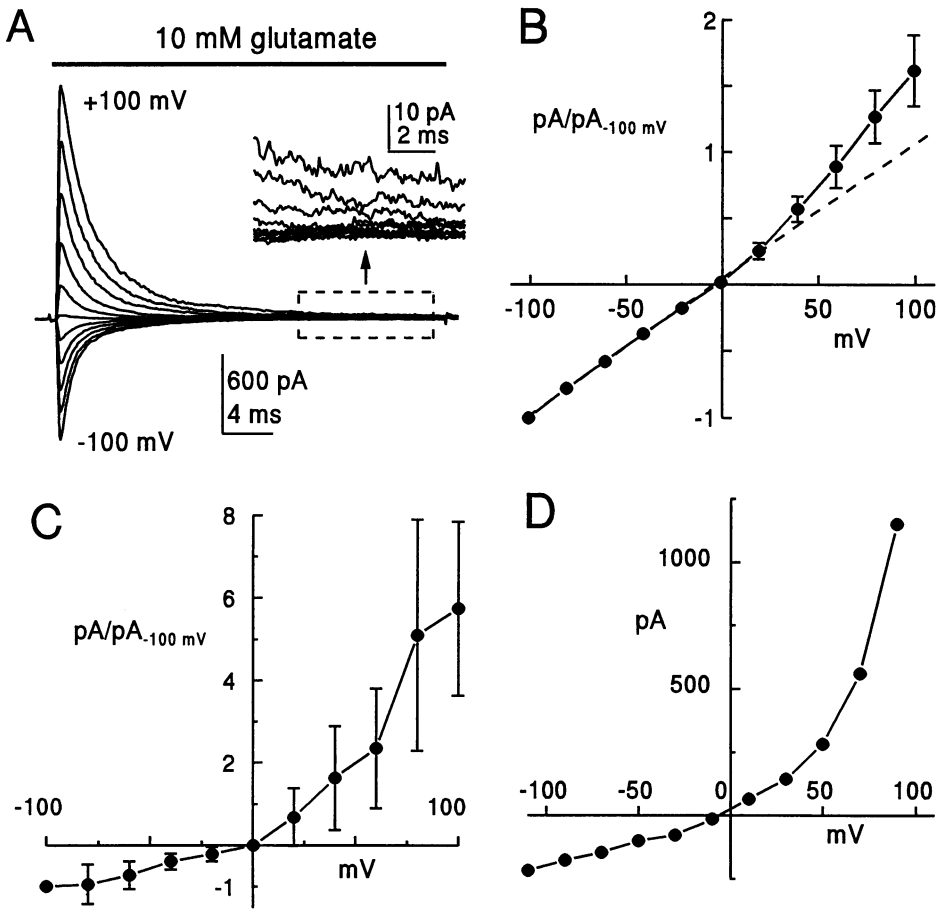

FIGURE 1 Currents activated by 10 mM glutamate at different holding potentials. (*A*) Glutamate was rapidly applied to an outside-out patch. Bars indicate the duration of drug application. Holding potentials were −100 to +100 mV in 20-mV increments. The region enclosed by the dashed line (- - -) is shown enlarged in the inset to illustrate the marked rectification of the steady-state currents. (*B*) I-V relation for peak current response to 10 mM glutamate. Peak currents evoked by 10 mM glutamate in five patches were normalized to the current at the potential nearest −100 mV and plotted against holding potential. Data were binned into 7-mV bins. To emphasize the extent of rectification, the dashed line (- - -) represents a linear regression over the points at the five most negative potentials. The estimated reversal potential from this fit is −0.43 mV. (*C*) I-V relation for the steady-state response in five patches after 30 ms of exposure to 10 mM glutamate. Data were binned as in (*B*) and normalized to the response at −100 mV. (*D*) Steady-state current obtained from a whole cell in response to voltage jumps (−100 to +90 mV, leak subtracted) in the presence of 1 mM glutamate, showing that outward rectification persists in intact cells.

but $\tau_{slow,\ des}$ showed no change. However, as illustrated in Fig. 2 *D*, %SLOW, the percentage of current contributed by the fast component, calculated as $A_S/(A_F+A_S)$ where A_F and A_S are, respectively, the amplitudes of the fast and slow exponential components extrapolated to the onset of the current response, decreases substantially with potential (Table 1). Thus, the more gradual onset of desensitization at positive potentials reflects an increase in the influence of one particular kinetic component.

After a brief exposure to glutamate, currents deactivate as channels close and ligand unbinds; the deactivation time provides a convenient estimate of the channel open time or burst duration. Deactivating currents were recorded after submillisecond pulses of glutamate. Brief pulses were necessary because the extent and rate of desensitization were too profound and rapid to permit measurement of deactivation after longer pulses. Representative traces at potentials from −100 mV to +100 mV are shown in Fig. 3 *A*. The single-exponential time constant of current deactivation (τ_{off}) increased significantly with holding potential, as illustrated in Fig. 3 *B*. However, like desensitization, glutamate deactivation currents could be fit with a sum of two exponentials, especially at positive potentials; 6 of 18 patches recorded at negative potentials gave clearly double-exponential decays, whereas 8 of 10 patches decayed biexponentially at positive potentials. Because deactivation is independent of agonist concentration (Raman and Trussell, 1992a, 1995), measurements of the time constants and %SLOW versus holding potential at three concentrations of glutamate were pooled and plotted in Fig. 3, *C* and *D*. As with desensitization, the major effect of depolarization is to increase the weight of the slower component of decay during deactivation (Table 1). The apparent rectification of desensitization and deactivation suggests that a kinetic parameter of channel gating is voltage dependent. Because deactivation shows strong voltage dependence, it may be that the effects of membrane potential on desensitization actually reflect voltage sensitivity of the microscopic rates of entry into or exit from the open state rather than the forward and backward rates associated with the desensitized state.

Several observations support this interpretation. Rectification was also seen in the response to 1 mM kainate, a weakly desensitizing agonist at the AMPA receptor, as shown in Fig. 4. The ratio of the 1 mM kainate-activated conductance at +70 mV to that at −70 mV was 2.21 ± 0.66 (N = 7). Although desensitization of kainate responses was weak, it may be that a significant fraction of the receptors desensitized faster than the rapid flow system could resolve, complicating the interpretation of voltage sensitivity of desensitization. To exclude this possibility, we also examined currents evoked by 100 μM kainate, a concentration that

TABLE 1 Quantification of rectification of currents at AMPA receptors in nMAG

Treatment	Negative potentials (−100 to −60 mV) mean ± σ (N)	Positive potentials (+60 to +100 mV) mean ± σ (N)	p
G_{MAX}, 10 mM glutamate	Normalized	1.61 ± 0.25 (5)	
τ_{des} (ms), 10 mM glutamate	1.45 ± 0.13 (6)	2.29 ± 0.25 (5)	**
$\tau_{fast,\ des}$ (ms), 10 mM glutamate	0.86 ± 0.08 (6)	1.18 ± 0.19 (5)	**
$\tau_{slow,\ des}$ (ms), 10 mM glutamate	3.39 ± 0.97 (6)	3.75 ± 0.58 (5)	ns
%SLOW, des, 10 mM glutamate	20 ± 5% (6)	36 ± 3% (5)	**
τ_{off} (ms), 0.3, 1, 10 mM glutamate	0.51 ± 0.11 (18)	0.87 ± 0.13 (10)	**
$\tau_{fast,off}$ (ms)	0.42 ± 0.10 (6)	0.53 ± 0.14 (8)	ns
$\tau_{slow,off}$ (ms)	1.71 ± 0.70 (6)	2.13 ± 0.77 (8)	ns
%SLOW, off	8 ± 5% (6)	23 ± 16% (8)	*
G_{MAX}, 1 mM kainate	Normalized	2.21 ± 0.66 (8)	
τ_{off} (ms), 1 mM kainate	1.15 ± 0.10 (7)	2.62 ± 0.36 (7)	**
$\tau_{fast,\ off}$ (ms), 1 mM kainate	0.76 ± 0.08 (5)	1.49 ± 0.51 (5)	*
$\tau_{slow,\ off}$ (ms), 1 mM kainate	1.16 ± 0.10 (5)	4.60 ± 1.64 (5)	**
%SLOW, off, 1 mM kainate	31 ± 10% (5)	43 ± 20% (5)	ns
G_{MAX}, 60 μM CTZ + 1 mM glutamate	Normalized	2.10 ± 0.71 (7)	
τ_{off} (ms), CTZ + glutamate	2.63 ± 1.07 (8)	6.92 ± 4.04 (7)	*
$\tau_{fast,\ off}$ (ms), CTZ + glutamate	1.13 ± 0.44 (7)	2.33 ± 1.18 (7)	*
$\tau_{slow,\ off}$ (ms), CTZ + glutamate	9.12 ± 6.87 (7)	19.0 ± 13.0 (7)	ns
%SLOW, off, CTZ + glutamate	23 ± 12% (7)	40 ± 10% (7)	*

CTZ, cyclothiazide.
*$p < 0.05$; **$p < 0.005$; ns, not significant.

produced a current which activated more slowly than the rate of solution exchange. Traces of currents at +90 and −90 mV evoked by 100 μM kainate are shown in Fig. 4 *B*. The degree of rectification was actually more pronounced in lower concentrations of kainate, as is evident by comparing the responses to 100 μM and 1 mM kainate in Fig. 4, *A* and *B*. For 100 μM kainate, the ratio of the conductance at positive to that at negative potentials was 6.90 ± 0.54 (N = 4). As was the case for glutamate, responses to kainate reversed near 0 (data not shown). Notably, the time constant of current deactivation, τ_{off}, after removal of kainate also exhibited a significant voltage dependence. In Fig. 4 *C*, the deactivating phases of the currents in Fig. 4 *A* have been normalized to illustrate the difference in the rate of termi-

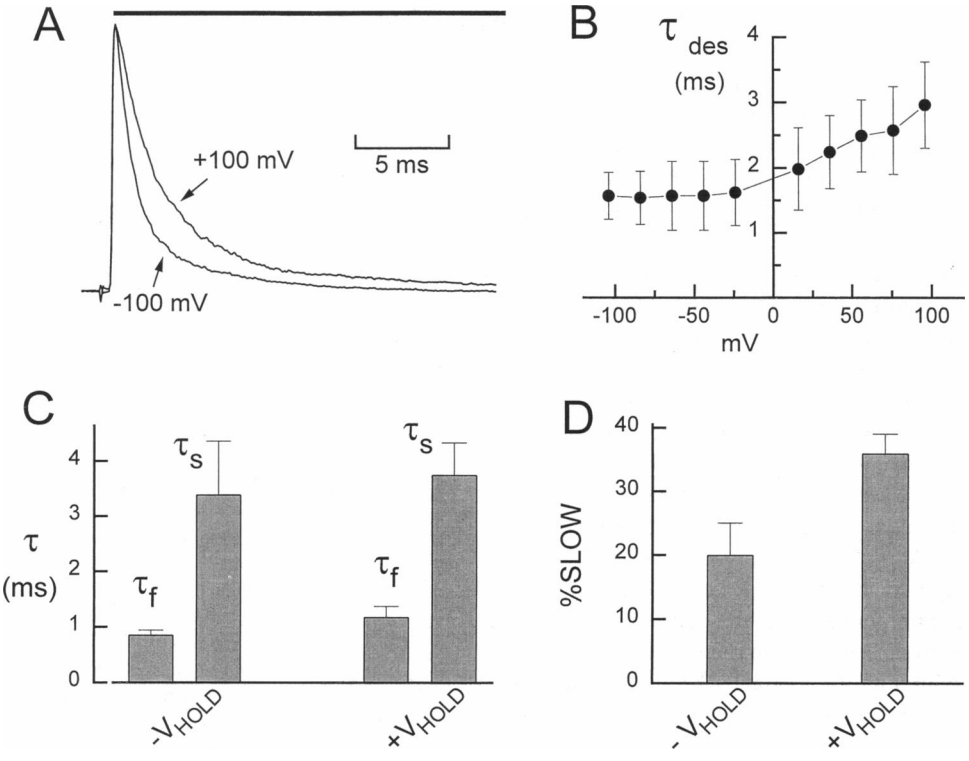

FIGURE 2 Rectification of glutamate-activated peak currents and decay constants. (*A*) Responses in Fig. 1 *A* at +100 and −100 mV are scaled to illustrate the apparent slowing of desensitization at positive potentials. Black bar shows duration of application. (*B*) Time constants of decay (τ_{des}) from single-exponential fits to the desensitizing phase of currents evoked by 10 mM glutamate, plotted against holding potential. Data from six patches in 12-mV bins. (*C*) Fast and slow time constants of decay plotted for patches held at −60 to −80 mV ($-V_{HOLD}$) or +60 to +80 mV ($+V_{HOLD}$). Data from five to six patches. (*D*) %SLOW (defined in text) for the data set shown in (*C*).

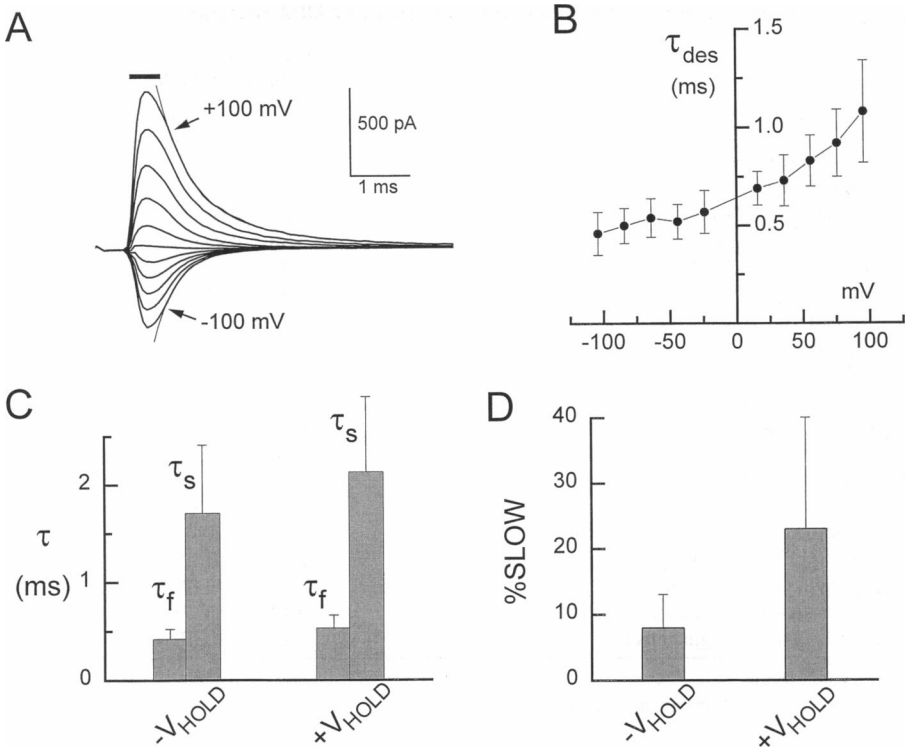

FIGURE 3 Deactivation rates are voltage dependent. (*A*) Applications (500-μs) of 10 mM glutamate to a single patch. Holding potentials were from −100 to +100 mV in 20-mV increments. Same patch as in Fig. 1. The traces at the extreme potentials are shown with single-(−100 mV) and double-exponential (+100 mV) fits superimposed on the deactivating current. For the fit to the +100-mV trace, $\tau 1$ = 0.54 ms, $\tau 2$ = 2.0 ms, and %SLOW = 13%, whereas the fit for −100 mV was a single exponential of τ = 0.40 ms. Black bar shows duration of application. (*B*) Single-exponential time constant of deactivation (τ_{off}) as a function of holding potential. Data from nine patches, in 14-mV bins. Measurements were pooled from applications of 10, 1, and 0.3 mM glutamate (see text). (*C*) Fast and slow time constants of decay plotted for patches held at −60 to −80 mV ($-V_{HOLD}$) or +60 to +80 mV ($+V_{HOLD}$). Data from six to eight patches. (*D*) %SLOW (defined in text) for the data set shown in (*C*).

nation of the currents at the two potentials. In five of seven patches, deactivation times of kainate-evoked currents were better fit with the sum of two exponentials. Both the brief and the long component increased significantly with membrane potential (Table 1). The rise time of responses to 100 μM kainate were not voltage sensitive: the 10–90% rise time of the currents at positive (+87 to +90 mV) potentials was only 1.06 ± 0.25 times that at negative (−93 to −90 mV) potentials (N = 4).

Additional evidence that the microscopic rate of entry into a desensitized state is not responsible for the observed voltage dependence came from experiments with cyclothiazide, a blocker of AMPA receptor desensitization (Patneau et al., 1993; Trussell et al., 1993; Yamada and Tang, 1993). As shown in Fig. 5 *A*, rectification of glutamate-activated current persisted in the presence of 60 μM cyclothiazide, a concentration sufficient to eliminate desensitization. Fig. 5 *B* displays the I-V relation for the maximal currents measured in eight patches exposed to 1 mM glutamate in the continued presence of 60 μM cyclothiazide. Regression over the points at the most negative potentials indicated that the reversal potential shifted slightly negative; this extrapolated reversal of −5 mV was used to calculate the conductance ratios for glutamate with cyclothiazide given in Table 1. In two of eight patches, cyclothiazide failed to abolish desensitization completely at the most positive holding potentials. Like the maximal currents, the time course of current deactivation continued to rectify in cyclothiazide, as shown in Fig. 5, *A* and *C*. Deactivating currents in cyclothiazide were best fit with the sum of two exponentials, although the $\tau_{slow,\ off}$ and %SLOW were quite variable. A

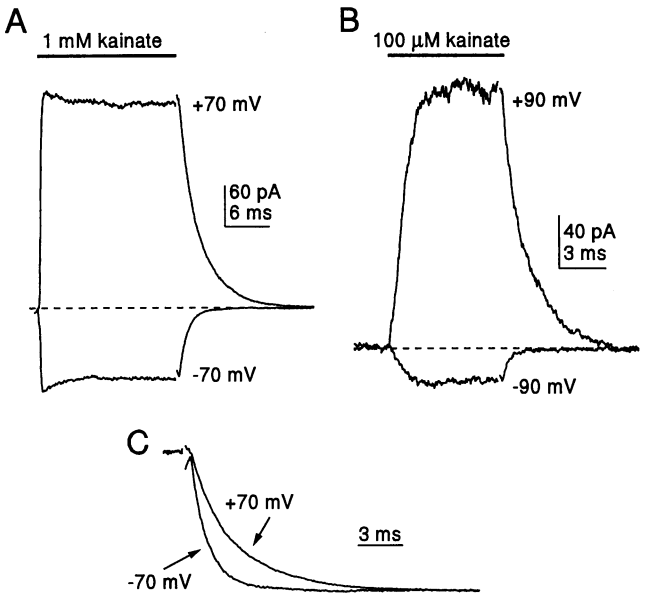

FIGURE 4 Rectification of kainate-evoked currents. (*A*) Kainate (1 mM) was applied to a patch held at −70 mV and +70 mV. The dashed line (- - -) indicates 0 current. Mean of 16 records. (*B*) Kainate (100 μM) was applied to a patch, different from the one in (*A*). Holding potentials were +90 and −90 mV. Mean of five records. (*C*) Current offsets from (*A*) are normalized to the maximal current in each trace in order to illustrate the slower deactivation at positive potentials. The time constants (in milliseconds) from a double-exponential fit to the deactivating phase and the relative contributions of the two components at −70 mV were $\tau_{fast,\ off}$ = 0.87, $\tau_{slow,\ off}$ = 1.81, %SLOW = 30%; at +70 mV, $\tau_{fast,\ off}$ = 1.23, $\tau_{slow,\ off}$ = 3.52, and %SLOW = 68%.

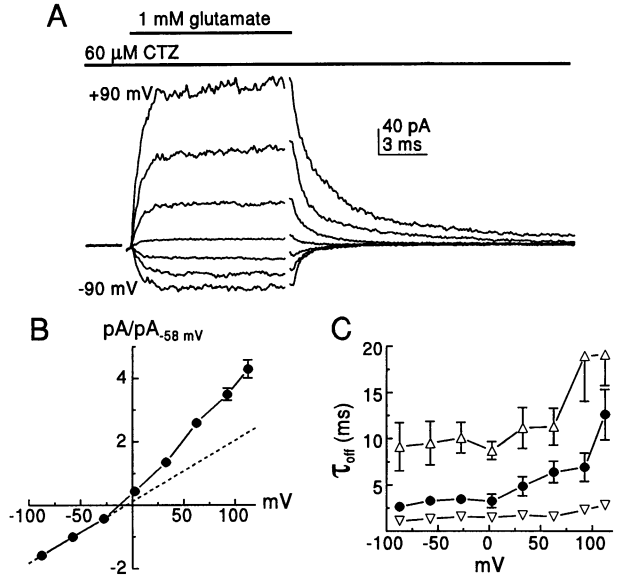

FIGURE 5 Rectification persists in the presence of cyclothiazide. (*A*) Responses of a patch to 1 mM glutamate in the presence of 60 μM cyclothiazide. Holding potentials were −90 mV to +90 mV in 30-mV steps. Each trace is an average of five records. (*B*) Maximal currents from eight patches were normalized to the current evoked at the holding potential nearest −58 mV (11-mV bins). The dotted line (· · ·) is a linear regression over the data at the three most negative potentials, and it has a slope of 0.019 and a *y*-intercept of 0.11. The reversal potential is therefore estimated at −5.8 mV. (*C*) Deactivation time constants versus holding potential; data from eight patches as in (*B*). Bars are standard errors. Filled symbols (●) represent values from single-exponential fits. Triangles show the time constants from double-exponential fits, with the inverted (▽) and upright (△) triangles representing the fast and slow components, respectively.

particularly slow component developed at the most extreme positive potentials, but at negative potentials the dominant decay time constant was quite fast (~1 ms; see Table 1). Also, despite the changes in the magnitude of the various time constants, the ratios of the deactivation time constants at positive and negative potentials shown in Table 1 were very similar for glutamate (τ_{off} at positive potentials ÷ τ_{off} at negative potentials = 2.35), glutamate with cyclothiazide (2.63), and kainate (2.28).

To identify any voltage dependence associated with transition out of the desensitized state, we examined the time course of recovery from desensitization at +50 mV. A previous study (Raman and Trussell, 1995) showed that recovery of nMAG AMPA receptors from desensitization at −70 mV occurs with a single-exponential time constant, τ_{rec}, of 16 ms. Patches held at +50 mV were conditioned with a 0.96-ms application of 1 mM glutamate. The amplitudes of responses to a second pulse of glutamate were measured at various intervals after the first pulse, and an index of recovery was obtained by finding the ratio of the amplitude of the second pulse to that of the first, conditioning pulse. Representative paired responses from a single patch are shown in Fig. 6 *A*. The average recovery is plotted against interval in Fig. 6 *B*. The time course of recovery could be fit with a single exponential with a τ_{rec} of 14.1 ms, indicating that the recovery rate was not strongly dependent on membrane potential.

The action of aniracetam

The drug aniracetam reduces but does not abolish desensitization and is associated with a prolongation of channel open time (Tang et al. 1991; Vycklicky et al., 1991). Glutamate-evoked currents were recorded in the presence of aniracetam to determine whether slowing of the kinetics of the receptors might also reduce the sensitivity of the receptors to voltage. Fig. 7 *A* shows responses of a patch to 1 mM glutamate plus 3 mM aniracetam at different potentials. Aniracetam markedly slowed the onset and reduced the extent of desensitization (*left and middle*) and slowed the deactivation rate after brief agonist pulses (*right*). Fig. 7, *B–D*, shows the effects of potential on current amplitude, extent of desensitization, and the time constants of desensitization and deactivation, averaged from four patches. Desensitization and deactivation were described with single-exponential fits for convenience in Fig. 7, as we could not consistently fit traces at all potentials with sums of two exponentials.

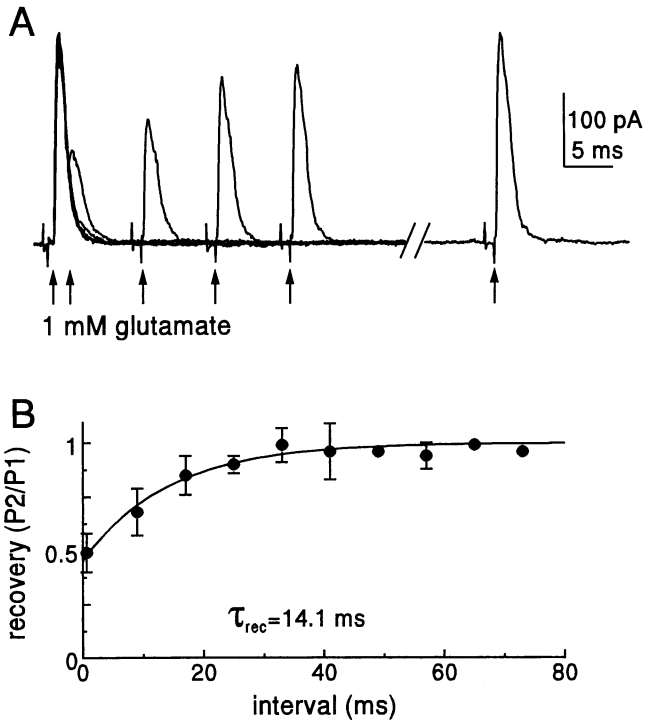

FIGURE 6 The time course of recovery from desensitization is independent of membrane potential. (*A*) Pairs of pulses of 1 mM glutamate were applied to a patch held at +50 mV. Arrows indicate the onset of application of glutamate. The first pulse was 0.96 ms and subsequent pulses were 1.3 ms. Intervals were 1, 9, 17, 25, and 65 ms. (*B*) In four patches, recovery was measured as ratio of the amplitude of the second pulse (P2) to that of the first pulse (P1) and was plotted against the interval between the pulses. The time course of recovery could be fitted with a single exponential with a time constant of 14.1 ms.

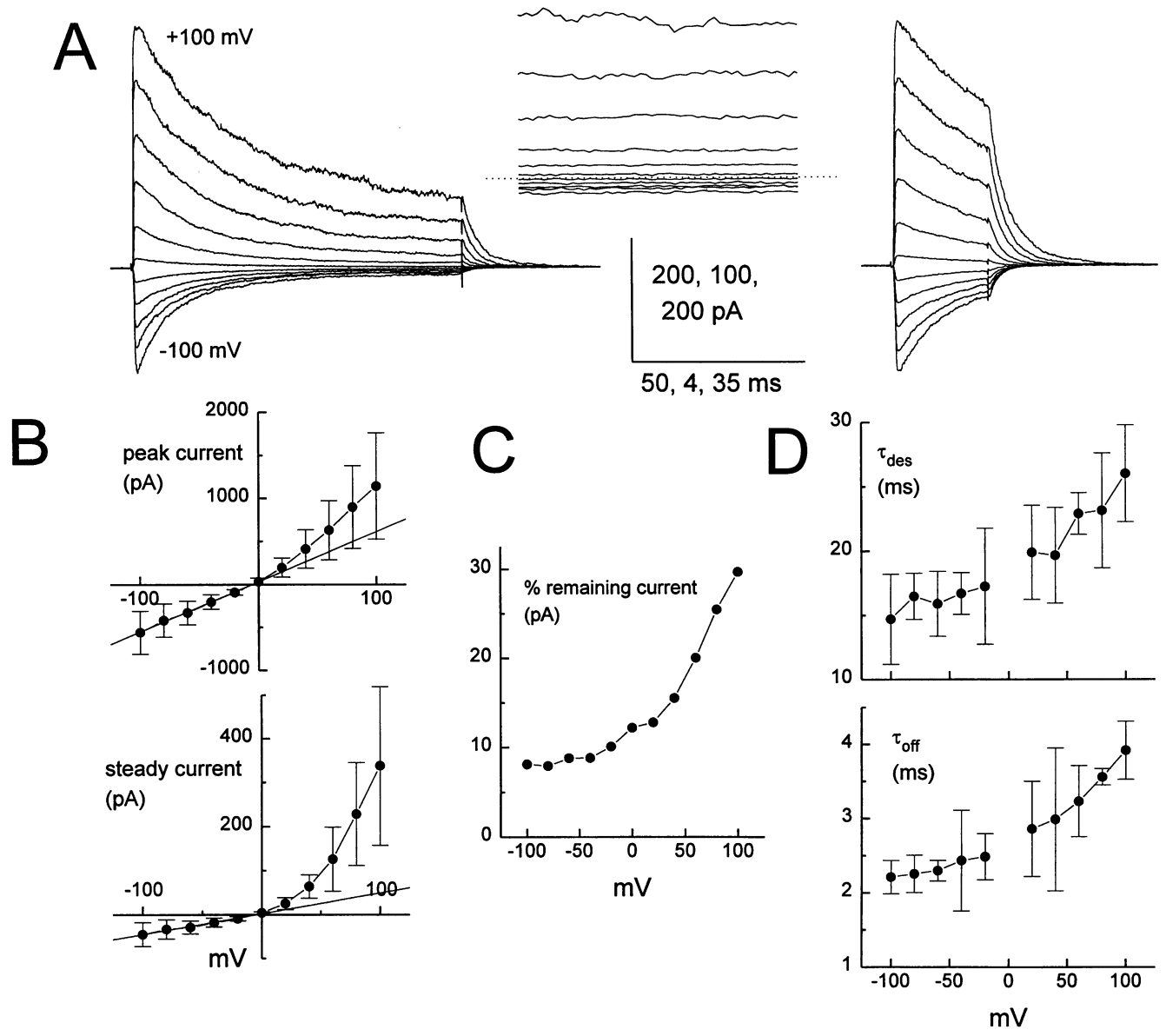

FIGURE 7 Effects of aniracetam on the voltage sensitivity of glutamate-evoked currents. (*A*) Left panel: superimposed traces obtained at different holding potentials showing responses to 200-ms steps of 1 mM glutamate plus 3 mM aniracetam. Holding potentials were −100 mV to +100 mV in 20-mV steps. Middle panel shows last 10 ms of the preceeding traces to illustrate the voltage sensitivity of steady-state current. Dotted line (· · ·) is at zero current. Right panel shows responses to 20-ms pulses of glutamate plus aniracetam to illustrate the deactivation of the current response. (*B*), (*C*), and (*D*) are I-V plots of the current amplitude (*B*), fractional steady-state current (*C*), obtained by dividing the bottom panel by the top panel of (*B*), and the single-exponential decay kinetics of desensitization and deactivation (*D*).

Using this simpler measure of decay kinetics, it is apparent that despite the nearly 10-fold increase in the decay times and the extent of desensitization seen in aniracetam, the fold increases in current and decay times at positive potentials were essentially identical to responses without the drug described above.

Voltage sensitivity of channel block and conductance

Experiments by Gu and Huang (1991) on spinal trigeminal neurons have indicated that extracellular Ca^{2+} reduces kainate-evoked currents through a channel-block mechanism. To examine whether removal or reduction of external divalents eliminates or diminishes rectification through AMPA receptor-channels of nMAG neurons, patches were chronically exposed to 1 mM kainate and the holding voltage was ramped from −120 to +120 mV in the presence and nominal absence of Ca^{2+} and Mg^{2+}. Control records were obtained with and without divalents but in the absence of kainate, and were subtracted from the corresponding experimental records. A representative I-V is shown in Fig. 8, illustrating that rectification is not abolished by reduction of divalents. However, in most trials, the magnitude of the

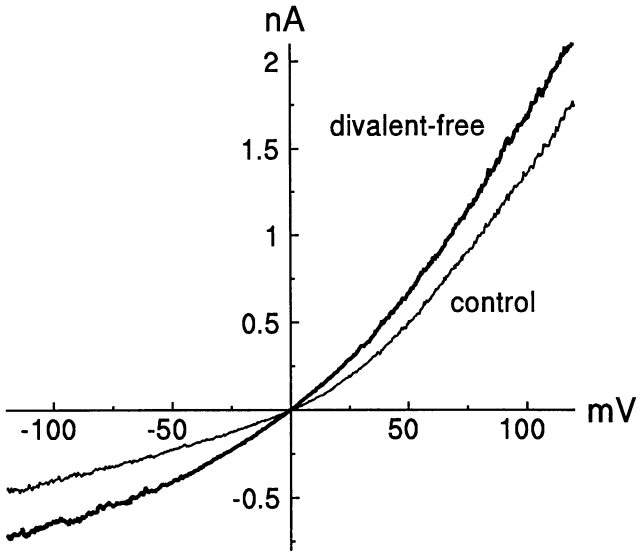

FIGURE 8 Rectification persists in low-divalent solutions. The thin line is an I-V relation obtained by applying a voltage ramp from −120 to +120 mV in the presence of 1 mM kainate and normal divalents; the thick line shows the kainate-activated currents in the nominal absence of divalents. Control records were subtracted to eliminate the contribution of leak currents, as described in the text.

currents was increased at all potentials, consistent with some blockade of channels by divalents. In four patches, rectification was measured as the ratio of the current amplitude at +120 to that at −120 mV. This ratio was 3.34 ± 1.05 in 3 mM Ca^{2+} and 1 mM Mg^{2+}, and 2.45 ± 0.52 in nominally divalent-free solutions, indicating that as the concentrations of extracellular divalent cations are lowered, the currents at negative potentials increased to a greater extent than did the currents at positive potentials. However, although this voltage dependence of divalent cation action is particularly apparent in the example shown, the difference was not significant in the population studied.

Voltage-dependent changes in peak current responses might arise from changes in peak open probability or in single-channel conductance. We estimated these parameters using nonstationary variance analysis of glutamate and kainate responses (see Methods section). Examples of repeated 30-ms applications of 10 mM glutamate to a patch held at −60 and +60 mV are shown in Fig. 9 *A*. The current variance, $\sigma^2(t)$ in pA^2, is plotted against the mean current, $I(t)$ in pA. The data could be fit with a parabola of the form,

$$\sigma^2(t) = iI(t) + I(t)^2/N + \sigma^2_n, \quad (1)$$

where i is the single-channel current, N is the total number of channels in the patch, and σ^2_n is the variance of the patch noise in the absence of glutamate. For cases in which the data set did not "roll over" sufficiently to allow an accurate parabolic fit, the data points were fit with a straight line of the form

$$\sigma^2(t) = iI(t) + \sigma^2_n, \quad (2)$$

The value of γ could be calculated from i/driving force, assuming a reversal potential of 0 mV. Sample traces and the variance versus mean relations for one patch held at symmetrical voltages are shown in Fig. 9 and indicate that γ is voltage dependent. At positive potentials (+51 to +72 mV), γ was 28.9 ± 7 pS (N = 5) vs. 19.3 ± 4.7 pS (N = 11) at negative potentials (−82 to −62 mV, data from Raman and Trussell, 1995). For the three patches that could be fit with parabolas, the open probability at the peak of the response, P_o, was calculated as I_{max}/iN, where I_{max} is the peak glutamate-evoked current. At positive potentials, P_o was 0.67 ± 0.10. In two patches, the responses did not roll over at positive potentials, indicative of a P_o less than 0.5. On average, P_o values obtained at positive potentials do not seem markedly different from the average P_o value of 0.54 measured at comparable negative potentials (Raman and Trussell, 1995).

Similar nonstationary variance analyses were performed on deactivation currents recorded upon rapid removal of 1 mM kainate from patches, as shown in Figure 10. The estimates of γ were considerably more variable for kainate than for glutamate. At negative potentials (−71 to −61 mV) γ was 14.7 ± 8.1 pS (N = 7), and at positive potentials (+60 to +78 mV) γ was 19.0 ± 7.9 pS (N = 6). In individual patches, the γ at positive potentials was always equal to or larger than the γ at negative potentials. Where possible, the P_o was calculated as for glutamate. In five of six patches, the P_o was 0.75 ± 0.11 at negative potentials, and in four of six patches P_o was 0.75 ± 0.20 at positive potentials. The P_o in the remaining patches was inferred to be below 0.5. Thus, as with glutamate, the P_os for kainate seemed similar at the different potentials.

DISCUSSION

Possible mechanisms of rectification in nMAG AMPA receptors

These data are consistent with a model in which transition rates between certain kinetic states are sensitive to voltage. The simplest interpretation is that the opening and closing rates of the channel (α and β of Scheme 1 below) are voltage sensitive; the attendant lengthening of the channel burst time should slow both deactivating and desensitizing currents (at least when the burst duration and desensitization time constants are rather similar), as observed for glutamate and kainate. Voltage dependence of gating is also consistent with the increase in rectification at lower kainate concentrations; stabilizing the open state will increase the apparent affinity of the receptor for agonist, thus shifting the dose-response curve to the left (Raman and Trussell, 1992a). A shift in the dose-response curve with membrane potential was also noted by Verdoorn and Dingledine (1988) for kainate responses in an oocyte expression system. It is unlikely that microscopic rates into or out of the desensitized state of the nMAG AMPA receptor are voltage dependent. This conclusion is based on the following observa-

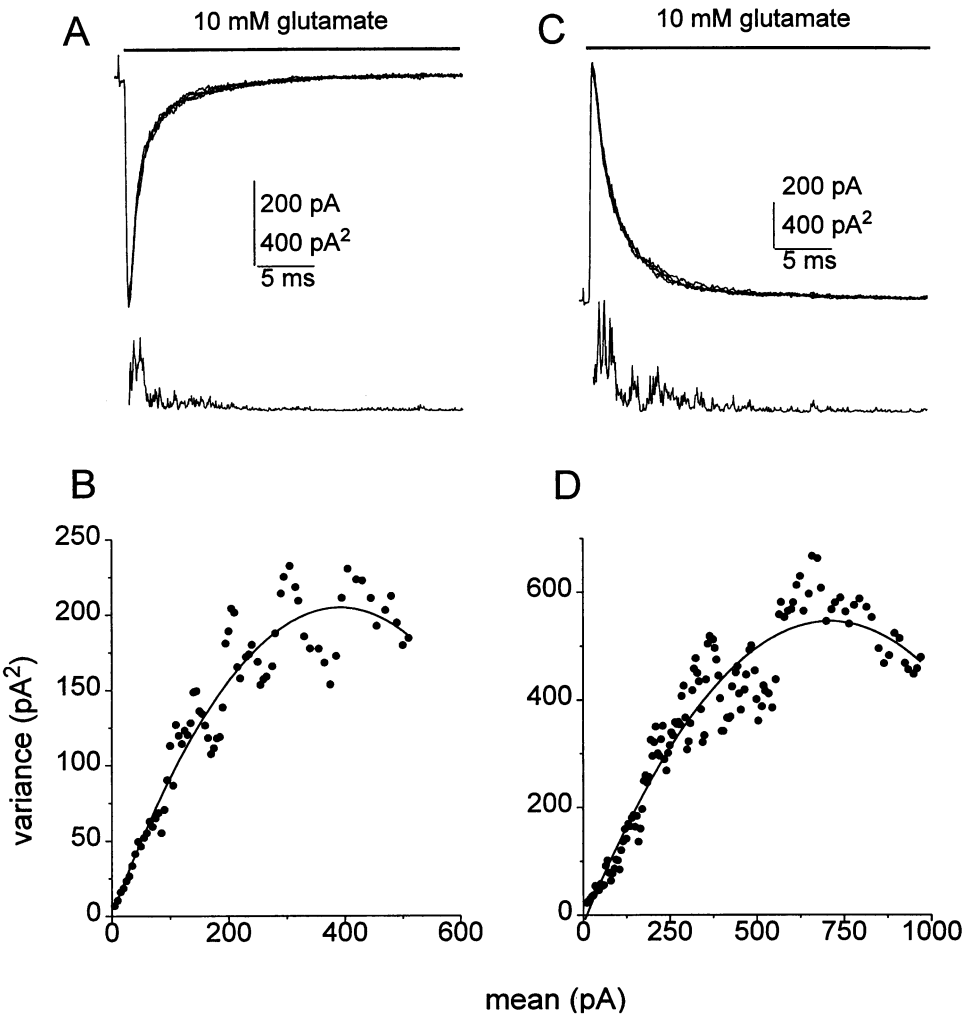

FIGURE 9 Nonstationary variance analysis of glutamate-gated currents. (*A*) Upper panel shows five replications of 10 mM glutamate applied to a patch held at −60 mV. Lower panel shows variance, $\sigma^2(t)$, of the five current records. (*B*) Variance versus mean plot for the patch shown in (*A*). Forty-eight records were included in the analysis. The solid line is a parabolic fit according to Eq. 1. The parameters i and N were estimated to be 1.04 pA and 753 channels, respectively, giving $p = 0.69$. (*C*) Same as in (*A*), except the holding potential was +60 mV. (*D*) Data obtained at +60 mV plotted as in (*B*). The parameters i and N were estimated to be 1.58 pA and 897 channels, respectively, giving $p = 0.74$.

tions: 1) rectification is seen with kainate, an agonist that is only weakly desensitizing in nMAG; 2) voltage sensitivity of deactivation is observed with either glutamate or kainate; 3) rectification is preserved during application of cyclothiazide; 4) stronger rectification is observed in lower kainate concentrations; 5) depolarization alters the relative magnitude, but not the value, of different rates describing the onset of desensitization; 6) the relative change in deactivation rates going from positive to negative potentials was the same for glutamate with and without cyclothiazide and for kainate; and 7) voltage sensitivity of recovery from desensitization is lacking. Although it remains possible that ligand binding or unbinding is voltage dependent, it is notable that rectification is not eliminated with relatively high agonist concentrations, which should saturate any effect of potential on the binding/unbinding equilibrium.

The effect of depolarization on both deactivation and desensitization is characterized predominantly by a shift in the weight of two exponential components. Possibly, channels have access to different open states, and depolarization increases the fraction of time that channels are in a longer-lived open state. However, without single-channel analysis, a full kinetic explanation of this behavior is not possible.

Two observations from this study militate against explaining voltage dependence by a purely kinetic mechanism. First, reduction of divalent cations did increase currents, and in some cases this effect was strongest at the more negative potentials. Second, nonstationary variance analysis of both kainate- and glutamate-evoked currents revealed a consistent increase in single-channel conductance upon depolarization, with little change in estimated peak open probability. The increase in channel conductance upon depolarization and the effects of divalent cations noted here and by Gu and Huang (1991) are probably related, in that rapid but partial channel block by Ca^{2+} might change the apparent channel conductance.

To reconcile these various observations, we suggest that depolarization favors both the slowing of channel gating

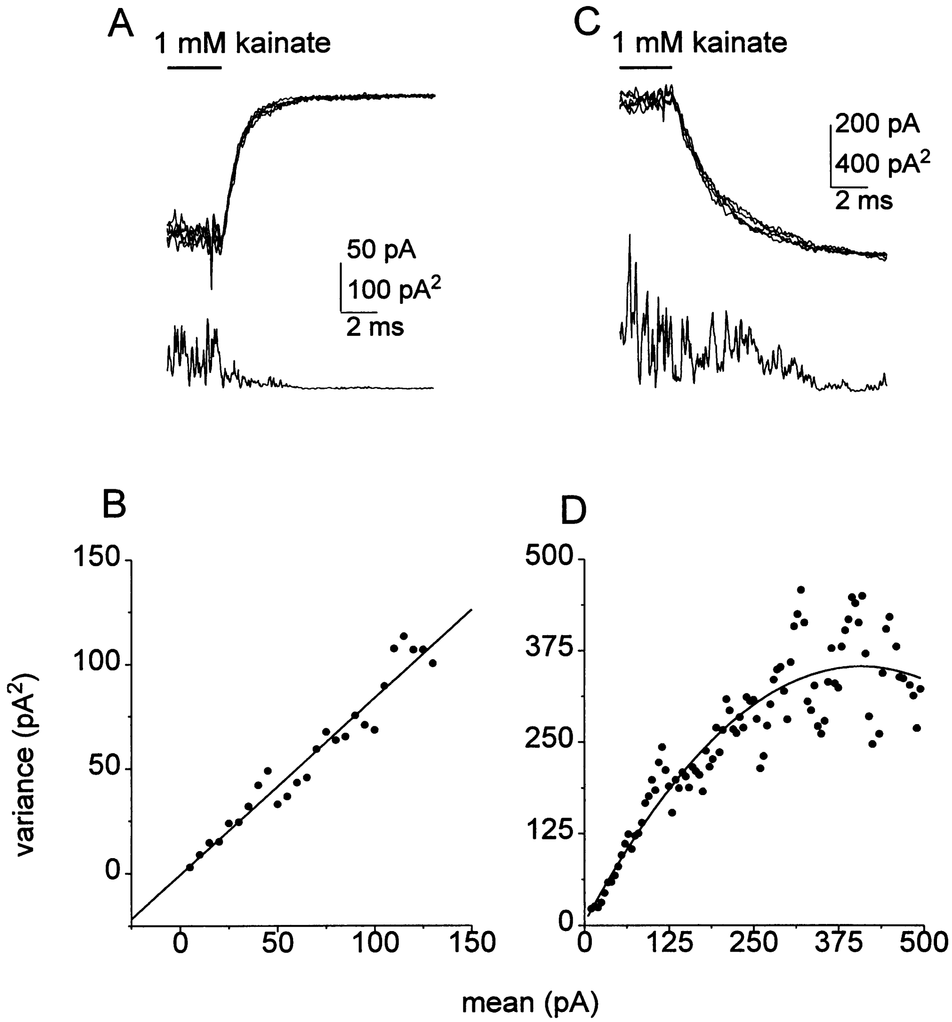

FIGURE 10 Nonstationary variance analysis of kainate-gated currents. (*A*) Upper panel shows five replications of 1 mM kainate applied to a patch held at −62 mV. Lower panel shows variance of the five kainate-activated current records. (*B*) Variance versus mean plot for the patch shown in (*A*). Thirty-two records were included in the analysis. The solid line is a linear regression (Eq. 2) over the data points and has a slope, *i*, of 0.84 pA and an intercept of −0.59. (*C*) Same as (*A*), except that the holding potential was +78 mV. (*D*) The data at +78 mV are plotted as in (*B*). Analysis includes 30 records. Solid line is a parabolic fit (Eq. 1) with i = 1.71 pA and N = 478 channels, giving p = 0.64.

and the rapid unblock of open channels by calcium ions. These effects are most apparent at different parts of the response to glutamate. Voltage dependence of single-channel conductance is probably the dominant cause of rectification in the peak current at high agonist concentrations. Indeed, the 50% increase in channel conductance measured using nonstationary variance analysis accounts well for the upward turn in the peak current I-Vs shown in Figs. 1 *B* and 6 *B*. After desensitization has occurred, the I-Vs rectify more sharply (Figs 1, *C* and *D*, and 6 *B*). A stronger effect of voltage-sensitive kinetics on steady-state currents than on peak currents is predicted from a simple model of gating:

$$\begin{array}{ccccc} C_0 & \overset{K_d}{\rightleftharpoons} & C_1 & \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} & O_1 \\ & & f \downarrow\uparrow b & & \\ & & D_1 & & \end{array}$$

Scheme 1

where C is a closed state, O is an open state, and D is a desensitized state, with subscripts indicating the number of bound agonist molecules. In this model, the equilibrium open probability given a high glutamate concentration is

$$\frac{\beta/\alpha}{1+\beta/\alpha+f/b} \tag{3}$$

and the peak open probability before desensitization approaches

$$\frac{\beta/\alpha}{1+\beta/\alpha} \tag{4}$$

Assuming a value of f/b of 100, a depolarization-induced increase in β/α from 5 to 10 would double the steady-state open probability yet would produce only a ~10% increase in the peak open probability. This explains the apparent lack of increase in open probability with depolarization observed using variance analysis: in that technique the measurement of a change in probability focuses on the peak, i.e., the extent of "roll-over" of the parabolic curve. Because the peak is less sensitive to voltage than the steady-state current, the major effect of voltage is to reduce the extent of desensitization (Fig. 7 *C*).

Voltage sensitivity of native AMPA receptors

The relative contributions to rectification of voltage-dependent kinetic steps, single-channel conductance, or channel block have not been extensively explored in other cell types. However, it seems likely that this behavior of native receptors varies among cell types. In many neurons, including cultured rat and mouse hippocampal neurons, CA1 pyramidal cells, CA3 pyramidal cells, dentate gyrus granule neurons, aspiny neurons, spiny hilar neurons from rat brain slices, and isolated rat and chick spinal neurons (Trussell and Fischbach, 1989; Smith et al., 1991; Thio et al., 1991; Colquhoun et al., 1992; Livsey et al., 1993; Patneau et al., 1993; Yamada and Tang, 1993), peak currents evoked by AMPA-receptor agonists applied with fast flow techniques show little or no rectification, at least when examined at potentials up to ~+50 mV. The deactivation rate after brief applications of glutamate in hippocampal CA1, CA3, and dentate gyrus cells, and in cerebellar Purkinje cells, is not voltage dependent (Colquhoun et al., 1992; Barbour et al, 1994), and in rat spinal neurons the desensitization rate does not vary with voltage (Smith et al., 1991). In our initial experiments, we found that the voltage dependence of the time constants of desensitization and their relative amplitude showed no consistent pattern from cell to cell. Upon signal averaging large current responses, it became clear that the time constants themselves did not change so much as the contribution of the slower component increased. This observation, and the fact that separation of similar kinetic components of desensitization is difficult with low agonist concentrations (Raman and Trussell, 1992a), indicates that more complete analyses might reveal a similar pattern of voltage-dependent kinetics in other cell types. Whether there was a kinetic element to rectification was also examined in hippocampal neurons by Jonas and Sakmann (1992) and in spinal trigeminal neurons by Gu and Huang (1991). Voltage steps were applied to neurons or patches in the chronic presence of AMPA or kainate. Current relaxation was observed during the jumps in the hippocampal neurons but not in the spinal trigeminal neurons. Recent studies from this laboratory show strong relaxations during voltage jumps with activation of nMAG AMPA receptors (Otis et al., 1994). Differences in voltage dependence of glutamate-mediated synaptic currents among cells with linear or outwardly rectifying AMPA receptor currents might suggest additional experimental protocols for identification of the subunit composition of native synaptic receptors.

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REFERENCES

Andersen, C. R., and C. F. Stevens. 1973. Voltage clamp analysis of acetylcholine produced end-plate current fluctuations at frog neuromuscular junction. *J. Physiol.* 235:655–691.

Barbour, B., B. U. Keller, I. Llano, and A. Marty. 1994 Prolonged presence of glutamate during excitatory synaptic transmission to cerebellar Purkinje cells. *Neuron.* 12:1331–1343.

Bochet, P., E. Audinat, B. Lambolez, F. Crepél, J. Rossier, M. Iino, K. Tsuzuki, and S. Ozawa. 1994. Subunit composition at the single-cell level explains functional properties of a glutamate-gated channel. *Neuron.* 12:383–388.

Bowie, D., and M. L. Mayer. 1995. Inward rectification of both AMPA and kainate subtype glutamate receptors generated by polyamine mediated ion channel block. *Neuron.* 15:453–462.

Burnashev, N., A. Khodorova, P. Jonas, P. J. Helm, W. Wisden, H. Monyer, P. H. Seeburg, and B. Sakmann. 1992. Calcium-permeable AMPA-kainate receptors in fusiform cerebellar glial cells. *Science.* 256:1566–1570.

Colquhoun, D., P. Jonas, and B. Sakmann. 1992. Action of brief pulses of glutamate on AMPA/kainate receptors in patches from different neurones of rat hippocampal slices. *J. Physiol.* 458:261–287.

Gilberston, T. A., R. Scobey, and M. Wilson. 1991 Permeation of calcium ions through non-NMDA glutamate channels in retinal bipolar cells. *Science.* 251:1613–1615.

Gu, Y., and L.-Y. M. Huang. 1991. Block of kainate receptor channels by Ca^{2+} in isolated spinal trigeminal neurons of rat. *Neuron.* 6:777–784.

Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* 391:85–100.

Hestrin, S. 1992. Activation and desensitization of glutamate-activated channels mediating fast excitatory synaptic currents in the visual cortex. *Neuron.* 9:991–999.

Hollmann, M., M. Hartley, and S. Heinemann. 1991. Ca^{++} permeability of KA-AMPA-gated glutamate receptor channels depends on subunit composition. *Science.* 252:851–853.

Iino, M., S. Ozawa, and K. Tsuzuki. 1990. Permeation of calcium through excitatory amino acid receptor channels in cultured rat hippocampal neurones. *J. Physiol.* 424:151–165.

Jonas, P., C. Racca, B. Sakmann, P. Seeburg, and H. Monyer. 1994. Differences in Ca^{2+} permeability of AMPA-type glutamate receptor channels in neocortical neurons caused by differential GluR-B subunit expression. *Neuron.* 12:1281–1289.

Jonas, P., and B. Sakmann. 1992. Glutamate receptor channels in isolated patches from CA1 and CA3 pyramidal cells of rat hippocampal slices. *J. Physiol.* 455:143–171.

Livsey, C. T., E. Costa, and S. Vicini. 1993. Glutamate-activated currents in outside-out patches from spiny versus aspiny hilar neurons of rat hippocampal slices. *J. Neurosci.* 13:5324–5333.

Magleby, K. L., and C. F. Stevens. 1972. A quantitative description of endplate currents. *J. Physiol.* 223:173–197.

McBain, C. J., and M. L. Mayer. 1994. *N*-methyl-D-aspartic acid structure and function. *Physiol. Rev.* 74:723–760.

Müller, T., T. Möller, T. Berger, J. Schnitzer, amd H. Kettenmann. 1992. Calcium entry through kainate receptors and resulting potassium-channel blockade in Bergmann glial cells. *Science.* 256:1563–1566.

Otis, T. S., I. M. Raman, and L. O. Trussell. 1994. Voltage-dependent gating of AMPA receptors on chick auditory neurons. *Soc. Neurosci.* Abstr. 20:485.

Otis, T. S., I. M. Raman, and L. O. Trussell. 1995. AMPA receptors with high Ca^{++} permeability mediate synaptic transmission in the auditory pathway. *J. Physiol.* 482:309–315.

Patneau, D. K., L. Vyklicky, Jr., and M. L. Mayer. 1993. Hippocampal neurons exhibit cyclothiazide-sensitive rapidly desensitizing responses to kainate. *J. Neurosci.* 13:3496–3509.

Raman, I. M., and L. O. Trussell. 1992a. The kinetics of the response to glutamate and kainate in neurons of the avian cochlear nucleus. *Neuron.* 9:173–186.

Raman, I. M. and Trussell, L. O. 1992b. Voltage-dependent kinetics of currents activated by glutamate or kainate. *Soc. Neurosci.* Abstr. 18:653.

Raman, I. M., and L. O. Trussell. 1995. The mechanism of AMPA receptor desensitization after removal of glutamate. *Biophys. J.* 68: 137–146.

Raman, I. M., S. Zhang, and L. O. Trussell. 1994. Pathway-specific variants of AMPA receptors and their contribution to neuronal signaling. *J. Neurosci.* 14:4998–5010.

Sheridan, R. E., and H. A. Lester. 1977. Rates and equilibria at the acetylcholine receptor of *Electrophorus* electroplaques. *J. Gen. Physiol.* 70:187–219.

Sigworth, F. J. 1980. The variance of sodium current fluctuations at the node of Ranvier. *J. Physiol.* 307:97–129.

Smith, D. O., C. Franke, J. Rosenheimer, F. Zufall, and H. Hatt. 1991. Glutamate-activated channels in ventral spinal cord cells. *J. Neurophysiol.* 66:369–378.

Tang, C.-M., Q. Shi, A. Katchman, and G. Lynch. 1991. Modulation of the time course of fast EPSCs and glutamate kinetics by aniracetam. *Science.* 254:288–290

Thio, L. L., D. B. Clifford, and C. F. Zorumski. 1991. Characterization of quisqualate receptor desensitization in cultured postnatal rat hippocampal neurons. *J. Neurosci.* 11:3430–3441.

Trussell, L. O., and G. D. Fischbach. 1989. Glutamate receptor desensitization and its role in synaptic transmission. *Neuron.* 3:209–218.

Trussell, L. O., S. Zhang, and I. M. Raman. 1993. Desensitization of AMPA receptors upon multi-quantal neurotransmitter release. *Neuron.* 10:1185–1196.

Verdoorn, T. A., N. Burnashev, H. Monyer, P. H. Seeburg, and B. Sakmann. 1991. Structural determinants of ion flow through recombinant glutamate receptor channels. *Science.* 252:1715–1718.

Verdoorn, T. A., and R. Dingledine. 1988. Excitatory amino acid receptors expressed in *Xenopus* oocytes: agonist pharmacology. *Mol. Pharmacol.* 34:298–307.

Vyklicky, Jr., L., D. K. Patneau, and M. L. Mayer. 1991. Modulation of excitatory synaptic transmission by drugs that reduce desensitization at AMPA/kainate receptors. *Neuron.* 7:971–984

Yamada, K. A., and C.-M. Tang. 1993. Benzothiadiazides inhibit rapid glutamate receptor desensitization and enhance glutamatergic synaptic currents. *J. Neurosci.* 13:3904–3915.

Zhang, S., and L. O. Trussell. 1994. Voltage dependence of the EPSC and desensitization of AMPA receptors by transmitter. *Soc. Neurosci.* Abstr. 20:1715.