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ACCELERATED COMMUNICATION

Quisqualate Activates *N*-Methyl-D-Aspartate Receptor Channels in Hippocampal Neurons Maintained in Culture

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SUMMARY

Whole-cell and single-channel patch-clamp recordings from hippocampal neurons in culture have been used to study the receptor channel selectivity of the glutamate analog quisqualate. The dose-response relationship of quisqualate acting at the *N*-methyl-D-aspartate (NMDA) receptor was measured as that portion of the whole-cell current activated by quisqualate that could be blocked by the addition of two NMDA antagonists, 5-fluoroindole-2-carboxylic acid, a competitive antagonist of the NMDA receptor-associated glycine site, and D-2-amino-5-phosphonovalerate, a competitive NMDA binding site antagonist. We found that quisqualate was 10-fold less potent than NMDA. In outside-out patches quisqualate activates single-channel events that range in conductance from 5 to 50 pS. The NMDA antagonists 5-fluoroindole-2-carboxylic acid and D-2-amino-5-phosphonovalerate completely blocked all of the 40–50-pS channel openings in the presence of quisqualate. These results indicate that quisqualate gates 40–50-pS events by activating NMDA receptor channels.

Glutamate is thought to be the primary neurotransmitter mediating excitatory synaptic transmission in the vertebrate central nervous system (1, 2). Glutamate receptors coupled directly to ion channels have been divided into three subtypes, based on their affinities for the selective glutamate agonists NMDA, quisqualate, and kainate (1). At the single-channel level, glutamate opens channels with conductances that range from about 140 fS to 50 pS (3–5). The three analogs of glutamate are more selective; each agonist preferentially activates one conductance class but also produces channel openings of additional conductances that are similar in size to those activated by the other agonists (4–7). Quisqualate primarily opens channels with conductances of 8–15 pS but also produces channel openings with conductances of 40–50 pS, the same size as the primary conductances activated by NMDA (4–8), suggesting that quisqualate may activate NMDA receptors. This is consistent with the results of binding studies that indicate that quisqualate binds to two distinct sites with different affinities (9); the lower affinity site is thought to be the NMDA receptor (10). The affinity of quisqualate for the NMDA receptor has been reported to be 2- to 23-fold less than that of NMDA, based on the ability of quisqualate to displace [^{3}H] glutamate (11, 12) and tritiated NMDA antagonists (13, 14) from NMDA-sensitive sites.

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Recently, competitive antagonists (15, 16) of the glycine site (17) associated with the NMDA receptor have been described. Because activation of the glycine site is apparently necessary for NMDA receptor channel gating (15, 18), we have used one of these antagonists in conjunction with D-APV, a competitive antagonist of the NMDA binding site, to determine whether the 40–50-pS events activated by quisqualate are due to NMDA receptor channel activation.

Materials and Methods

Details of the techniques used to culture CA1 hippocampal neurons have been published elsewhere (19). Briefly, the CA1 regions of hippocampi from 1–3-day-old rats were incubated in papain (20 units/ml; Worthington Biochemical Corp., Freehold, NJ). The tissue was dissociated by trituration in complete growth medium, based on minimum essential medium and fetal bovine serum (see Ref. 19), and was plated onto glass coverslips coated with collagen (Biomedical Technologies, Stroughton, MA) and poly-D-lysine (Collaborative Research, Bedford, MA).

Whole-cell and outside-out patch recordings were obtained at room temperature (21–25°) from neurons maintained in culture for 1–2 weeks, using an Axopatch-1B-patch-clamp amplifier. Currents were low-pass filtered at 2 kHz (−3 dB Bessel) and digitally sampled at 125 Hz (whole-cell) or 10 kHz (outside-out). Open times were measured at 50% amplitude. Patch pipettes were filled with (mM): Cs methanesulfonate, 140; NaCl, 10; HEPES, 5; EGTA, 10; Mg-ATP,4; GTP, 1; adjusted to pH 7.4 with CsOH. Neurons were constantly superfused

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; D-APV, D-2-amino-5-phosphonovalerate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraccetic acid; FIC, 5-fluoroindole-2-carboxylic acid.

with an extracellular solution containing: NaCl, 155 mM; KCl, 3 mM; $CaCl_2$, 2 mM; HEPES, 5 mM; glucose, 10 mM; tetrodotoxin, 0.3 μM (Calbiochem, San Diego, CA); picrotoxin, 50 μM; and strychnine, 1 μM (Sigma Chemical Co., St. Louis, MO). Solutions containing quisqualate (Cambridge Research Biochemicals, Valley Stream, NY), NMDA, D-APV (Tocris, Buckhurst Hill, UK, or Cambridge Research Biochemicals), glycine (Bio-Rad, Richmond, CA), or FIC (Aldrich, Milwaukee, WI) were dissolved in the extracellular solution and applied through multi-barrelled perfusion pipettes similar to those described by Johnson and Ascher (17). One barrel always contained control extracellular solution, which ensured rapid wash-out after drug application.

Amino acid analysis by Cambridge Research Biochemicals did not detect any glutamate contamination of the synthetic quisqualate used. They report that glutamate contamination was less than 0.0025% or 2.5 nM in 100 μM quisqualate, too low to activate NMDA receptors at the frequencies reported below (although see Discussion).

Results

Whole-cell currents. Whole-cell currents activated by NMDA (30 μM) and glycine (10 μM) were completely and reversibly suppressed when the superfusate was switched to one containing NMDA (30 μM), D-APV (20 μM), and FIC (200 μM) (Fig. 1A). To be diagnostic for NMDA receptor-mediated currents, however, this combination of antagonists must not block quisqualate receptors. The ability of the same concentrations of FIC and D-APV to block quisqualate-activated currents was tested in the presence of 2 mM magnesium but with no added glycine. At −60 mV, 2 mM magnesium should greatly attenuate any residual current through NMDA receptors (20). FIC and D-APV had no discernible antagonist action on quisqualate currents under these conditions (Fig. 1C $n = 4$). In separate experiments, neither the addition of FIC to solutions containing quisqualate and D-APV nor the addition of D-APV to solutions containing quisqualate and FIC had any effect on the amplitudes of the evoked currents. Thus, at these concentrations, these antagonists completely and selectively block activation of NMDA receptor channels and can, then, be used to test the ability of quisqualate to activate NMDA receptor channels at the whole-cell and single-channel levels.

The current activated by quisqualate (300 μM) and glycine (10 μM) was partially inhibited by either or both (Fig. 1B) D-APV and FIC. To determine equipotent concentrations of NMDA and quisqualate for use in single-channel experiments (see below), we examined the low concentration portion of the dose-response relations for the two agonists in a constant concentration of glycine (10 μM). The quisqualate dose-response relation at the NMDA receptor was determined by measuring the amplitude of the quisqualate current blocked by the NMDA antagonist solution (see Fig. 1B). In each cell, the amplitude of the antagonized component was normalized to the current amplitude evoked by 30 μM NMDA, so that the responses of different neurons could be averaged. The quisqualate dose-response curve is parallel to the NMDA curve and is shifted 10-fold to the right, indicating that quisqualate, in this dose range, is 10-fold less potent at the NMDA receptor than NMDA. The dose-response curves are illustrated in Fig. 2.

Single-channel events. The whole-cell dose-response relationships indicate that 10 μM NMDA and 100 μM quisqualate, both in the presence of glycine (10 μM), should be approximately equipotent in activating NMDA receptors. At these concentrations, both NMDA and quisqualate activated 40–50-pS channel events in outside-out patches (Fig. 3, A and C). Channel openings of 40–50 pS were completely eliminated, leaving only small conductance events when D-APV and FIC were added to NMDA ($n = 21$) or quisqualate ($n = 12$) and glycine was deleted (Fig.3, B and D). This is illustrated in Fig. 3 in the current histograms that were constructed using 4–6 sec epochs from patches in each of the different recording conditions. In many patches, each drug solution was applied several times, to ensure that desensitization or rundown of the 40–50-pS events could not account for the results.

If the proportion of the different species of glutamate channels contained in an outside-out patch is representative of that in a whole neuron, then the ratio of charge transfer through NMDA and non-NMDA channels activated by a given concentration of an agonist should be the same as the ratio of whole-cell current amplitudes activated by the same concentration of agonist. This was tested with 10 μM NMDA and 100 μM quisqualate, both in the presence of 10 μM glycine. With both agonists, most of the charge transfer was through 40–50-pS events, as is apparent from the sample records illustrated in Fig. 3, A and C. Charge transfer was calculated by multiplying the value of each point in a current histogram by its current amplitude and was, thus, transformed into a measure of charge (length of time at a given current amplitude multiplied by the current amplitude) (Fig. 4). This distribution was then divided into the portion due to 40–50-pS openings and that due to all smaller openings. Each portion of the distribution was then integrated, to obtain total charge transfer. The examples in Fig.

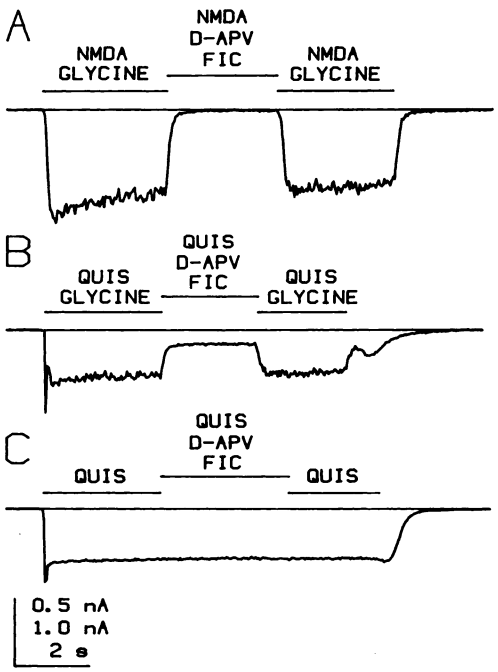

Fig. 1. Whole-cell responses to NMDA and quisqualate are antagonized by NMDA receptor channel antagonists. A, Response of a hippocampal neuron in culture to the serial application of NMDA (30 μM) and glycine (10 μM), then NMDA (30 μM), D-APV (20 μM), and FIC (200 μM), and back to the first solution. B, Response of another neuron to quisqualate (300 μM) and glycine (10 μM), then quisqualate (300 μM), D-APV (20 μM), and FIC (200 μM), and back to the first solution. C, The same experiment as in B in a different neuron, without added glycine and in the presence of 2 mM magnesium. All other recordings were in the absence of added magnesium (magnesium contamination approximately 0.2 μM). Holding potential = −60 mV. Current calibration of 0.5 nA applies for A and B. *QUIS*, quisqualate.

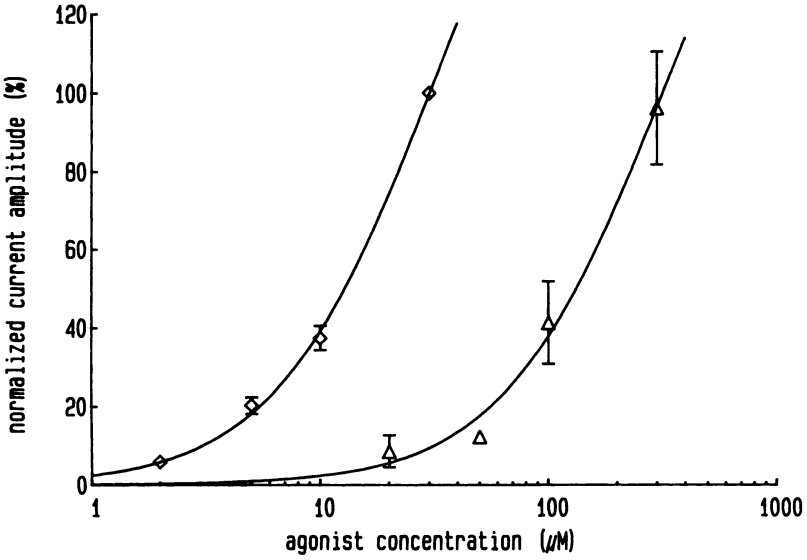

Fig. 2. Dose-response relationships of NMDA and quisqualate at the NMDA receptor. The steady state current amplitude of responses to NMDA were normalized to that evoked by 30 μM NMDA in each cell. The quisqualate-induced NMDA receptor responses were taken as the current amplitude blocked by the NMDA antagonists (20 μM D-APV and 200 μM FIC), normalized to the current evoked by 30 μM NMDA. The *smooth curve* was fitted to the NMDA data using an iterative nonlinear least squares fitting routine (in the software package GraphPAD), with the equation $I = I_{max}/(1+(EC_{50}/[agonist])^{HS})$, where *HS* is the Hill slope. The slope and maximum were allowed to vary while the minimum was constrained to zero and the EC_{50} was set at 30 μM, as found by others (25, 29). Hill slope = 1.28. The quisqualate data were fitted with the same method, assuming an EC_{50} of 300 μM (Hill slope = 1.28). Each point is the mean ± standard deviation of the responses from three to seven neurons. The lack of error bars indicates error smaller than the plotting symbol.

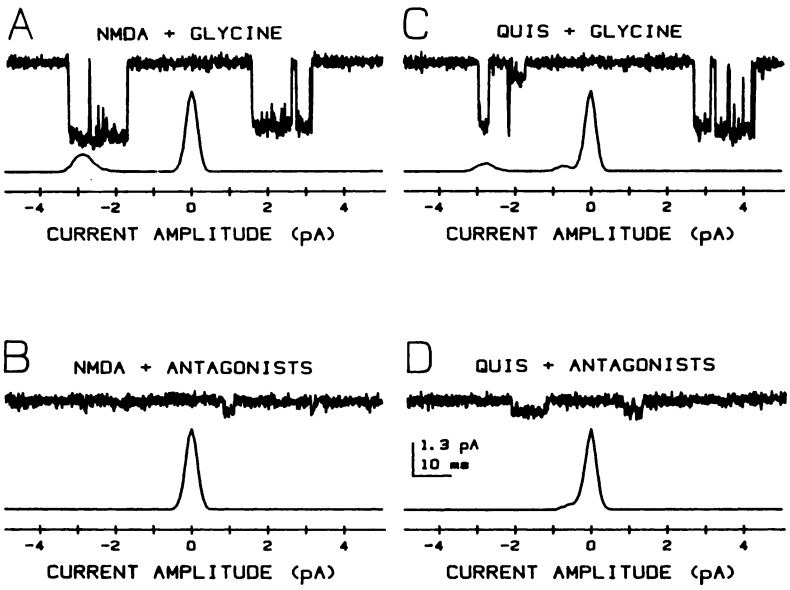

Fig. 3. Large conductance single-channel events gated by NMDA and quisqualate are blocked by NMDA antagonists. Each panel (A–D) is composed of a representative single channel record (*top*) and a current histogram constructed from the values of each digitized point of current. The height of the histogram at a given current value is a measure of the total time spent at that current. Each histogram represents 4–6 sec of data and is normalized by the peak height of the baseline noise. Concentrations (in μM): NMDA, 10; glycine, 10; quisqualate (*QUIS*), 100; D-APV, 20; FIC, 200.

4 show that >99% (mean ± SD = 98 ± 4%; n = 10 patches) of the charge transfer activated by 10 μM NMDA was due to NMDA receptor activation, whereas 81% (mean ± SD = 71 ± 30%; n = 14 patches) of the charge transfer activated by 100 μM quisqualate was through NMDA channels. In whole-cell recordings, the proportion of "NMDA current" to total current evoked by 100 μM quisqualate was 53% (SD = 16%; n = 4).

Discussion

Our results show that a combination of selective NMDA antagonists, one acting at the NMDA binding site and one acting at the glycine binding site, can completely prevent activation of 40–50-pS single-channel events by NMDA and quisqualate. This indicates that quisqualate gates these channels by binding to the NMDA receptor. The antagonists are selective for responses mediated by NMDA receptors, because neither diminishes the whole-cell response to quisqualate either in the presence of the other antagonist or when both are added in the presence of 2 mM magnesium, which, at −60 mV, should almost completely block current through NMDA receptors (20). At the single-channel level, however, NMDA and quisqualate activate a very low but significant probability of NMDA receptor channel events in the presence of either FIC or D-APV. This is because both antagonists are competitive inhibitors (15, 21) and glycine, which is required for channel opening (15, 18), is an ever-present contaminant. Thus, agonist and glycine will occasionally bind to an unoccupied receptor at the same time, resulting in an opening. One of the competitive antagonists alone, then, is not capable of completely blocking NMDA receptor-mediated single-channel events.

Several observations from physiological and binding studies suggest that quisqualate can activate NMDA receptors. 1) In cerebellar Purkinje cells, which do not respond to NMDA, quisqualate does not activate 40–50-pS events in outside-out patches (8). 2) Magnesium, which blocks NMDA channels at negative membrane potentials (22, 23), can decrease whole-cell currents activated by quisqualate (6). 3) The density and distribution of the low affinity quisqualate binding site match the distribution of NMDA receptors (10). 4) In the presence of NMDA, the proportion of low affinity quisqualate binding sites

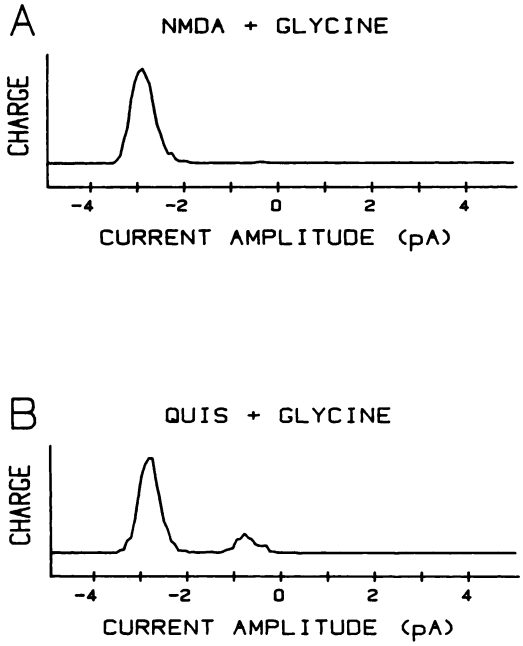

Fig. 4. Charge transfer through large conductance channels activated by NMDA and quisqualate. The baseline peaks of current histograms in Fig. 3, A and B, were fitted with Gaussian distributions, which were then subtracted to leave current values during channel openings. The value of each point of the current histogram (a measure of time spent at that current) was multiplied by its current amplitude, the product being a measure of charge. QUIS, quisqualate.

decreases (10). 5) Quisqualate displaces NMDA-sensitive [^{3}H] glutamate binding, with K_i values 2–4-fold higher than those for NMDA (11, 12). 6) The selective NMDA antagonists [^{3}H] 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (13) and D-[^{3}H]APV (14) are displaced by quisqualate, with an IC_{50} 6- and 23-fold higher, respectively, than the corresponding values for NMDA. Our finding that quisqualate is 10-fold less potent at the NMDA receptor than NMDA is in agreement with these results.

Recently Cha *et al.* (24) have reported that synthetic quisqualate is contaminated with glutamate to an extent that was dependent on the source. Quisqualate from Cambridge Research Biochemicals (our supplier) was found to be 0.13% glutamate, by high pressure liquid chromatographic analysis (24). At this level of contamination, 1.8 mM quisqualate would be required to reach the EC_{50} concentration of glutamate at the NMDA receptor (2.3 μM; see Ref. 25), and yet our apparent EC_{50} of quisqualate at the NMDA receptor was 300 μM. Activation of the NMDA receptor, then, cannot be due, in main, to glutamate contamination. If the dose-response curve is corrected for the current due to glutamate contamination of 0.13%, the EC_{50} of quisqualate at the NMDA receptor is shifted from 300 to 380μM.

In the present study, we have shown that the proportion of charge transfer through NMDA receptor channels activated by quisqualate in outside-out patches is similar to the percentage of the whole-cell current amplitude due to NMDA receptor channels activated by the same concentration of quisqualate. The large variation in the single-channel recordings is not unexpected, because of the presumed relatively small number of channels in each patch. The single-channel recordings also have a larger percentage of charge transfer through NMDA channels, on average, than the whole-cell recordings. This is at least in part due to including those small conductance events that occurred concurrently with large amplitude openings in the high conductance portion of the charge distribution. In addition, because quisqualate receptors are clustered in neuronal membranes (26, 27), most outside-out patches will contain a smaller proportion of quisqualate receptors than expected from whole-cell recordings. Differences in desensitization rates in whole cell and outside-out patches cannot be ruled out.

We have used high concentrations of quisqualate throughout these experiments, in order to activate large conductance channel openings at a frequency high enough to reliably determine the effects of antagonists and, therefore, identify the receptor channel species responsible. It is clear from the dose-response curves in Fig. 2 (see also Ref. 17), however, that, at concentrations of quisqualate that produce little desensitization of responses resulting from activation of quisqualate receptors (< 1 μM; Ref. 28), quisqualate will gate very few NMDA channels. At the whole-cell level, these concentrations of quisqualate, especially in the absence of glycine, will result in relatively pure non-NMDA responses.

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