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N-Methyl-D-aspartate activates different channels than do kainate and quisqualate

(excitatory amino acids/glutamate receptors/phencyclidine receptor/*N*-methyl-D-aspartate channel block/oocyte expression system)

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ABSTRACT **In the mammalian central nervous system, the excitatory amino acid transmitter L-glutamate activates three pharmacologically distinguishable receptors, the *N*-methyl-D-aspartate (NMDA), kainate, and quisqualate receptors. The present paper addresses the issue of whether these three receptors operate independent channels or whether they share channels that may have several conductance substates. The *Xenopus* oocyte provides a system for expression of exogenous mRNAs that permits detailed study of receptor structure and function. In oocytes injected with rat brain mRNA, NMDA has a stoichiometry of channel activation different from that for kainate and quisqualate. NMDA activates its own channels as indicated by simple summation or near-summation of currents evoked by NMDA with those evoked by quisqualate or kainate. Deviations from summation are ascribable to lack of selectivity in which an agonist at one receptor acts as a weak antagonist at another receptor. A further indication of separate channels is that block of NMDA channels by Mg^{2+} or phencyclidine has no effect on kainate or quisqualate responses evoked during the block. Interactions of kainate and quisqualate are more complex, but they can be explained by lack of complete specificity of these agonists for their own receptors.**

The excitatory amino acid transmitter glutamate activates three receptors defined by the actions of the selective agonists *N*-methyl-D-aspartate (NMDA), kainate, and quisqualate (1, 2). Recently, it was suggested that these receptors may be distinct recognition components coupled to a common channel (3, 4). In cultured hippocampal or cerebellar neurons, single channels were found to exhibit multiple conductance states that were preferentially activated by the different agonists. In some cases, transitions between these conductance states indicated that they were different states of a single ion channel. The subsequent observation that currents evoked by NMDA, kainate, and quisqualate failed to summate linearly in cultures of chick spinal cord motoneurons was considered support for the concept of shared channels (5), although lack of ligand specificity as demonstrated here could equally well explain the data. Evidence against shared channels was that block of NMDA responses by phencyclidine (PCP) receptor ligands, which are putative channel blockers, had no effect on kainate and quisqualate responses evoked during the block (6, 7). The specificity of block was observed even under conditions in which the PCP receptor ligand appeared to be trapped within the channel by removal of agonist. If these ligands are indeed channel blockers, as is suggested by the voltage dependence of their block in neurons (6, 7) and in oocytes (8), then independence of NMDA-activated channels is clear.

NMDA-activated responses involve permeability increases to Na^+, K^+, and Ca^{2+} and are blocked by Mg^{2+}, whereas kainate- and quisqualate-activated responses involve permeability increases to Na^+ and K^+ but not to Ca^{2+} and are unaffected by Mg^{2+} (9, 10). Although a simple view of ion channel activity would be that different permeabilities indicate different channels, it was suggested that permeability could differ during high and low conductance states of a single molecular complex (11). The single channel study in which Mg^{2+} concentration was varied (3) did not test the possibility that the permeabilities differ during the extreme conductance states of a single channel. A later study of cultured cerebellar granule cells, which included both whole cell current and single channel measurements, gave evidence that the three agonists activate separate channels (12).

A test of separate vs. shared channels is comparison of responses evoked by each agonist alone with responses evoked by two agonists applied simultaneously. If two ligands open the same channel, the current induced during their simultaneous application should be less than the sum of currents induced when they are applied separately, i.e., the responses should occlude. The degree of occlusion will depend on the degree of receptor occupancy and on the fraction of time that the channel is open when the receptor is occupied. If either agonist opens the channel all the time, the two agonists together would not be able to open it any more. But even with partial receptor occupancy, occlusion could be observed. For example, if two agonists act independently and each opens the channels half the time, application of the two agonists together would produce an open probability of 0.75 rather than 1. In chick motoneurons, linear summation of responses to NMDA and non-NMDA agonists was observed, but results with non-NMDA agonists were consistent with a single kind of non-NMDA receptor–channel complex (13).

A simple system in which to assess summation is the *Xenopus* oocyte in which the channels in question have been expressed. The present study of *Xenopus* oocytes injected with rat brain mRNA indicates that channels activated by NMDA are distinct from those activated by kainate or quisqualate, since currents evoked by NMDA and the other ligands show summation (quisqualate) or near summation (kainate). Deviations from summation are ascribable to lack of selectivity of kainate and NMDA such that each acts as a weak antagonist at the other's receptor. Furthermore, selective block of NMDA channels by Mg^{2+} or PCP has no effect

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Abbreviations: NMDA, *N*-methyl-D-aspartate; PCP, phencyclidine; APV, D-2-amino-5-phosphonovaleric acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione.

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on kainate or quisqualate responses evoked during the block. A preliminary report of these data has appeared (14).

MATERIALS AND METHODS

RNA was prepared from the fore- and midbrains of adult (300 g) male Sprague–Dawley rats by using the guanidium isothiocyanate (Fluka) and CsCl density gradient method of Ullrich *et al.* (15). mRNA was then selected by oligo-(dT)-cellulose chromatography (16). This poly(A)$^+$ RNA was dissolved in water and stored at −70°C until use.

Ovarian lobes were dissected from anesthetized *Xenopus laevis* and incubated 2 hr at 22°C in Ca^{2+}-free ND96 medium (82.5 mM NaCl/2 mM KCl/1 mM $MgCl_2$/5 mM Hepes·NaOH, pH 7.5) (17) supplemented with sodium pyruvate at 2.5 mmol per liter, to which penicillin (100 units/ml), streptomycin (1 mg/ml), and collagenase (2 mg/ml) (Sigma, type IA) were added. After transfer to Ca^{2+}-containing ND96, stage V and VI oocytes (18) were defolliculated and injected with mRNA (50 ng per cell). Oocytes were maintained at 16.5°C in Leibovitz's L-15 medium (0.7 strength) (Sigma) supplemented with 5 mM Hepes (pH 7.6), penicillin (100 units/ml), and streptomycin (1 mg/ml). For assays, oocytes were placed in a bath with a volume of about 0.1 ml and perfused with Mg^{2+}-free amphibian Ringer's solution (116 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/5 mM Hepes, pH 7.2). All drugs were dissolved and applied in this medium. D-2-amino-5-phosphonovaleric acid (APV) was purchased from Cambridge Research Biochemicals (Cambridge, U. K.), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) was from Tocris Neuramin (Essex, U.K.). All other chemicals were purchased from common commercial sources.

Cells were voltage clamped at a holding potential of −60 mV with two beveled electrodes filled with 1 M KCl (1–2 MΩ) (19, 20). All compounds were bath-applied with an access time of <0.4 sec; solutions were washed out within 2 sec as shown by visual inspection of dye application.

RESULTS

Oocytes injected with brain mRNA show responses to glutamate agonists that differ in pattern depending on the agonist applied (19). Maintained responses to NMDA were essentially undetectable in the absence of glycine, so in most experiments 10 μM glycine was included in NMDA solutions. Responses induced by concentrations of NMDA greater than about 20 μM (with 10 μM glycine) were characterized by an initial peak of membrane current that decayed with a time constant of 1–2 sec to a steady level that remained unchanged for minutes (Fig. 1*A Inset*). This peak–plateau pattern was presumably due to desensitization. Similar to its action in neurons (21), glycine enhanced the NMDA response without effect on the Hill coefficient n (0.93 ± 0.05 with 10 μM and 1.03 ± 0.05 with 0.1 μM glycine, mean ± SEM) or apparent receptor affinity K_d (29.3 ± 1.5 μM with 10 μM and 31.3 ± 2.3 μM with 0.1 μM glycine) (Fig. 1*A*). Kainate-induced currents developed more slowly, were nondesensitizing, and were usually much larger than those induced by NMDA (Fig. 1*C Inset*). In most oocytes, quisqualate evoked two kinds of response. One was small, of short latency, smooth, and showed desensitization only at high quisqualate concentration (Fig. 1*B*); it presumably corresponds to the activation of the "classical" quisqualate neuronal receptor after its expression by the oocyte system. The other response was of long latency (about 30 sec) and consisted of large oscillations (data not shown); it is apparently second messenger-mediated (19, 22, 23). In the present report we consider only the earlier quisqualate responses in oocytes in which the later responses were small or absent.

Dose–response curves for quisqualate- and kainate-induced currents are shown in Fig. 1; Hill coefficients were calculated to be 2.6 ± 0.1 and 2.4 ± 0.1, respectively, values that indicate a degree of cooperativity. Apparent receptor affinities were 0.21 ± 0.06 μM for quisqualate and 56.1 ± 2.6 μM for kainate.

Summation experiments involving NMDA and quisqualate revealed no detectable interaction (Fig. 2). Simultaneous application of NMDA and quisqualate (at concentrations above their K_d values and evoking near maximal responses) resulted in an inward current that was the simple sum of the currents evoked by each alone (first three responses in Fig. 2 *A* and *B*). This result strongly indicates that each agonist activates independent channels. Block of NMDA responses by APV, a competitive antagonist at the NMDA receptor, had little effect on quisqualate responses with or without NMDA present (Fig. 2*B*, last three responses). This result indicates pharmacological specificity, but it does not demonstrate separate channels. High concentrations of APV did

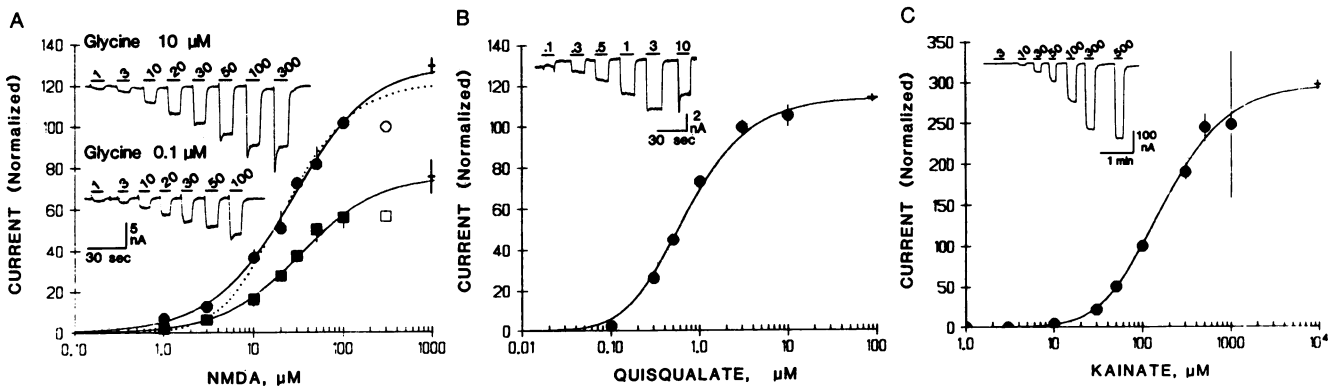

FIG. 1. Responses to glutamate agonists of *Xenopus* oocytes injected with rat brain mRNA. The dose–response curves for NMDA (*A*), quisqualate (*B*), and kainate (*C*) are shown. (*Insets*) Examples of inward currents during the periods indicated by the solid bars at each concentration (in μM). Each agonist was applied at least three times to each oocyte at each concentration and the currents were averaged. These values were normalized and pooled for computer fitting following the equation $I = I_{max}(A/A + K_d)^n$, where I is the observed response and A is the concentration of agonist. I_{max}, the maximum response, K_d, the apparent affinity constant, and n, the Hill coefficient, were fit as free parameters. The best fits are shown together with averaged data points (± SEM, 4–12 oocytes). The estimated I_{max} values are also indicated. In the case of NMDA (*A*) dose–response curves were calculated in the presence of 0.1 μM (■; 6 oocytes) or 10 μM (●; 12 oocytes) glycine and normalized to the response to 300 μM NMDA (○, □), but those points were omitted for curve fitting because of desensitization. Increasing the glycine concentration increased I_{max} but did not affect the K_d (estimated mean ± SEM; 29.3 ± 1.5 μM and 31.3 ± 2.3 μM) or n (0.93 ± 0.05 and 1.03 ± 0.05). The dotted line in this plot indicates the inferiority of the best fit achieved assuming $n = 2$. The calculated Hill coefficients were 2.6 ± 0.1 for quisqualate and 2.4 ± 0.1 for kainate; K_d values were 0.21 ± 0.06 μM for quisqualate and 56.1 ± 2.6 μM for kainate.

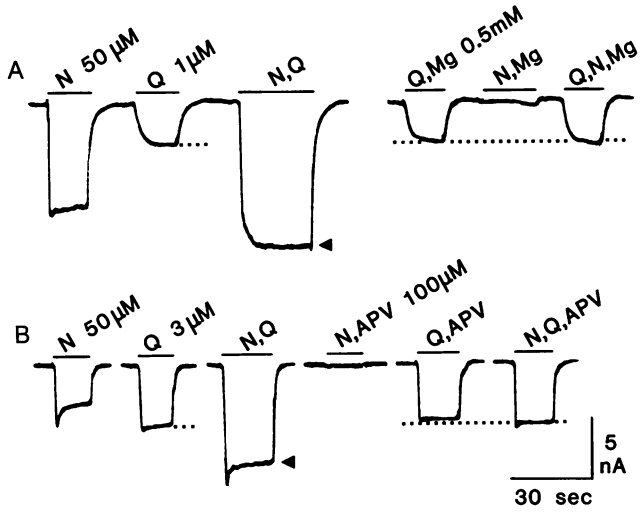

FIG. 2. Linear summation of NMDA and quisqualate responses. These experiments were performed with oocytes injected with a different mRNA preparation that gave small responses to NMDA so that the NMDA responses were of similar size to the quisqualate responses, which were always small. (*A*) The amplitude of the steady-state response to NMDA (50 μM plus 10 μM glycine) and to quisqualate (1 μM) applied together was equal to the sum of the steady-state responses of the two perfused separately (arrowhead, first three responses). The quisqualate response was not reduced by Mg^{2+} at a concentration that did block the NMDA-induced current (fourth and fifth responses). The response to simultaneous perfusion of the same quisqualate, NMDA, and Mg^{2+} concentrations was equal to that to quisqualate alone (sixth response). (*B*) A different oocyte. The procedure was the same as in *A*, but the quisqualate concentration was higher (3 μM) and the NMDA blocker used was APV (100 μM), which at this concentration decreased the quisqualate response <10%. N, NMDA; Q, quisqualate.

slightly antagonize quisqualate action (about 10% at 100 μM APV; Fig. 2*B*). The presence of Mg^{2+} at a concentration sufficient to block NMDA responses had no effect on quisqualate responses (Fig. 2*A*, fourth and fifth responses). Simultaneous application of Mg^{2+}, NMDA, and quisqualate gave the same size response as quisqualate alone (Fig. 2*A*, last response). As Mg^{2+} blocks NMDA channels (9, 10), this result is inconsistent with channel sharing by these two receptors.

Kainate- and NMDA-evoked currents summated with a small deficit (about 20% of the steady-state current with near-saturating NMDA and kainate close to its K_d; Fig. 3 *A* and *B*, first three responses). At least half of this deficit can be accounted for by weak antagonism of NMDA at the kainate receptor, and the remainder is ascribable to kainate antagonism at the NMDA receptor. NMDA antagonism at the kainate receptor was indicated by the effect of NMDA on the kainate-induced current under conditions in which NMDA receptors or channels were blocked by specific inhibitors. APV largely blocked currents evoked by 100 μM NMDA and produced a modest reduction in currents evoked by 50 μM kainate (Fig. 3*A*, fourth and fifth responses). When kainate, NMDA, and APV were applied together, the response was smaller (about 10%) than the response to kainate with APV; thus NMDA reduced the kainate currents, presumably by a direct action at the kainate receptor. Furthermore, responses to 50 μM kainate were reduced about 15% by 100 μM NMDA in the presence of the putative channel blocker PCP at a concentration sufficient to greatly reduce the NMDA currents; PCP alone had no effect on kainate currents (Fig. 3*A*, last three records). [The absence of the early peak in the response to NMDA, kainate, and PCP applied together is explained by trapping of PCP in the channels after the earlier application of NMDA and PCP (8).]

Similar results were obtained when Mg^{2+} was used instead of PCP as a channel blocker; responses to 50 μM kainate were reduced about 15% by NMDA in the presence of enough Mg^{2+} to block the NMDA responses, whereas Mg^{2+} alone had no effect on kainate responses (Fig. 3*B*).

NMDA antagonism at kainate receptors accounted for about half of the deficit from simple summation at moderate concentrations of kainate; the residuum was about 10% of the total current or 20% of the NMDA current (Fig. 3 *A* and *B*). The discrepancy was ascribable to a blocking action of kainate at NMDA receptors. When both kainate and NMDA were applied at near saturating concentrations, summation was still incomplete (Fig. 3 *C* and *D*, first three responses). The deficit was not due to reduction in the kainate response by NMDA since the response to 500 μM kainate, 100 μM NMDA, and 100 μM APV applied together was equal to the response to 500 μM kainate and 100 μM APV, whereas the response to 100 μM APV and 100 μM NMDA was negligible. The deficit could be explained by antagonist action of kainate at NMDA receptors. At these concentrations, 100 μM NMDA and 500 μM kainate, the NMDA response was reduced by 50%.

Further evidence for kainate's acting as a weak antagonist at the NMDA receptor was obtained by the use of the fairly selective kainate blocker CNQX (24) in experiments corresponding to those using specific NMDA antagonists. CNQX at 10 μM reduced the response to 500 μM kainate by about 80% but decreased the response to 100 μM NMDA by only 20% (Fig. 3*D*, fourth and fifth responses). The deficit in the response to kainate plus NMDA as compared to the sum of the responses to kainate and NMDA applied separately was the same fraction of the NMDA response (*ca.* 50%) in the presence or absence of CNQX, although the kainate responses differed 5-fold in amplitude (Fig. 3*D*). Thus, at these concentrations of kainate and NMDA, as in Fig. 3*C*, the entire deficit in summation was accounted for by kainate block of NMDA receptors.

Antagonism of kainate at NMDA receptors was directly demonstrated in oocytes injected with mRNA from the NCB-20 cell line; these oocytes exhibited responses to NMDA but not to kainate (20). The degree of block of NMDA responses in these oocytes was the same as that inferred from summation of NMDA and kainate responses in oocytes expressing both receptors.

NMDA antagonism at kainate receptors was recently demonstrated in retinal ganglion cells (25). In the retina as well, PCP has no effect on NMDA reduction in kainate responses, although PCP block of NMDA responses differs from that in the oocytes in that it is voltage independent.

A number of oocytes ($n = 4$) expressed kainate receptors without quisqualate receptors. In these cells, quisqualate reduced kainate responses. In one cell, block by 1 μM quisqualate was fitted by a competitive relation, while inhibition by 10 μM quisqualate appeared more complex. Reduction in kainate currents by quisqualate has been seen in goldfish horizontal cells (26) and chick spinal neurons (5).

An additional interaction could be observed when quisqualate and kainate were applied simultaneously to oocytes expressing both quisqualate and kainate responses, although the maximal quisqualate responses were small compared to the maximal kainate responses. In these cells, application of quisqualate at a concentration near saturation for steady-state responses (1 μM) together with kainate at a low concentration (10 μM) gave a response greater than the sum of the two currents (Fig. 4, first, third, and fourth responses). Since quisqualate reduced kainate responses, this result suggests that kainate can facilitate quisqualate responses. The response to 100 μM kainate, twice its K_d, and 1 μM quisqualate was less than the response to 100 μM kainate alone, but whether there was still facilitation of the quis-

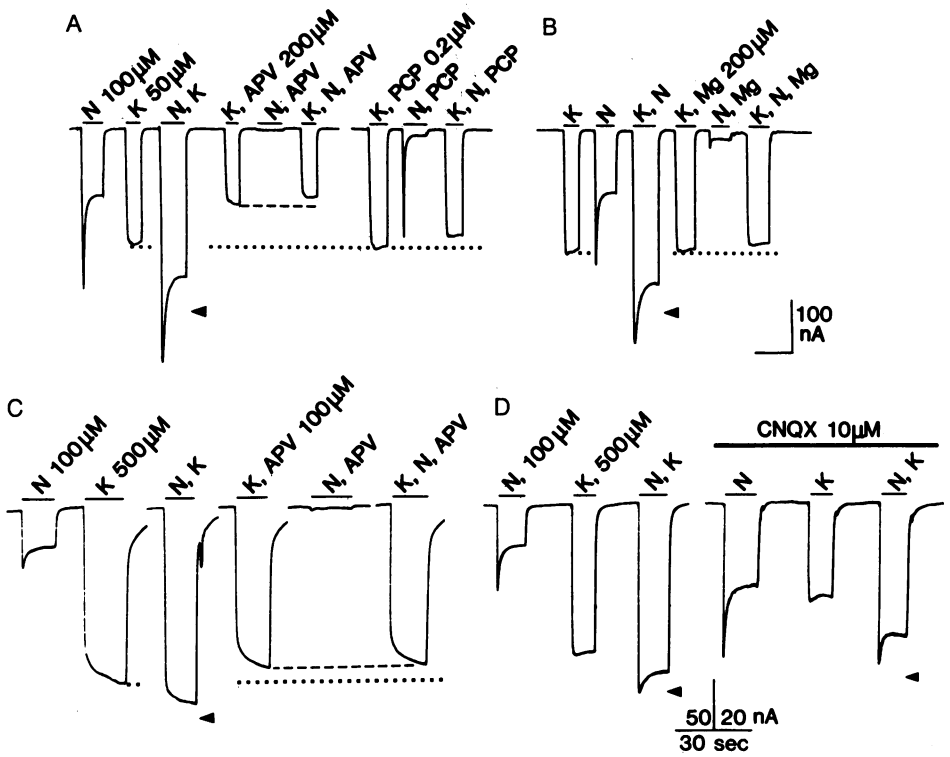

FIG. 3. Summation of NMDA and kainate responses. (*A*) The amplitude of the steady-state responses to NMDA (plus 10 μM glycine) at an almost saturating concentration (100 μM) and to kainate (50 μM) perfused together was somewhat smaller than the sum of the two responses (at steady state) evoked separately (arrowhead, first three responses). APV at a concentration that almost completely blocked the NMDA response significantly reduced the kainate response (about 30%). Application of NMDA, kainate, and APV together produced a slightly smaller response (about 10%), indicating that NMDA is a weak antagonist at the kainate receptor (second three responses). PCP had no effect on the kainate response at a concentration of PCP adequate to block the NMDA response almost completely. (The onset of PCP block was slow and use-dependent, and the initial peak when NMDA and PCP were applied together was only slightly smaller than the peak of the response to NMDA alone.) The steady-state response to NMDA, kainate, and PCP together was reduced compared to the response to kainate alone, again indicating reduction of kainate responses by NMDA (last three responses; the absence of the initial peak in the last response was due to PCP trapped in the channels after its application with NMDA in the preceding response). (*B*) A similar experiment showing incomplete summation of NMDA and kainate responses and reduction of the kainate response by NMDA when the latter's response was largely blocked by Mg^{2+}, a putative channel blocker. (*C*) Responses to near-saturating concentrations of kainate (500 μM) and NMDA (100 μM) summated with some occlusion (first three responses). APV (100 μM) caused a small reduction in the kainate current (fourth response). NMDA added to the APV plus kainate caused no further reduction in the kainate current (sixth response), while the APV and NMDA caused almost no current (fifth response), indicating that NMDA had no effect on the kainate current. Thus the deficit in the third, summated response is ascribable to kainate causing a *ca.* 50% reduction in the NMDA current. (*D*) A different oocyte. The last three responses, during CNQX application, were recorded at higher gain. CNQX (10 μM) reduced the response to NMDA by about 20% but that to kainate by 80%. The response to NMDA and kainate applied together in the presence of CNQX showed a deficit that was the same fraction of the NMDA current as the response to the two drugs in the absence of CNQX. Thus, kainate at 500 μM produced a 50% reduction in currents evoked by 100 μM NMDA, whereas 100 μM NMDA had negligible effect on responses to 500 μM kainate. N, NMDA; K, kainate.

qualate response cannot be determined because of its small size (Fig. 4, sixth and seventh responses, recorded at lower gain). A high concentration of quisqualate (10 μM) depressed the response to 100 μM kainate (Fig. 4, sixth and eighth responses) and to 10 μM kainate as well (Fig. 4, third and fifth responses), but again presence of facilitation of the quisqualate response was indeterminate. The differential actions of NMDA and APV on kainate and quisqualate responses indicate that their receptors are distinct. Also indicating separate receptors is the wide variation that we observed in the ratio of maximum amplitudes of kainate and quisqualate responses. However, the small size of quisqualate responses and lack of complete specificity of the agonists do not allow the conclusion that kainate and quisqualate receptors activate separate channels.

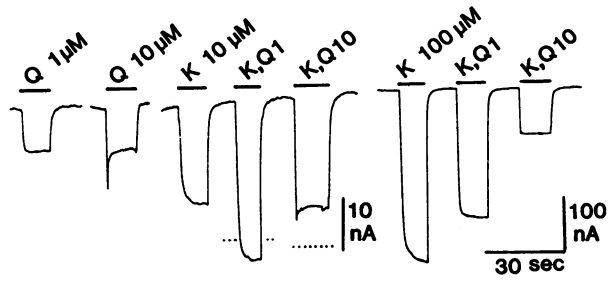

FIG. 4. Summation of quisqualate (Q) and kainate (K) responses. Quisqualate and kainate were applied separately and together at the indicated concentrations. The response to 1 μM quisqualate and 10 μM kainate applied together was larger than the sum of the responses to their separate application (dotted line). The response to 100 μM kainate was reduced by 1 μM quisqualate. Quisqualate at 10 μM reduced responses to both 10 and 100 μM kainate.

As noted above, PCP receptor ligands appear to act as NMDA channel blockers but with much slower off-rates than are observed for Mg^{2+}, so that PCP that is trapped in the channel by adding and then removing NMDA can be detected on reapplication of agonist (6–8). A channel "plugged" by trapped PCP should no longer be available for activation by kainate or quisqualate receptors. As predicted, kainate- and quisqualate-induced currents were unaffected by prior treatment with NMDA and PCP at a concentration adequate to largely block the NMDA response (Fig. 5). Application of NMDA speeded recovery from PCP block by allowing PCP

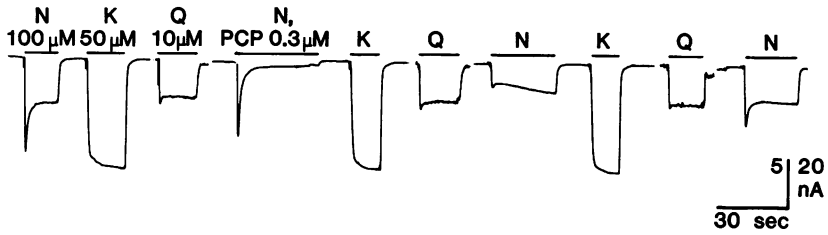

FIG. 5. Failure of PCP trapped in NMDA channels to occlude kainate or quisqualate responses. Responses to the three agonists applied separately were followed by application of NMDA and PCP together. After washing out the NMDA and PCP solution, the kainate and quisqualate responses were unaffected; subsequent application of NMDA showed the NMDA response still to be markedly depressed due to trapped PCP. The response largely recovered during the NMDA application, but there was no effect on subsequent kainate and quisqualate responses. The last application of NMDA showed almost complete recovery of the response. Gain was increased during quisqualate application, and then decreased again at the breaks in the record. Position along the time axis was not changed. N, NMDA; K, kainate; Q, quisqualate.

to exit the channel, but kainate and quisqualate had no effect on recovery (data not illustrated).

DISCUSSION

The results of the summation experiments strongly support the classical concept that NMDA, kainate, and quisqualate interact with separate receptors and that at least NMDA receptors form or are coupled to their own channels. For NMDA- and quisqualate-evoked currents, summation was ideal. The failure of simple summation in the case of NMDA plus kainate is readily explicable in terms of weak antagonism of the ligands at each other's receptors. Quisqualate and kainate have their own receptors, but the data do not demonstrate that these receptors have independent channels. Further support for independence of NMDA channels is provided by the experiments involving specific blockers of NMDA-evoked currents. Inhibition of the NMDA response by the putative channel blocker PCP had no effect on kainate and quisqualate responses, and recovery from PCP block was unaffected by the presence of kainate or quisqualate. As in neurons (6, 7), the specificity of block was observed even under conditions in which PCP appeared to be trapped in the channel. Moreover, Mg^{2+}, perhaps a more accepted channel blocker (9, 10), had no effect on quisqualate currents when applied with NMDA. Furthermore, the reduction in kainate responses when Mg^{2+} and NMDA were applied together could be explained entirely by NMDA antagonism at kainate receptors. Although Mg^{2+} blocks and unblocks more rapidly than PCP, it seems unlikely that kainate and quisqualate responses would be unaffected by Mg^{2+} block when a shared channel was opened by NMDA.

The presence of a common type of subunit in the different receptor–channel complexes is not excluded by our data. Conversely, expression of only one or two of the excitatory amino acid receptors in neurons (13, 27) or oocytes injected with some mRNA preparations, which we have observed, does not exclude channel sharing when more receptors are present. Since the mRNA used in these experiments is from a combination of fore- and midbrain, it is unlikely that channel sharing is common. Resolution of these questions will require identification and sequencing of the mRNAs encoding the different receptor–channel complexes. Expression of these messages in the oocyte system should prove valuable in validating their identification and in site-directed mutagenesis to manipulate their structure in functionally significant ways. Moreover, the pharmacological findings obtained here will remain relevant to studies of their role in neural function.

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