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Characterization of Excitatory Amino Acid Receptors Expressed by Embryonic Chick Motoneurons *in vitro*

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We have examined the effect of L-glutamate and other excitatory amino acids on embryonic chick motoneurons maintained in cell culture along with other types of spinal cord cells. When the motoneuron membrane is clamped at −50 mV, glutamate induces a dose-dependent inward current. Although the dose-response curve is hyberbolic with an ED_{50} of 78 μM, glutamate apparently activates 2 types of receptors on motoneurons. The first, G_1, is activated by *N*-methyl-D-aspartate (NMDA) and aspartate and inhibited by 2-amino-5-phosphonovaleric acid (2-APV). The second, G_2, is activated by kainate and quisqualate and is not inhibited by 2-APV. At −50 mV, 38% of the glutamate current is due to activation of G_1 receptors and the remaining 62% to G_2 activation. In contrast to motoneurons grown with other spinal cord cells, sorted motoneurons grown in isolation apparently exhibit only G_2 receptor-mediated currents.

Both G_1 and G_2 currents reverse polarity between −10 and −5 mV. However, they could be distinguished when the membrane was hyperpolarized. G_2 currents increased but G_1 currents decreased when the membrane potential was increased beyond −50 mV. Consistent with the mixed agonist action of glutamate, glutamate currents remained nearly constant on hyperpolarization. No evidence was obtained that the G_2 class of receptors on motoneurons could be subdivided: Quisqualate and kainate apparently compete for the same sites; gamma-glutamylglycine blocked quisqualate as effectively as it blocked kainate currents when the different potencies of the 2 agonists were taken into account.

Analysis of current fluctuations indicates that the glutamate-activated G_1 channel conductance (24 pS) and mean channel open time (3.7 msec) are more than twice as great as the G_2 channel conductance (10 pS) and open time (1.7 msec). The mean open time of G_2 channels activated by kainate (5.5 msec) is nearly 3 times longer than the open time of the same G_2 channels activated by glutamate. This disparity may account for the observation that the maximal kainate current is 3 times greater than the maximal glutamate G_2 current.

In the preceding paper (O'Brien and Fischbach, 1986a), we described techniques for labeling embryonic chick motoneurons in the lumbar lateral motor column and for growing them *in vitro* with or without other spinal cord cells. Such cultures provide an opportunity to examine the effect of interneurons on the distribution of motoneuron L-glutamate receptors. We have chosen to focus on L-glutamate because numerous studies have implicated this amino acid or a closely related compound as the transmitter mediating excitatory interactions between spinal cord interneurons and motoneurons *in vivo* (for recent reviews, see Fonnum, 1984; Foster and Fagg, 1984; Puil, 1981; Watkins and Evans, 1981).

Glutamate depolarizes motoneurons, but the precise mechanism of its action remains the subject of considerable debate. Studies of motoneurons in the adult cat spinal cord have found the depolarization to be associated with an increase in membrane conductance, a decrease in conductance, or no change at all (Bernardi et al., 1972; Engberg et al., 1979). These apparent conflicts have been resolved somewhat by recent experiments indicating that glutamate acts as a mixed agonist at more than one type of receptor.

Receptors for excitatory amino acids have been subdivided into several classes based on the relative potencies of various agonists, the selective action of antagonists, and the affinities of radiolabeled ligands (Foster and Fagg, 1984; Watkins and Evans, 1981). The details are not yet clear because of the dearth of potent antagonists and the complexity of the tissues examined. Nevertheless, two broad classes have been defined. One is activated preferentially by *N*-methyl-D-aspartate (NMDA) and inhibited by 2-amino-5-phosphonovaleric acid (2-APV), α-aminoadipate, and Mg^{2+}. The other is preferentially activated by kainate and quisqualate and is not blocked by 2-APV or Mg^{2+}. For convenience we shall refer to these receptors as G_1 and G_2, respectively. Voltage-clamp studies of cat motoneurons *in vivo* and of mouse spinal cord cells grown in cell culture indicate that kainate- and quisqualate-induced currents increase as the membrane is hyperpolarized, whereas NMDA-induced currents do not (Engberg et al., 1979; MacDonald and Porietis, 1982; MacDonald and Wojtowicz, 1982; MacDonald et al., 1982; Mayer and Westbrook, 1984). Indeed, *V–I* curves constructed in the presence of NMDA show a region of negative slope conductance between about −40 and −80 mV. The decrease in membrane conductance is, in large part, due to a voltage-dependent block of the ion channel by Mg^{2+} rather than to an effect of the membrane potential gradient on the channel itself (Mayer et al., 1984; Nowak et al., 1984). Consistent with its action as a mixed agonist, the glutamate *V–I* relation is intermediate between those characteristic of NMDA and kainate (Mayer and Westbrook, 1984).

In this paper we show that G_1 and G_2 receptor–ionophore complexes are present on identified embryonic chick motoneurons grown in heterogenous spinal cord cell cultures. In addition, we have characterized G_1 and G_2 receptors by studying a wide range of agonist concentrations and by analyzing current fluctuations to estimate mean channel conductance and mean channel open time associated with each. The importance of studying identified cells is emphasized by our finding that only 1 type of glutamate response (G_2) can be evoked in sorted motoneurons grown in the absence of interneurons or glia. A preliminary

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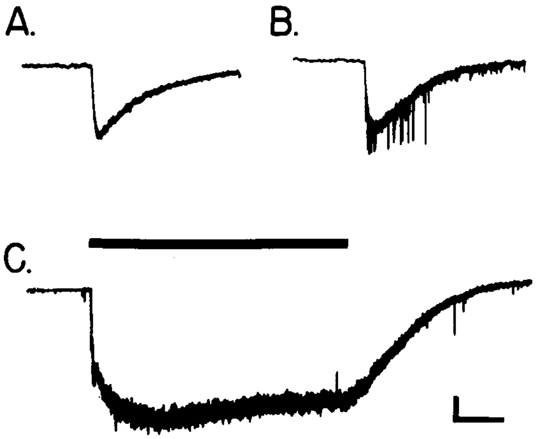

Figure 1. L-Glutamate currents in identified motoneurons. Labeled motoneurons were identified by fluorescence microscropy 6 d after plating. *A* and *B*, Glutamate, 10^{-4} M, applied by a 0.4 sec pressure pulse (beginning with the rising phase of the inward current). Note the fast inward currents superimposed on the response in *B*. *C*, Glutamate application was continued for the duration of the overlying bar. Calibration bars: 50 pA, 2 sec.

account of our results has been presented (O'Brien and Fischbach, 1983).

Materials and Methods

Cell cultures

Techniques for labeling motorneurons *in vivo* by retrograde transport of Lucifer Yellow–wheat germ agglutinin or fluorescein isothiocyanate–wheat germ agglutinin conjugates and for identifying them *in vitro* were described in the preceding paper (O'Brien and Fischbach, 1986a). Spinal cord cells dissociated from 6 d embryos were plated on collagen-coated glass coverslips containing multinucleated myotubes and grown in Eagle's Minimum Essential Medium supplemented with chick embryo extract (2%, vol/vol) and heat-inactivated horse serum (10%, vol/vol). Electrophysiological experiments were performed on the 5th or 6th day after plating.

Electrophysiology

L-Glutamate and other agonists were applied to motoneurons at various concentrations by pressure ejection from pipettes broken off to 2–5 μm tip diameter and positioned about 100 μm from the cell bodies. In some experiments, receptor antagonists were mixed with agonists and applied from the same pipette. L-Glutamate, NMDA, kainate, and quisqualate were obtained from Sigma Chemicals. Glutamate diethyl ester, gamma-D-glutamylglycine (γGG), and 2-amino-5-phosphonovalerate were obtained from Cambridge Research Biochemicals. All experiments were performed at room temperature (24°C), while the cells were bathed in a simple salt solution containing 120 mM NaCl, 5.4 mM KCl, 3.6 mM $CaCl_2$, 0.16 mM $MgCl_2$, 0.64 $MgSO_4$, 0.8 mM NaH_2PO_4, 6 mM glucose, and 12 mM HEPES (pH 7.4).

Membrane currents were recorded with a Dagan 8900 amplifier equipped with a 10^{10} Ω feedback resistor. The preparation of patch-clamp electrodes and techniques for whole-cell recording were also similar to those described previously. Except where noted in the text, the electrodes were filled with an "intracellular" solution of 140 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM EGTA-KOH, and 10 mM HEPES (pH 7.4). They measured 4–5 × 10^6 Ω prior to cell contact, and the access resistance increased 2- to 4-fold after intracellular communication was established. As most of the amino acid-induced currents were less than 200 pA, series resistance compensation was not employed. The total cell capacitance determined by integrating the discharge of current following a small voltage step was about 40 pF, and the time constant of decay of the capacitance current was less than 1 msec. Amino acid-induced currents were filtered at 1 kHz and displayed on a Tektronix oscilloscope and a Gould chart recorder.

The ability to space-clamp the membrane exposed to a pulse of L-glutamate depends on the electrotonic length of the dendrites. During the first week in culture, when the cells are small, square current pulses injected into the soma generate electrotonic potentials that rise and fall along a single exponential (O'Brien and Fischbach, 1986a). Therefore, a large portion of the neuritic arbor must be electronically "close" to the cell body (Rall, 1977).

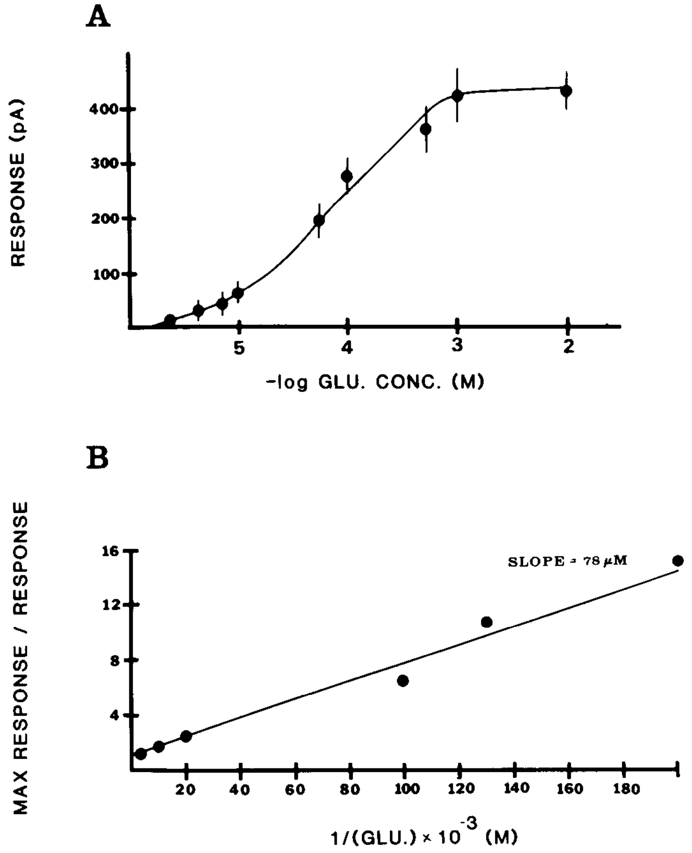

Figure 2. Glutamate dose–response curve. *A*, Semilog plot. Each point represents the mean ± SEM for at least 12 cells from 3 platings. Each cell was tested at 1 concentration. *B*, Same data, linearized in the form of a Lineweaver-Burk plot. The maximum glutamate induced current was 425 pA. The slope of this plot equals the ED_{50} for glutamate (78 μM). Lines with slopes corresponding to 50 and 100 μM are shown for comparison.

Current fluctuations

Amino acid currents were analyzed online with a PDP 11/23 computer. One channel was utilized for low-gain DC recording to determine mean currents. A second, high-gain bandpass-filtered (0.5 Hz–1.2 kHz; Krohn Hite) channel was used to analyze current fluctuations. Each channel was sampled 1024 times at a rate of 2 kHz. Extracellular Ca^{2+} was reduced (0.1 mM) and Mg^{2+} was increased (5 mM) to minimize synaptic activity. Nevertheless, each 1024 point sample was displayed on an oscilloscope to reject segments that contained superimposed synaptic currents.

To estimate mean channel open time, power spectra of 10–30 acceptable 1024 point segments were calculated with a Fast Fourier Transform subroutine and averaged. The mean spectrum (1024 points averaged 32 times) of current fluctuations recorded from the same motoneuron in the absence of agonist was subtracted from the mean spectrum obtained in the presence of agonist. The final spectrum was fit by eye with a computer-generated Lorentzian curve of the form $S(F) = S(0)/1 + (f/f_c)^2$, where f_c is the frequency at which the power was reduced by 50% and $S(0)$ is the extrapolated power at $f = 0$. The mean channel open time, τ, was calculated from the relation $\tau = \frac{1}{2}\pi f_c$.

Single-channel conductance, γ, was estimated by dividing the variance of the agonist-induced conductance fluctuations by the mean current. The current variance of the 1024 point sample and the mean of the same points were converted to conductances by dividing by (V_H −

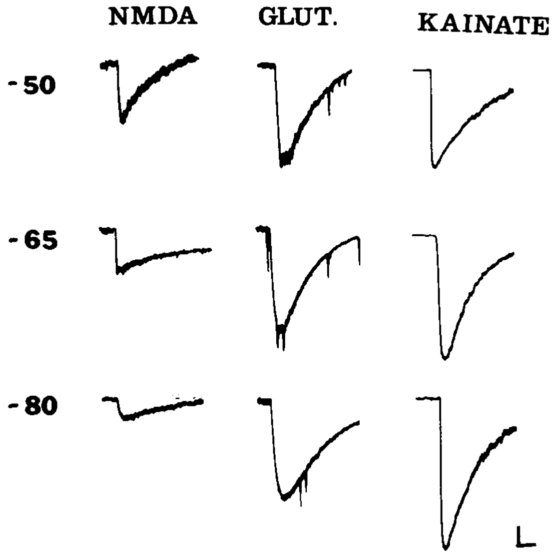

Figure 3. Voltage dependence of currents induced by excitatory agonists. The holding potential is shown to the *left* of each set of oscilloscope traces. All agonists were applied at 10^{-3} M. Calibration bars: 50 pA and 2 sec for NMDA and glutamate; 100 pA and 2 sec for kainate.

$V_R)^2$ and $(V_H - V_R)$, respectively, where V_H is the holding potential and V_R is the reversal potential of the response. The mean holding current and the current variance in the absence of agonist were subtracted from each sample.

Results

Glutamate activates 2 receptor types

Virtually all motoneurons dissociated from 6 d embryos survive for at least 1 week when they are plated (along with interneurons and glia) on a "monolayer" of myotubes (O'Brien and Fischbach, 1986a). When a motoneuron (a neuron containing fluorescent granules) was clamped at −50 mV, glutamate always evoked an inward current. Figure 1*A* shows a typical response following pressure ejection of 0.1 mM L-glutamate from an electrode positioned 100 μm away from the soma. The current rose rapidly during the 0.4 sec pressure pulse and decayed exponentially after the pulse was terminated. Spontaneously occurring, fast inward currents were occasionally observed while recording at room temperature, and glutamate often increased their frequency (Fig. 1*B*). Since they were not observed in cultures of sorted motoneurons, the fast currents presumably result from synaptically released transmitter. The increase in frequency probably reflects a presynaptic action of glutamate, but we did not investigate whether the increase was due to generation of spikes in presynaptic neurons or to local depolarization of the terminals. Lowering Ca^{2+} to 0.1 mM and raising Mg^{2+} to 5 mM eliminated the synaptic currents but did not alter the amplitude or time course of the slow, underlying current. Therefore, the glutamate response we measured was due to a direct action on the postsynaptic membrane rather than to the release of transmitter from presynaptic nerve terminals.

The current induced by 10^{-4} or 10^{-3} M glutamate showed little decay during prolonged application (Fig. 1*C*). The mean decrease in current at the end of 20-sec-long pulses was only 17.4% ($n = 12$) of the peak value. Although prolonged currents reflect the diffusion of glutamate to distal receptors as well as desensitization of more proximal ones, it is unlikely that the initial peak current we used to quantitate the response was associated with significant desensitization.

A glutamate dose–response (peak inward current) curve is shown in Figure 2*A*. The Lineweaver-Burk plot shown in Figure 2*B* could be fit with a straight line with a slope that corresponded to an ED_{50} of 78 μM. The excellent fit over the entire concentration range suggests that, if multiple binding sites exist for glutamate, they must have similar affinities. A Hill plot of the same data (not shown) had a limiting slope of 1.08, which indicates that 1 glutamate molecule interacts with each receptor to produce the observed inward current.

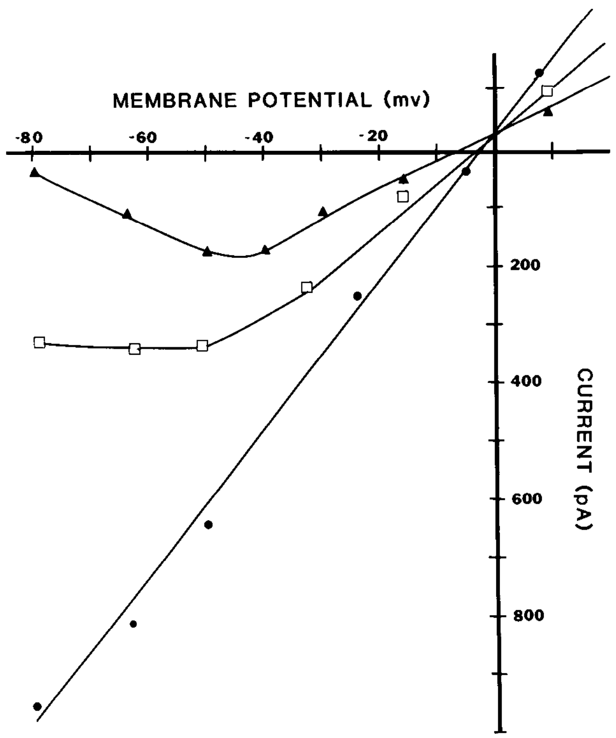

Figure 4. Voltage–current relations in the presence of excitatory amino acid agonists. Same motoneuron was tested with NMDA (*closed triangles*), glutamate (*open squares*), and kainate (*closed circles*).

Dose–response curves similar to that shown in Figure 2 were constructed with NMDA, aspartate, kainate, and quisqualate. These agonists generated different maximal responses (see below), but, in each case, the curve followed a simple hyperbolic relation without any indication of multiple binding sites or cooperativity. The ED_{50}'s were as follows: NMDA, 50 μM; aspartate, 100 μM; kainate, 80 μM; and quisqualate, 1 μM.

The reversal potentials of the various agonists could not be used to distinguish between different receptors.[2] We were able to demonstrate a clear reversal of the glutamate current in 11 of 16 neurons. Failures resulted from an inability to inject enough current to shift the potential adequately in the face of the large increase in membrane conductance associated with depolarization. The mean reversal potential for glutamate was −8 ± 2 mV (mean ± SD, $n = 11$). The reversal potentials for NMDA (−5 ± 4 mV, $n = 5$), and kainate (−4 ± 6 mV, $n = 4$) were virtually identical to that of glutamate. Considering the intracellular and extracellular concentrations of Na^+ and K^+, the reversal potential of a current due to equal increases in conductances to these ions is −6 mV.[3] Although the dose–response

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[2] In these experiments, 15 mM NaCl was added to the intracellular electrode solution, and the KCl concentration was reduced by 10 mM. Large currents were required to depolarize most of the neurons tested beyond −20 mV, so series resistance compensation was employed.

[3] One assumption of this calculation is that the cells are adequately perfused with the pipette solution. To test this assumption, we applied 10^{-4} M GABA to motoneurons penetrated with patch electrodes containing different concentrations of Cl^-. For intracellular Cl^- concentrations of 21, 56, and 150 mM, the observed GABA reversal potentials (−43, −27, and −2 mV) were close to those calculated assuming complete perfusion of the cell (−48, −24, and +2 mV). Four cells were tested in each case.

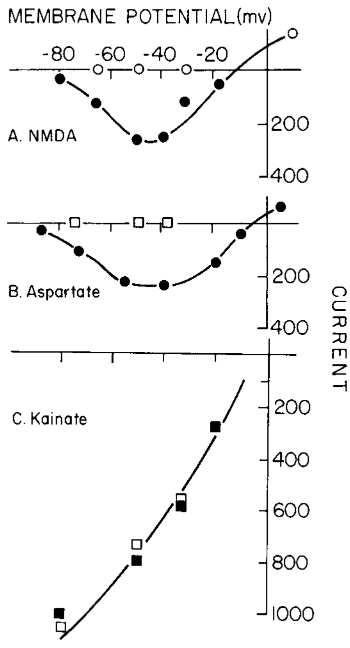

Figure 5. Specificity of 2-APV. In each case (*A–C*), a motoneuron was tested with the indicated amino acid (*filled symbols*) and again with the amino acid plus 1 mM 2-APV applied simultaneously by pressure ejection (*open symbols*). 2-APV blocks the response to NMDA and aspartate at all levels of membrane potential but does not affect kainate currents.

curves and the similar reversal potentials of the excitatory amino acids are consistent with a single site of action, the effect of membrane hyperpolarization on the various currents suggests that more than one receptor–ionophore complex is involved. The chart recorder traces in Figure 3 and the graph in Figure 4 show that glutamate currents did not increase with hyperpolarization, as would be expected from a simple conductance increase mechanism. Kainate and NMDA currents behaved quite differently; kainate-induced current increased with hyperpolarization, whereas the NMDA current decreased. The voltage dependence of the glutamate current lies midway between that of NMDA and kainate, implying that glutamate activates both types of channel. Indeed, the current–voltage relation in the presence of both kainate and NMDA was similar in shape to that in the presence of glutamate alone (Fig. 4).

Table 1. Inhibition of G_2 agnoists by γGG

	Peak current (pA)		
Agonist	Agonist alone	Agonist + γGG (10 mM)	% Inhibition
Quisqualate, 10^{-5} M (n = 11)	320 ± 49	59 ± 13	82
Kainate, 10^{-3} M (n = 9)	1120 ± 381	170 ± 35	84
Glutamate, 10^{-3} M, + 2-APV, 10^{-3} (n = 18)	300 ± 85	84 ± 19	72
GABA, 10^{-5} M (n = 11)	288 ± 51	295 ± 76	0

Effect of γGG on currents induced by G_2 agonists and by GABA. Each motoneuron was voltage-clamped at −50 mV and tested with a saturating concentration of the indicated agonist. Each entry represents the mean ± SD. The cells were in culture for 5 d.

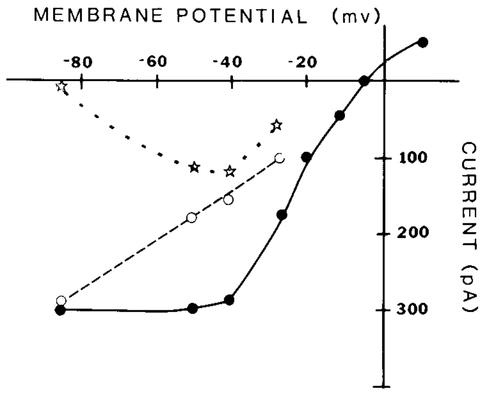

Figure 6. Glutamate voltage–current curves in the presence and absence of 2-APV. *Closed circles,* 10^{-3} M glutamate applied alone; *open circles,* glutamate applied along with 1 mM 2-APV. Subtraction of 2-APV-resistant currents from the total glutamate currents, recorded at the same membrane potential, results in a curve (*stars*) that is similar to that of NMDA (see Figs. 4*A*, 5).

Evidence that the 2 conductances activated by glutamate are associated with different receptors (binding sites) was obtained with 2-APV, a relatively potent, competitive antagonist of NMDA. The evidence for the competitive nature of the 2-APV inhibition *in vivo* is based on dose–ratio plots over a wide range of agonist and antagonist concentrations (Evans et al., 1982). We determined that 2-APV is a selective antagonist of NMDA action on embryonic chick motoneurons. As shown in Figure 5, 2-APV at 1.0 mM blocked the effect of saturating concentrations of NMDA and aspartate at all levels of membrane potential tested between −30 and −70 mV, whereas no effect was observed on saturating or half-maximal concentrations of kainate over the same range of membrane potentials.

The same concentration of 2-APV reduced, but did not entirely block, glutamate currents. As shown in Figure 6, the glutamate current remaining in the presence of 2-APV increased on hyperpolarization in a manner similar to that of kainate currents. Subtracting the 2-APV-resistant glutamate current from the total current results in a nonlinear curve identical to that characteristic of NMDA (Fig. 6). Based on a series of 58 motoneurons tested with or without 2-APV, we estimate that 38% of the current induced by a saturating concentration of glutamate at resting potential is due to activation of the 2-APV-sensitive (G_1) receptor and 62% is due to activation of the 2-APV-resistant (G_2) receptor.

Competition between glutamate and NMDA for the G_1 receptor was shown by applying saturating concentrations of both agonists simultaneously. Because glutamate and NMDA have similar ED_{50}'s, a mixture of the 2 at equal concentration should result in activation of 50% of the receptors by each if they compete for the same site. The net current should not be the sum of their individual currents. In fact, when they were applied together the net current was not additive (Fig. 7*A*). In 3 motoneurons tested in this manner, 10^{-3} M glutamate produced a net current of 415 + 35 pA (mean ± SD), whereas application of glutamate and NMDA together at 10^{-3} M resulted in a net current of only 388 ± 50 pA.

To determine whether the 2-APV-independent glutamate current was due to activation of the same receptors activated by kainate, we compared currents induced by equal concentrations of kainate and glutamate applied separately and together, all in the presence of 1 mM 2-APV. Although the peak currents induced by glutamate and kainate are quite different, their ED_{50}'s are virtually identical. As shown in Figure 7*B*, the currents were not additive. The peak kainate current was 1200 ± 308 pA

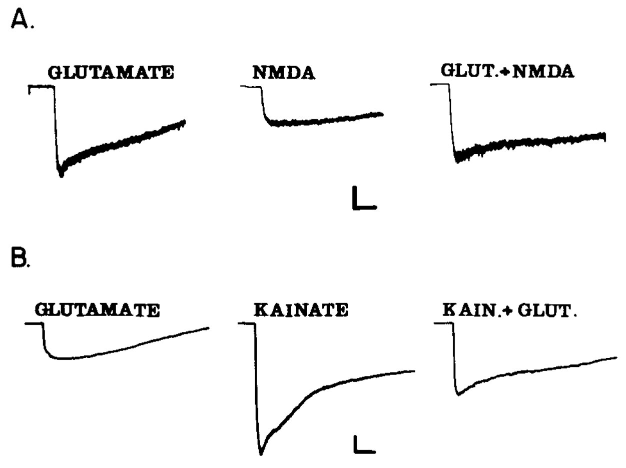

Figure 7. Competition between glutamate and NMDA and between glutamate and kainate. *A*, NMDA (10^{-3} M), glutamate (10^{-3} M), and a combination of the 2 were applied to the same motoneuron from 3 different pipettes. The individual currents do not add. *B*, Another motoneuron tested with glutamate (10^{-3} M) and kainate (10^{-3} M) and a combination of the 2 (all in the presence of 1 mM 2-APV). Here again the individual currents do not add. Calibration bars: *A*, 100 pA, 1 sec; *B*, 200 pA, 2 sec.

(mean ± SD; $n = 14$) and the peak 2-APV-resistant glutamate current was 308 ± 98 pA ($n = 11$). Application of glutamate and kainate simultaneously resulted in a peak current of 870 ± 225 pA ($n = 20$), which is comparable with the mean of the individual responses (790 pA). Therefore, nearly all of the 2-APV-independent glutamate current represents activation of the same receptor–ionophore complex as that activated by kainate.

Quisqualate-induced inward currents increased linearly on hyperpolarization, suggesting selective activation of G_2 receptors. However, it has been suggested that quisqualate and kainate activate different receptors *in vivo* (McLennan and Lodge, 1979; Watkins and Evans, 1981). Indeed, we found that quisqualate acts at far lower concentration ($ED_{50} = 1$ µM) than kainate and generates a much smaller peak current (250 pA) at saturating concentrations. Because of the very different maximal effects of quisqualate and kainate, we could perform a detailed analysis of their interaction. Application of increasing concentrations of quisqualate along with a single saturating concentration of kainate resulted in a steady decrease of the kainate-induced current to a plateau level appropriate for receptor activation by quisqualate alone (Fig. 8, upper graph). Conversely, when the quisqualate concentration was kept constant at 10^{-5} M and the concentration of kainate was increased, the total current approached the level induced by kainate alone (Fig. 8, lower graph). Thus, it appears that in chick motoneurons kainate and quisqualate compete for the same binding site.

The distinction between quisqualate and kainate receptors *in vivo* is, in part, based on 2 antagonists. Glutamate diethyl ester (GDEE) inhibits the increase in motoneuron spike frequency evoked by iontophoretic application of quisqualate but not kainate (McLennan and Lodge, 1979). In our system, however, GDEE at concentrations as high as 5 mM had no detectable effect on quisqualate, kainate, or glutamate currents. The second antagonist, γGG, inhibits the response of various neurons in the intact spinal cord to iontophoretically applied NMDA and kainate but not to quisqualate (Davies et al., 1981; Francis et al., 1980). We found that γGG was indeed an effective antagonist of G_1 receptors, although the concentration required was higher than 2-APV. However, γGG at a concentration of 10 mM markedly inhibited responses to saturating concentrations of quisqualate, as well as kainate and glutamate, in the presence of 2-APV (Table 1). While this is a rather high concentration of γGG, it had no effect on resting membrane potential, conductance, or response to inhibitory transmitters such as GABA (Table 1) and glycine. Therefore, γGG appears to be a nonselective excitatory amino acid antagonist.

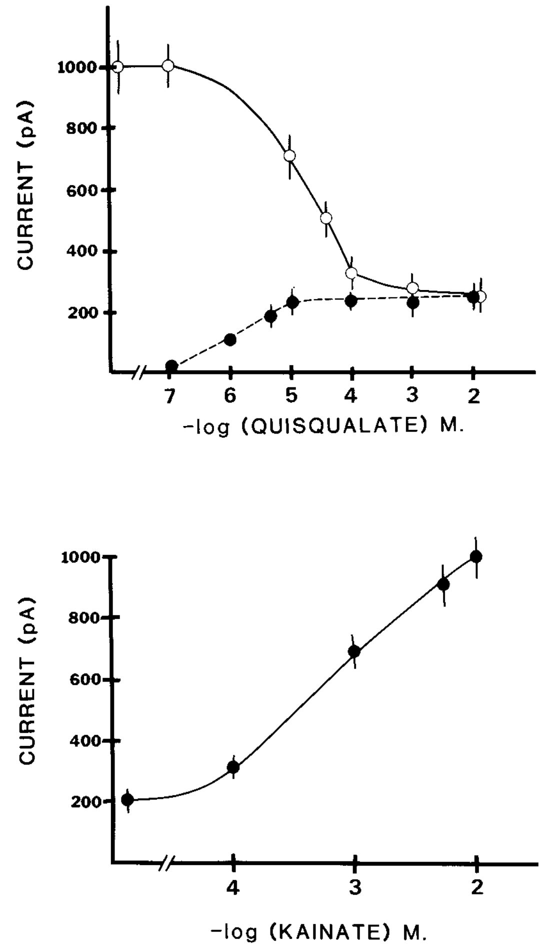

Figure 8. Competition between kainate and quisqualate. *Upper graph*, A series of motoneurons was tested with increasing concentrations of quisqualate (*filled circles*) or with the same concentrations of quisqualate plus 10^{-3} M kainate (*open circles*). Each motoneuron was tested with a single concentration. Each point represents the mean ± SEM. At saturating concentrations of quisqualate, the total kainate plus quisqualate current equaled that of quisqualate alone. *Lower graph*, Kainate dose was increased in the presence of 10^{-5} M quisqualate. The current evoked by 10^{-2} M kainate in the presence of quisqualate was similar to the maximum current evoked by kainate alone.

Channel conductance and open time

We further explored the properties of G_1 and G_2 ion channels by analyzing the current fluctuations induced by the various excitatory agonists. When the agonists were applied at low concentration (producing inward currents only 5–10% of the maximum), the relation between the mean current and the variance of the current fluctuations was linear. Assuming that Poisson statistics apply, and after converting currents to conductances, the slope of this line provides an estimate of mean channel conductance (γ). Figure 9 shows an analysis of glutamate currents in the presence and absence of 1 mM 2-APV. In the presence of 2-APV, the slope estimates the G_2 channel conductance activated by glutamate. Subtraction of the variance obtained in the presence of 2-APV from the total variance yields a line whose slope estimates the G_1 channel conductance activated by glutamate. These slopes differ markedly. In the cell illustrated, the

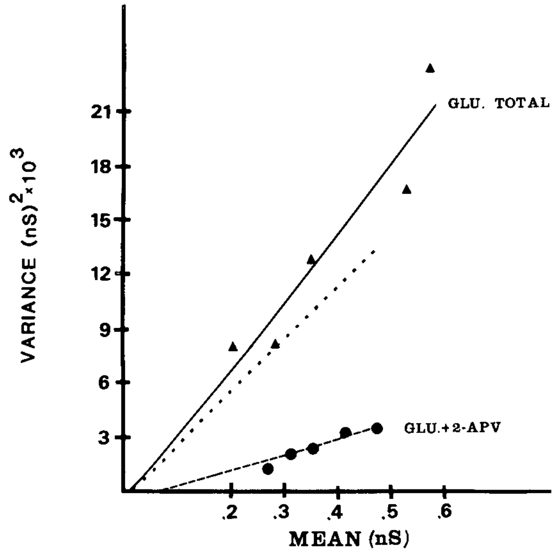

Figure 9. Relationship between mean glutamate conductance and variance of conductance fluctuations in the absence (*closed triangles*) and presence (*filled circles*) of 2-APV. The *dotted line* was obtained by subtracting 2-APV-resistant conductance variance from the total variance at each level of mean current. All values were obtained during 2 prolonged pressure pulses (one with 10^{-5} M glutamate and the other with 10^{-5} M glutamate plus 1 mM 2-APV). Holding potential, −50 mV; T = 24°C.

G_1 channel conductance was 30 pS, while the G_2 channel conductance was only 8 pS.

Results obtained from 5 mononeurons activated by glutamate are summarized in Table 2. The conductances of channels activated by the selective agonists aspartate and kainate were nearly identical to those activated by glutamate binding to the corresponding receptors. Thus, the channel conductance is independent of the activating agonist. In each case, the G_1 conductance was nearly 3 times greater than that of G_2.

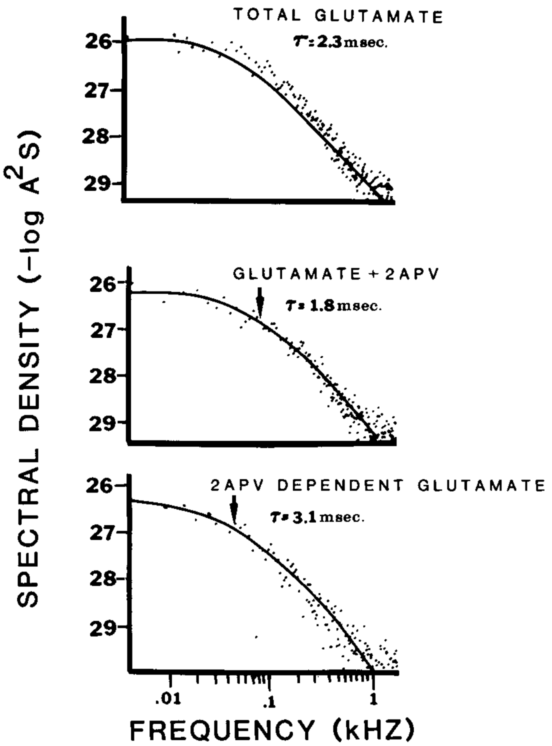

Figure 10. Power spectra generated by 10^{-5} M glutamate (*upper curve*) and by glutamate + 1 mM 2-APV (*middle curve*) in the same motoneuron. *Lower curve* was obtained by subtracting the glutamate plus 2-APV spectrum from the total glutamate spectrum; *upper curve* could not be fit precisely with a single Lorentzian. The *arrows* in the *middle* and *lower panels* indicate the frequency at which the power was reduced by ½ (f_c). Holding potential, −50 mV; T = 24°C. Plotted points above 1 kHz were ignored.

Power spectra of glutamate current fluctuations were calculated in 11 motoneurons. Five could not be fit with a single Lorentzian curve (Fig. 10, upper curve). For each of these cells, we also measured glutamate-induced fluctuations in the presence of 1 mM 2-APV. In the presence of the G_1 antagonist, all of the spectra were accurately fit by a single Lorentzian curve (Fig. 10, middle curve). Since we have evidence for only 2 classes of receptors present on chick motoneurons, we assume that this spectrum represents activation of G_2 receptors. Subtraction of the G_2 spectrum from the total glutamate spectrum provides an estimate of the spectrum due to glutamate activation of G_1 receptors (Fig. 10, lower trace). Table 2 summarizes the results of all 5 cells. The mean open time (τ) of G_2 channels activated by glutamate was 1.7 msec, while the mean open time of G_1 channels was 3.6 msec. Estimates of τ in the 6 cells, whose spectra were fit by a single Lorenztian (in the absence of 2-APV), fell midway between these values. These spectra probably contain contributions from both types of receptor, which could not be distinguished by curve fitting.

Table 2. Channel properties of G_1 and G_2 receptors activated by different agonists

	G_1		G_2	
	γ (pS)	τ (msec)	γ (pS)	τ (msec)
Glutamate	24 ± 3.2 (n = 5)	3.6 ± 0.7 (n = 5)	10.5 ± 2.1 (n = 5)	1.7 ± 3 (n = 5)
Kainate	—	—	8.9 ± 1.2 (n = 7)	5.5 ± 1.1 (n = 7)
Aspartate	28 ± 5 (n = 6)	3.1 ± 0.7 (n = 6)	—	—

Amino acid-induced current fluctuations were analyzed as described in the text. Aspartate and kainate are selective G_1 and G_2 agonists, respectively. The G_2 component of the glutamate response was obtained in the presence of 1 mM 2-APV, and the G_1 component was obtained by subtraction (derived from the 5 motoneurons whose spectra could not be fit with a single Lorentzian curve). Each entry represents the mean ± SD. T = 24°C.

Although kainate and glutamate both activate the G_2 receptor, the maximum current induced by kainate is approximately 4 times greater (see above). This disparity might be due to a difference in the channel properties of the G_2 receptor when activated by the different agonists. Although the G_2 channel conductance is the same for kainate and glutamate plus 2-APV, the spectra generated by their agonists were quite different. Kainate spectra cut off at lower frequencies (Fig. 11), indicating that the mean open time of G_2 channels activated by kainate is prolonged compared with those activated by glutamate. The data shown in Table 2 indicate that the G_2 channel activated by kainate remains open 3.2-fold longer, on average, than the same channel activated by glutamate. This difference is similar to the difference in peak G_2 currents evoked by the 2 agonists. The maximal G_1 currents evoked by aspartate and glutamate are similar. Table 2 shows that the open times of G_1 channels activated by aspartate and glutamate are similar as well.

Sorted motoneurons

The independence of G_1 and G_2 receptors was demonstrated by simultaneous application of selective agonists. This finding was

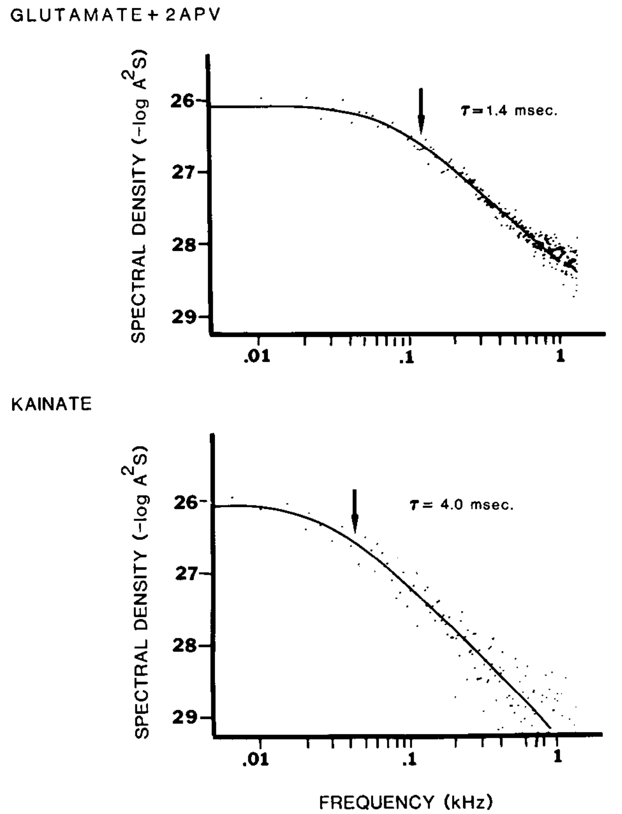

Figure 11. Power spectra of currents generated by 10^{-5} M glutamate in the presence of 1 mM 2-APV and by 10^{-5} M kainate. *Arrows* indicate the half-power frequency. $V_H = -50$ mV; T = 24°C. Plotted points above 1 kHz were ignored.

underscored by our study of sorted motoneurons. Current–voltage plots of the response of sorted motoneurons to glutamate were nearly linear (Fig. 12; compare with Fig. 4), suggesting that few, if any, G_1 receptors are present. In fact, the glutamate current in sorted motoneurons was not inhibited by 2-APV at any membrane potential (Fig. 12) examined. The same result was obtained in 12 other motoneurons from 3 different sorts. Finally, sorted motoneurons did not respond to saturating concentrations of NMDA or aspartate in the presence of 1 mM Mg^{2+} or in Mg^{2+}-free medium (*n* = 20; 3 sortings).

As expected from analysis of macroscopic currents, analysis of current fluctuations indicated that sorted motoneurons have few, if any, G_1 receptors. Spectra obtained from 5 sorted cells could all be fit with a single Lorentzian curve corresponding to a mean channel open time of 2.0 ± 0.3 msec. Ratios of the variance and the mean of glutamate currents in the same cells indicated a single-channel conductance of 13.6 pS. These values are in good agreement with corresponding estimates of glutamate-activated G_2 channels of motoneurons in mixed spinal cord cell cultures (see Table 2).

Discussion

Our voltage-clamp analysis of membrane currents has shown that glutamate activates 2 types of receptor on embryonic chick motoneurons in culture. One, which we have termed G_1, is activated selectively by NMDA and aspartate, and is inhibited completely by 2-APV. The G_1 current decreases when the membrane is hyperpolarized beyond about −40 mV. We have confirmed that the *V–I* relationship becomes more linear when Mg^{2+} is eliminated from the intracellular solution and the bath (R. J. O'Brien and G. D. Fischbach, unpublished observations). The other, termed G_2, is activated selectively by kainate and quisqualate and is not inhibited by 2-APV. The G_2 current increases on hyperpolarization even in the presence of Mg^{2+}. In each of these details, our results are similar to those reported by Mayer and Westbrook (1984), who studied cultured mouse spinal cord neurons.

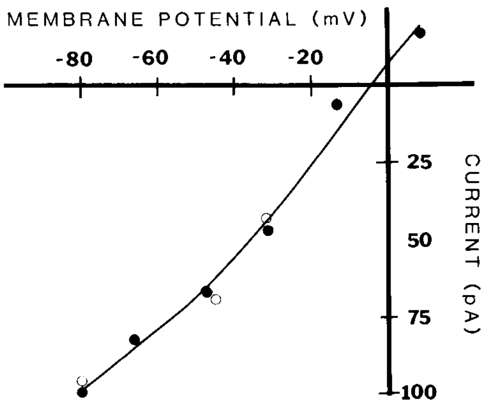

Figure 12. Voltage–current relation of a sorted motoneuron in the presence of 10^{-3} M glutamate (*closed circles*). Motoneurons labeled with fluorescein–wheat germ agglutinin were separated from other spinal cord cells with a FACS IV cell sorter (see O'Brien and Fischbach, 1986a). *Open circles* were obtained following pressure ejection of 10^{-3} M glutamate plus 1 mM 2-APV.

Recent studies of ^{3}H-glutamate binding to subcellular fractions enriched with postsynaptic densities prepared from rat brain (Fagg and Matus, 1984) are consistent, on a qualitative level, with the electrophysiological findings reported here. Although ^{3}H-glutamate binds to an apparently homogeneous class of sites with a K_d of 0.34 μM and a Hill coefficient close to 1.0, only 58% of the specific binding was inhibited by NMDA. The remaining 42% was inhibited by quisqualate or kainate. In some respects, there is quantitative agreement as well. The K_i's reported by Fagg and Matus (1984) for quisqualate (1 μM) and kainate (30 μM) are close to our estimates of the ED_{50}'s of quisqualate (1 μM) and kainate (80 μM) for activation of G_2 receptors. On the other hand, their reported K_i for NMDA (7.2 μM) is only one-seventh our estimate of the ED_{50} for NMDA activation of G_1 receptors, and the affinity of glutamate was 200 times greater than our estimate of the ED_{50} (78 μM). The reasons for these discrepancies are not clear, although a parallel with studies of the ACh receptor can be drawn. At equilibrium, ^{3}H-ACh binds with an affinity that is at least 3 orders of magnitude greater than the ED_{50} determined at short intervals (Boyd and Cohen, 1980; Neubig et al., 1982). The disparity in this case is due to the fact that, at equilibrium, the K_d of ^{3}H-ACh reflects the affinity of desensitized receptors. It is interesting in this regard that G_1 receptors desensitize more rapidly than do G_2 receptors (Mayer and Westbrook, 1985; Zorumski and Fischbach, 1985).

Because the effects of aspartate in the intact spinal cord are inhibited only 30–60% by 2-APV (Davies et al., 1981), it has been suggested that aspartate, like glutamate, acts as a mixed agonist. Our finding that aspartate currents are completely inhibited by 2-APV differs from the *in vivo* result. In addition, the voltage dependence of aspartate currents in cultured chick motoneurons mimics that of NMDA, whereas in cat motoneurons its response more resembles that of glutamate (Engberg et al., 1978). It may be that receptors expressed *in vivo* and *in vitro* have different specificities or that chick receptors differ from those of frog and cat. However, it is difficult to compare membrane currents recorded in cultured neurons with ventral root potentials and spike frequencies measured *in vivo*. It is possible, for example, that the aspartate response observed *in vivo* is due in part ot the release of an endogenous transmitter that is capable of activating both types of postsynaptic receptors. Indeed, in our cultures, aspartate induced a greater increase in the frequency of synaptic currents than any other agonist tested (R. J. O'Brien and G. D. Fischbach, unpublished observations).

Our experiments in which kainate and quisqualate were applied simultaneously provide strong evidence that they compete for the same binding site, with quisqualate having about 100-fold greater affinity. The much higher affinity of quisqualate for the G_2 receptor might account for previous reports that γGG inhibits kainate but not quisqualate *in vivo* (Collingridge et al., 1983; Francis et al., 1980). We found that γGG blocks quisqualate as effectively as it blocks kainate, when the different affinities of these agonists are taken into account.

We have emphasized 2 broad classes of excitatory amino acid receptors. Together they account for all of the inward current induced by glutamate in chick motoneurons dissociated from 6 d embryos and maintained in mixed spinal cord cell culture for 1 week. It is important to define the preparation precisely, because it seems likely that amino acid receptors will be further subdivided as the cells mature or as more selective ligands are introduced. Evidence for greater receptor diversity in other regions of the CNS has already appeared. For example, a high-affinity kainate binding site with a K_d in the nanomolar range has been identified in the CA3 region of hippocampus (Monaghan et al., 1983), and kainate can activate dendrites in the same region at concentrations as low as 10 nM (Robinson and Deadwyler, 1981). Such low concentrations have no effect on motoneurons either *in vivo* or *in vitro*. Moreover, work in other laboratories suggests that the pharmacology of receptors for excitatory amino acids present on axons and presynaptic nerve terminals may be different from that of the postsynaptic receptors described here (Fagg and Matus, 1984; Harris and Cotman, 1983).

We found that the single-channel conductance of G_1 receptors is nearly 3 times greater than the channel conductance associated with G_2 receptors regardless of the agonist employed. Our estimate of the G_1 channel conductance (ca. 25 pS), based on noise analysis, is less than the values obtained in mouse spinal cord cells (50 pS: Nowak et al., 1984) and in insect muscle fibers (120 pS: Cull-Candy et al., 1980) by direct single-channel recording. The G_2 channel conductance is only about 10 pS at -50 mV, so single channels will be difficult to detect. Despite the considerable difference in G_1 and G_2 channel conductance, currents that flow through each of them reverse polarity near -5 mV, so they both probably transfer cations nonselectively.

As with the ACh receptor (Katz and Miledi, 1973), the mean open time of G_1 and G_2 channels depends on the agonist. For example, the mean open time of the G_2 channel activated by kainate is 3 times longer than the open time of the same channel activated by glutamate. The maximum current induced by a 0.4 sec pulse of kainate is also nearly 3 times greater than the maximum current induced by a pulse of glutamate. Kainate and glutamate apparently compete for G_2 sites, so, at maximal concentrations, they must occupy the same number of receptors. Therefore, the magnitude of the peak inward current is determined by the conductance of each channel and the number of channels open at the peak. The latter depends on both the mean channel open time and the probability of channel opening. If the probability of a liganded G_2 channel opening is low, then the increased macroscopic current induced by kainate compared with glutamate might reflect the relatively long open time of the kainate-gated channel. The close agreement between the difference in mean channel open times and the difference in macroscopic currents is consistent with this explanation.

Activation of a motoneuron by a saturating dose of glutamate on day 6 *in vitro* results in approximately 400 pA of inward current. This is far less than the peak currents recorded in voltage-clamped rat spinal cord neurons maintained in culture for several weeks (Mayer and Westbrook, 1984). Given G_2 and G_1 channel conductances of 26 and 10 pS, respectively, the value of 400 pA at -50 mV implies that the motoneuron soma and major processes (within range of the pressure ejection pulse) may contain only 800 G_2 and 190 G_1 receptors. The number of these receptors might be significantly larger if the probability of channel opening is indeed as low as we hypothesized above. In spite of this caveat, the small number of activatable channels present on innervated motoneurons *in vitro* and the presence of only 1 type receptor on uninnervated (sorted) motoneurons imply that receptor modulation may play an important role in synaptogenesis and synaptic plasticity. Before examining this issue (O'Brien and Fischbach, 1986c), however, we determined that both G_1 and G_2 receptors are involved in synaptic transmission (O'Brien and Fischbach, 1986b).

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