

Direct effects of metabotropic glutamate receptor compounds on native and recombinant *N*-methyl-D-aspartate receptors

ANIS CONTRACTOR, ROBERT W. GERAU, IV, TIM GREEN, AND STEPHEN F. HEINEMANN

Molecular Neurobiology Laboratory, The Salk Institute for Biological Studies 10010 North Torrey Pines Road, La Jolla, CA 92037

Contributed by Stephen F. Heinemann, May 11, 1998

ABSTRACT The actions of glutamate in the central nervous system are mediated through interaction with fast activating ionotropic receptors and G protein-coupled metabotropic glutamate receptors (mGluRs). Studies of these receptors have relied on the availability of agonists and antagonists selective for each receptor class. Compounds that were thought to be selective for mGluRs have been extensively used to study the role of these receptors in the brain. Their use has implicated mGluRs in a wide range of physiological and pathological processes including the modulation of *N*-methyl-D-aspartate (NMDA) receptors and NMDA receptor-dependent processes. We report that some of the most commonly used mGluR compounds act as antagonists on NMDA receptors at concentrations commonly used to activate or block mGluRs. In addition, several of the drugs also act as agonists at higher concentrations due at least in part to high levels of contaminant amino acids. Our results indicate that caution should be used when using these drugs to study the roles of mGluRs in various NMDA-dependent processes. The antagonist effects were dependent on the concentration of the NMDA receptor coagonists, preventing reappraisal of previously published work.

Since the first evidence that glutamate could act on G protein-coupled receptors (1), numerous physiological roles have been proposed for the metabotropic glutamate receptors (mGluRs) (2). Given the diversity and ubiquitous distribution of the mGluRs, in addition to the variety of transduction mechanisms to which they couple, it is not surprising that they are implicated in many neuronal processes. To study the role of mGluRs, it has been necessary to develop ligands that are selective for these receptors. A number of compounds are available that have been used to study both recombinant and native receptors (see reviews in refs. 3–5). While the mGluR subtype specificity of these compounds has been studied extensively, the cross-receptor reactivity of these drugs is less well characterized. Despite this, many studies have focused on the roles of mGluRs in processes that also involve *N*-methyl-D-aspartate (NMDA) receptors; in particular in the induction of hippocampal long-term potentiation (6–9) and glutamate-induced excitotoxicity (10–13). These studies have relied on the assumption that the mGluR drugs have no direct effects on NMDA receptors. Here we present the observation that many of the commonly used mGluR agonists and antagonists also act on recombinant and native NMDA receptors. The lack of specificity of these drugs may be the basis for contradictory results reported from different laboratories.

MATERIALS AND METHODS

Compounds used in this study were as follows: (1*S*, 3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid [(1*S*,3*R*)-ACPD; lot

no. 18/6373]; (RS)-3,5-dihydroxyphenylglycine (DHPG; lot nos. 6/6854, 6/6386, 6/6385); (S)-DHPG (lot no. 4/5694); (S)- α -methyl-4-carboxyphenylglycine (MCPG; lot no. 12/6070, 12/6309); *trans*-azetidine-2,4-dicarboxylic acid (*trans*-ADA; lot no. 2/3119); (S)-3-hydroxyphenylglycine (3-HPG; lot no. 7); and 1-aminoindan-1,5-dicarboxylic acid (AIDA; lot no. 6/6903) purchased from Tocris-Cookson (Bristol, UK). *trans*-ADA (lot no. EKG-396B) NMDA and 7-chlorokymurenic acid were from Research Biochemicals (Natick, MA). All other materials were obtained from Sigma. All drug solutions were prepared fresh on the day of the experiment using Milli-Q water (Millipore). Amino acid analysis was performed at the University of Michigan Protein Structure and Carbohydrate Structure Facility on the following drugs: (RS)-DHPG, lot no. 6/6385; (S)-HPG, lot no. 7; DL-2-amino-4-phosphonobutyric acid (DL-AP4), lot no. 50H3844; *trans*-ADA, lot no. EKG-396B.

Xenopus Oocyte Recordings. *Xenopus* oocytes were prepared and maintained as described (14). At 12–78 hr after cRNA injection, oocytes were placed individually in a recording chamber and perfused at a rate of \approx 3 ml/min with calcium-free Barth's solution (pH 7.4) composed of 88 mM NaCl, 1.1 mM KCl, 2.4 mM NaHCO₃, 1.8 mM BaCl₂, and 15 mM Hepes. Two-electrode voltage clamp recordings were performed by using an Axoclamp 2A (Axon Instruments, Foster City, CA). Oocytes were voltage clamped at -70 mV and data were acquired and analyzed by using software from Axon Instruments.

HEK293 Cell Recordings. Standard calcium phosphate coprecipitation techniques were used to transiently transfect HEK293 cells with plasmids containing NR1-1a (15) and NR2A (generously supplied by S. Nakanishi, Kyoto University) and a plasmid carrying the coding sequence for the CD8 surface antigen (generously supplied by B. Seed, Massachusetts General Hospital, Boston). 48 hr after transfections, cells were labeled with anti-CD8 antibody-coated beads (Dyna, Great Neck, NY). Standard whole-cell or perforated-patch whole-cell patch-clamp techniques were used to record from cells by using an Axopatch 200A amplifier (Axon Instruments). Cells were constantly perfused with Hepes-buffered extracellular solution (pH 7.4), composed of 135 mM NaCl; 5 mM KCl, 1.8 mM BaCl₂, 20 mM sucrose, 10 mM glucose, and 5 mM Hepes. The pipette solution for whole-cell recording was composed of 140 mM gluconolactone, 140 mM NaOH, 10 mM Hepes, 11 mM EGTA, and 10 mM NaCl (pH 7.4). For perforated patch recordings the pipette tip was filled with standard gluconate solution and then back filled with an amphotericin containing solution prepared by sonicating 3 μ l of amphotericin B stock solution [1.5 mg of amphotericin B

Abbreviations: NMDA, *N*-methyl-D-aspartate; mGluR, metabotropic glutamate receptor; (1*S*,3*R*)-ACPD, (1*S*, 3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid; DHPG, (RS)-3,5-dihydroxyphenylglycine; MCPG, (S)- α -methyl-4-carboxyphenylglycine; *trans*-ADA, *trans*-azetidine-2,4-dicarboxylic acid; 3-HPG, (S)-3-hydroxyphenylglycine; AIDA, 1-aminoindan-1,5-dicarboxylic acid; DL-AP4, DL-2-amino-4-phosphonobutyric acid.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1998 by The National Academy of Sciences 0027-8424/98/958969-6\$2.00/0
PNAS is available online at <http://www.pnas.org>.

(Sigma) in 20 μ l of dimethyl sulfoxide (Sigma)] in 1 ml of sodium gluconate pipette solution.

Single-Channel Recording. For single-channel recordings, cells were perfused with HEPES-buffered extracellular solution containing 1 mM Ca^{2+} in place of the BaCl_2 . Standard techniques were used to make outside-out patch-clamp recordings and channel openings were induced by application of drugs into the bath. Data records were stored on digital audiotape and later filtered at 1–2 kHz and continuously sampled at 10–20 kHz onto computer by using a 1401 plus interface (Cambridge Electronic Design, Cambridge, UK). The records were analyzed by using SCAN, a time course fitting program, kindly provided by David Colquhoun (University College London, UK) and histograms constructed for the distribution of the channel current amplitudes and open times (16).

Hippocampal Neuronal Cultures. Hippocampal neurons were cultured using methods described (17). Neurons were continuously perfused by the standard HEPES-buffered extracellular solution containing 1 mM Ca^{2+} and whole-cell and outside-out patch recordings made from these cells.

Membrane Preparation and Radioligand Binding Assays. Cells transfected with NR1–1a and NR2A, prepared as for electrophysiological recording but without the CD8 plasmid, were harvested after 24–48 hr. Membranes were prepared essentially as described in ref. 18, but using a glycerol containing buffer in all steps (10% glycerol/40 mM HEPES, pH 8.0). For competition experiments using [^3H]MDL 105,519 (69.0 Ci/mmol; Amersham; 1 Ci = 37 GBq), samples were incubated in HEPES buffer (10% glycerol/10 mM HEPES, pH 8.0) in a final volume of 0.5 ml, for 1 hr at 0°C. [^3H]MDL 105,519 (0.5 nM) was used, and nonspecific binding was defined as that not displaced by 1 mM glycine. For competition experiments using [^3H]CGP 39653 (48.9 Ci/mmol; NEN) samples were incubated in 10 mM HEPES (pH 8.0), 5 mM CaCl_2 . 3nM [^3H]CGP was used, and nonspecific binding was defined as that not displaced by 1 mM glutamate. For both drugs, bound and unbound radioligands were separated by vacuum filtration onto GF/B filters (Whatman), presoaked in water, followed by two 4 ml washes in ice-cold HEPES buffer (10 mM, pH 8.0). All assays were performed in triplicate. Curves were fitted using the Hill equation, and K_i values calculated from IC_{50} s using published K_d s for the two radioligands [K_d MDL, 3.7 nM (19); K_d CGP, 6 nM (20)].

RESULTS

We tested for possible direct interactions of the group I mGluR agonist (RS)-DHPG with recombinant NMDA receptors composed of NR1–1a and NR2A subunits expressed in transfected HEK293 cells. We found that coapplication of DHPG (100 μM) with NMDA (30 μM) and glycine (10 μM) reversibly inhibits NMDA induced currents by $25.9 \pm 3.7\%$ (mean \pm SEM, $n = 4$, $P < 0.001$; Fig. 1A). DHPG similarly reduced NMDA/glycine currents in *Xenopus* oocytes injected with NR1–1a/NR2A cRNAs ($29.1 \pm 6.9\%$, $n = 5$, $P < 0.05$; Fig. 1B). Untransfected HEK293 cells and uninjected oocytes show no response to mGluR compounds as determined by calcium imaging and voltage-clamp experiments, respectively (data not shown).

We further assessed the actions of a wide range of mGluR agonists and antagonists on NMDA receptors expressed in oocytes. The compounds tested and their reported pharmacology (3) were as follows: MCPG (nonselective mGluR antagonist), 3-HPG (group I agonist), (1S, 3R)-ACPD (nonselective mGluR agonist), *trans*-ADA (group I agonist), DL-AP4 (group III agonist), and AIDA (group I antagonist). Surprisingly, we found that all of these mGluR-“selective” compounds in some way affected the function of recombinant NR1–1a/NR2A NMDA receptors in the absence of exogenously expressed mGluRs (Fig. 1 C–F). The extent to which

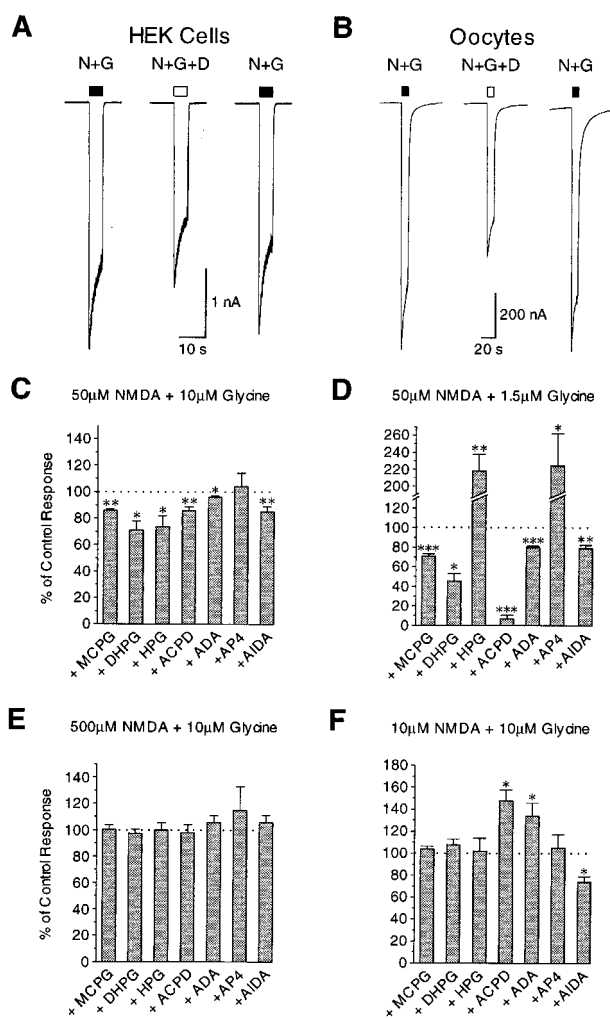


Fig. 1. NMDA-induced responses are modulated by mGluR compounds in the absence of exogenously expressed mGluRs (A). Whole-cell responses from HEK293 cells transfected with NR1–1a and NR2A NMDA receptor subunits. Currents are induced by application of 30 μM NMDA (N) and 10 μM glycine (G) before, during, and after coapplication of DHPG (D) (100 μM), to cells voltage clamped at -70 mV. (B) Whole-cell response induced by 50 μM NMDA and 10 μM glycine from a *Xenopus* oocyte injected with NR1–1a/NR2A RNAs. NMDA currents were recorded before, during and after coapplication of DHPG (100 μM). (C) Responses from oocytes due to coapplication of various mGluR agonists and antagonists with 50 μM NMDA and 10 μM glycine expressed as a percentage of the control response. The concentrations of the drugs tested were: (S)-MCPG, 100 μM ; (RS)-DHPG, 100 μM ; (S)-3-HPG, 100 μM ; (1S, 3R)-ACPD, 100 μM ; *trans*-ADA, 100 μM ; DL-AP4, 1 mM; AIDA, 200 μM . (D–F) Responses from coapplication of the mGluR compounds at these concentrations with NMDA and glycine at the concentrations indicated. See Table 1 for number of observations (asterisks denote significance using a one-sample Student's *t* test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

the different compounds inhibited and/or potentiated NMDA-mediated currents was highly dependent on the concentrations of the agonists NMDA and glycine. Thus, at an approximate EC_{50} concentration of NMDA (50 μM ; see Fig. 2B) with near-saturating glycine (10 μM), MCPG, DHPG, 3-HPG, (1S,3R)-ACPD, *trans*-ADA, and AIDA all significantly inhibited the NMDA/glycine-induced currents (Fig. 1C and Table 1). When the glycine concentration was reduced to 1.5 μM (the approximate EC_{50} for this subunit composition; see Fig. 2A), the inhibition by MCPG, DHPG, (1S,3R)-ACPD, *trans*-ADA and AIDA was increased, while the NMDA-induced currents in the presence of 3-HPG and DL-AP4 were

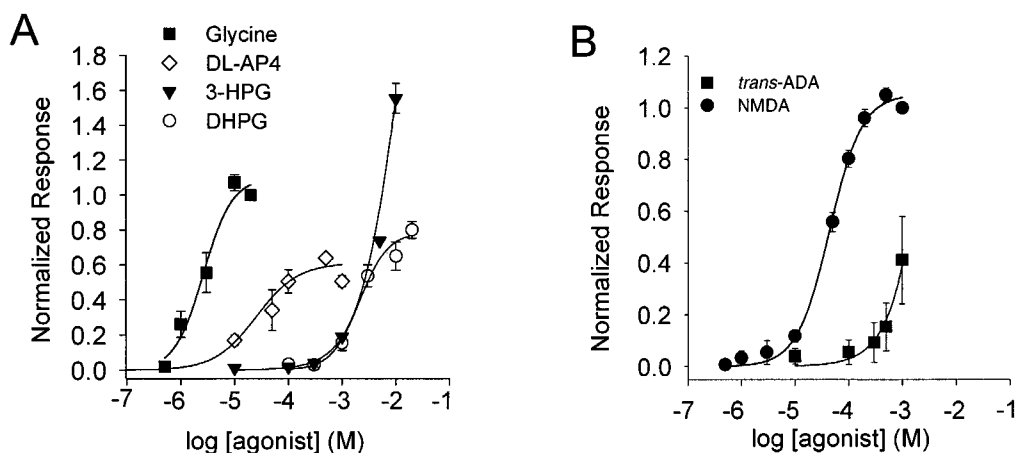


FIG. 2. mGluR compounds identified as coagonists with either NMDA or glycine. (A). Dose response curve to NMDA receptor coagonists in the presence of 50 μ M NMDA in oocytes injected with NR1-1a/NR2A. Dose response curves were generated using glycine ($n = 3$), (RS)-DHPG ($n = 3$), (S)-HPG ($n = 3$), and DL-AP4 ($n = 3$). Data were fitted to the Hill equation (with the exception of 3-HPG, which was fitted using an arbitrary value for I_{max}). Values obtained from the fits were: glycine, $EC_{50} = 2.6 \mu$ M, $n_H = 1.6$; DL-AP4, $EC_{50} = 25 \mu$ M, $n_H = 1.1$; DHPG, $EC_{50} = 21 \text{ mM}$, $n_H = 1.7$. (B). Dose response curves for NMDA ($n = 3$) and *trans*-ADA ($n = 5$) in the presence of 10 μ M glycine. Data for the NMDA dose response were fitted to the Hill equation. Values obtained from the fit are $EC_{50} = 44 \mu$ M, $n_H = 1.4$.

greatly potentiated (Fig. 1D and Table 1). At these agonist concentrations, the inhibition by 100 μ M (1S,3R)-ACPD was nearly complete. At saturating NMDA (500 μ M) and glycine (10 μ M) none of the compounds had any significant effect (Fig. 1E and Table 1). Currents induced by low NMDA concentrations (10 μ M) and saturating glycine (10 μ M) were potentiated by (1S,3R)-ACPD and *trans*-ADA, inhibited by AIDA and unaffected by MCPG, DHPG, 3-HPG and DL-AP4 (Fig. 1F and Table 1). The concentrations of mGluR compounds used here were chosen to reflect those commonly used when evaluating the roles of mGluRs in the brain (for review see ref. 3). When we tested oocytes injected with NR1-1a and NR2B, we found that DHPG did not cause any significant reduction in the current response to NMDA (50 μ M) and glycine (1.5 μ M), but caused a potentiation of the current when the glycine concentration was reduced to 100 nM ($118 \pm 2.9\%$, $n = 8$, $P < 0.001$). In this case we used lower concentrations of glycine to reflect the lower EC_{50} of glycine for this receptor subunit combination (21).

To test whether the potentiation of NMDA responses we had seen by some of the drugs was due to agonist actions of these compounds, increasing concentrations of the mGluR compounds were applied to oocytes expressing NR1-1a/NR2A in the presence of either NMDA (50 μ M) alone or glycine (10 μ M) alone. We found that both DL-AP4 and DHPG acted as partial coagonists with NMDA of NR1-1a/2A channels. These drugs gave EC_{50} s of 25 μ M and 2.1 mM, respectively, and maximum responses of 0.5 and 0.8 relative to saturating glycine responses (Fig. 2A). At higher concentra-

tions (1 mM and 5 mM), DL-AP4-induced currents were smaller than those induced by 500 μ M (not shown), suggesting that the drug also has some inherent antagonist activity (22). In contrast, 3-HPG produced a larger maximum response than glycine. Concentrations of 3-HPG (10 mM) that were clearly submaximal elicited nearly 2.5 \times the current induced by saturating concentrations of glycine (Fig. 2A). The NMDA receptor glycine site antagonist 7-Chlorokynurenic acid (5 μ M) blocked responses to NMDA (50 μ M) + glycine (10 μ M) ($78.8 \pm 9.8\%$, $n = 3$) and also to NMDA (50 μ M) + DHPG (1 mM) ($83.9 \pm 8.7\%$, $n = 3$). When the mGluR compounds were applied to oocytes expressing NMDA receptors in the presence of 10 μ M glycine, only *trans*-ADA induced significant inward currents. This suggested that this drug or a contaminant in the drug is a coagonist with glycine of NR1-1a/NR2A channels (Fig. 2B). MCPG, (1S,3R)-ACPD and AIDA did not induce currents at concentrations of up to 20 mM when coapplied with NMDA (not shown). To further examine the ability of DHPG or contaminants present in the drug to activate NMDA receptors, we performed single-channel recordings from outside-out patches excised from the soma of HEK cells transfected with NR1-1a/NR2A subunits. Consistent with the results from whole cell recordings, we found that coapplication of NMDA (5 μ M) and DHPG (1 mM) activated single-channel openings (Fig. 3B). These channels had a mean conductance of $44.2 \pm 0.7 \text{ pS}$ and a mean open time of $3.1 \pm 0.1 \text{ ms}$ ($n = 3$ patches) at -70 mV , similar to the conductance ($46.8 \pm 1.8 \text{ pS}$) and open time ($3.7 \pm 0.2 \text{ ms}$) in the presence of NMDA and glycine ($n = 3$; Fig. 3A). These data agree with

Table 1. Percentage of control responses as shown in Fig. 1 (measured before and after mGluR ligand coapplication), mean \pm SEM

[Agonist]	10 μ M Glycine (saturating)						1.5 μ M glycine (EC_{50})	
	10 μ M NMDA		50 μ M NMDA (EC_{50})		500 μ M NMDA (saturating)		50 μ M NMDA (EC_{50})	
	Mean \pm SEM	<i>n</i>	Mean \pm SEM	<i>n</i>	Mean \pm SEM	<i>n</i>	Mean \pm SEM	<i>n</i>
MCPG	104 \pm 3	3	86 \pm 1	4	100 \pm 4	7	71 \pm 2	6
DHPG	108 \pm 5	3	71 \pm 7	5	97 \pm 3	7	46 \pm 8	3
3-HPG	102 \pm 12	3	74 \pm 8	4	100 \pm 6	7	218 \pm 20	7
1S,3R-ACPD	147 \pm 10	3	86 \pm 3	6	98 \pm 6	7	7 \pm 4	7
<i>trans</i> -ADA	134 \pm 12	5	96 \pm 1	4	106 \pm 5	7	80 \pm 1	4
DL-AP4	105 \pm 12	7	104 \pm 10	5	115 \pm 18	7	224 \pm 38	7
AIDA	74 \pm 5	4	85 \pm 4	7	106 \pm 5	4	79 \pm 3	4

Data are for *n* determinations. Responses that were significantly different from control ($P < 0.05$), are indicated in boldface with *P* values as given in Fig. 1.

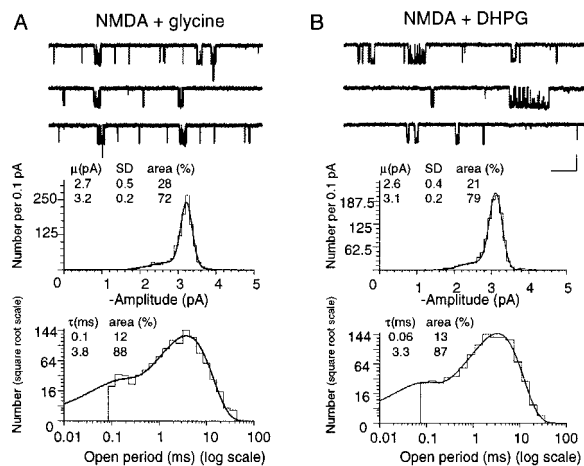


FIG. 3. Single-channel recordings in outside-out patches from HEK293 cells transfected with NR1-1a/NR2A show that (RS)-DHPG acts as a coagonist with NMDA. (A) Single-channel openings induced by application of 5 μ M NMDA and 5 μ M glycine to an outside-out patch held at a membrane potential of -70 mV. Typically 500–1,500 channel openings were measured for each recording and the amplitude and open period histograms constructed and fitted with the sum of two Gaussian components or two exponential components, respectively. For this recording the main conductance level had a single-channel conductance of 46 pS and a mean open time of 3.3 ms (B) Single-channel openings recorded in the same patch as illustrated in A., in the presence of 5 μ M NMDA and 1 mM DHPG. The main conductance level had a single-channel conductance of 44 pS and a mean open time of 2.9 ms. Calibration bars = 100 ms (x axis) and 3 pA (y axis).

NMDA receptor single-channel openings reported previously for recombinant heteromeric NMDA receptors composed of these receptor subunits (23).

To assess whether our results from oocytes and HEK cells also apply to native NMDA receptors, single-channel recordings were made from cultured hippocampal neurons. Outside-out patches excised from the soma of hippocampal neurons contained NMDA receptor channels of the expected conductance (44.6 ± 1.0 pS) and open time (3.5 ± 0.8 ms) at -70 mV ($n = 3$) in response to NMDA (5 μ M) and glycine (5 μ M) (Fig. 4A). Coapplication of DHPG (100 μ M) and NMDA (5 μ M) induced NMDA receptor-channel openings in the absence of added glycine (mean conductance 45.1 ± 0.5 pS and mean open time 3.1 ± 0.04 ms at -70 mV; $n = 3$) (Fig. 4B); no channel openings were observed when NMDA was applied alone (data not shown). Similarly, *trans*-ADA (300 μ M) coapplied with glycine (10 μ M) induced single-channel currents with a similar mean conductance (44.2 ± 1.3 pS), but slightly longer mean open time (5.1 ± 0.1 ms) ($n = 3$) (Fig. 4C). Single-channel openings were also observed in the absence of any conventional NMDA receptor agonists when *trans*-ADA (300 μ M) and DHPG (300 μ M) were coapplied (mean conductance, 42.6 ± 1.4 pS and mean open time 5.4 ± 1.0 ms ($n = 3$) at -70 mV) (Fig. 4D).

All of these agonist effects could be direct effects of the drugs or due to contaminant glutamate, aspartate, and glycine. Amino acid analysis performed on one batch of the compounds that showed agonist effects revealed levels of contaminant glutamate, aspartate, and glycine sufficient to account for all the agonist actions seen here. In the samples sent for analysis, contaminant levels of glutamate ranged from 0.01–0.26%, contaminant aspartate from 0.2–0.5%, and contaminant glycine from 0.3–1.0%. It should be noted that whilst the antagonist effects of these compounds on the NMDA receptor were seen at concentrations normally used to study mGluRs, the compounds only exhibited significant agonist properties at concentrations above the range normally used on mGluRs.

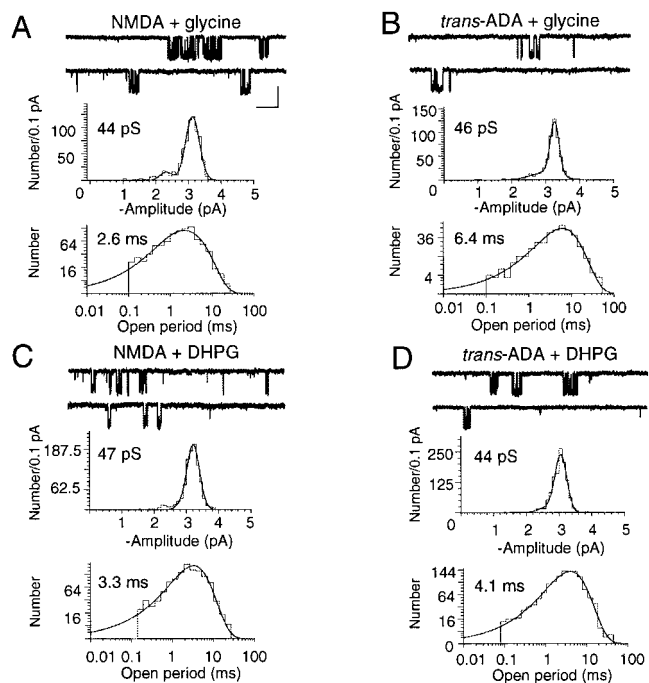


FIG. 4. Effect of mGluR compounds on native NMDA receptors in cultured hippocampal neurons. (A) Single-channel recording from an outside-out patch held at -70 mV made from the soma of these cells. Channel openings were induced by application of 5 μ M NMDA and 5 μ M glycine. Amplitude and open time histograms were fitted as for Fig. 3 (μ \pm SD; area; 2.3 ± 0.2 pA, 10.4% and 3.1 ± 0.2 pA, 89.6%) (τ : area; 1.0 ms, 29.0% and 3.2 ms, 71.0%). (B) Single-channel openings in an outside-out patch induced by 5 μ M NMDA and 100 μ M DHPG (2.8 ± 0.2 pA, 4.5% and 3.2 ± 0.2 pA, 95.5%) (3.3 ms). (C) Single-channel openings in the presence of 300 μ M *trans*-ADA and 10 μ M glycine (3.0 ± 0.4 pA, 30.1% and 3.3 ± 0.1 pA, 69.9%) (3.0 ms, 14.3% and 6.9 ms, 85.7%). (D) Single-channel openings induced by 300 μ M *trans*-ADA and 300 μ M DHPG (2.8 ± 0.3 pA, 18.6% and 3.1 ± 0.2 pA, 81.4%) (1.5 ms, 6.8% and 4.2 ms, 93.2%). For A–D the main conductance level and channel open periods are as indicated on the figure. Calibration bars = 100 ms (x axis) and 3 pA (y axis).

To determine whether these ligands were interacting with either of the agonist binding sites on the NMDA receptor, we performed radioligand displacement experiments. Fig. 5A shows displacement curves for glycine, DHPG and MCPG against the glycine site antagonist [3 H]MDL-105,519. The K_i s for displacement by glycine and DHPG were 6.0 ± 1.0 μ M and $1,200 \pm 170$ μ M, respectively ($n = 3$ in each case). No specific binding of [3 H]MDL-105,519 to mock-transfected HEK293 cell membranes was observed (data not shown). The K_i s were calculated using the Cheung-Prusoff equation (24) and the published K_d for [3 H]MDL-105,519 of 3.7 nM (19). MCPG did not displace the ligand at concentrations up to 1 mM. Displacement curves for the same three ligands against the glutamate site antagonist [3 H]CGP-39,653 are shown in Fig. 5B. DHPG showed significant displacement, with an estimated K_i of 336 ± 37 μ M ($n = 3$), using the published K_d for [3 H]CGP-39,653 of 6 nM for this subunit combination (20). MCPG and glycine showed only partial displacement at concentrations up to 1 mM. The DHPG displacement of [3 H]MDL-105,519 can be explained by glycine contamination in the DHPG stock, as can its agonist activity at higher concentrations. In addition, the displacement by DHPG of [3 H]CGP-39,653 reflects the glutamate/aspartate contamination present in this drug. The binding assays failed to reveal the mechanism of MCPG inhibition, suggesting that it might act as a noncompetitive antagonist at a site separate from the glutamate and glycine binding sites.

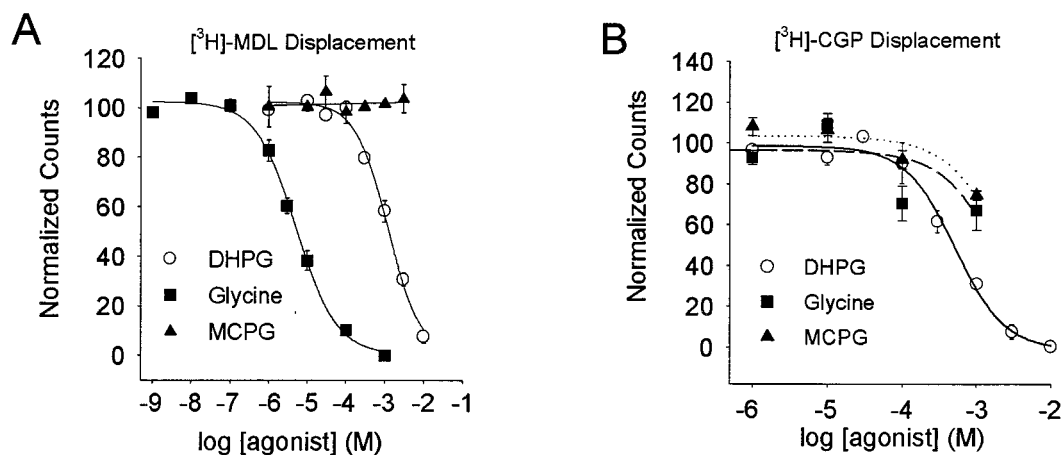


FIG. 5. Displacement of glutamate and glycine site ligands by mGluR compounds (*A*) Normalized displacement curves for the indicated compounds against [^3H]MDL 105,519 (0.5 nM), determined as the average of three experiments. K_i s for DHPG and glycine are given in the text. (*B*) Normalized displacement curves for the indicated compounds against [^3H]CGP 39,653 (3 nM), determined as the average of three experiments. The K_i for DHPG is given in the text. Displacements were performed as described in *Materials and Methods*.

To test whether MCPG also had direct effects on native receptors, we made whole-cell recordings from cultured hippocampal neurons. When bath-applied with NMDA (50 μM) and glycine (1.5 μM), MCPG (500 μM) significantly inhibited whole-cell currents by $14.1 \pm 4.1\%$ ($n = 6$, $P < 0.01$) (Fig. 6*A*). As MCPG is an mGluR antagonist, its effects on NMDA responses in this case cannot be attributed to mGluRs unless these cells contain tonically active mGluRs. Application of a saturating concentration of NMDA (500 μM) in the absence

of added glycine gave a significant whole-cell current (227 ± 47 pA at -70 mV, $n = 4$). Coapplication of 500 μM MCPG inhibited this current by $63.4 \pm 4.7\%$ ($n = 4$, $P < 0.01$) (Fig. 6*B*). Consistent with our observations from oocytes, we found that responses to saturating NMDA and 10 μM glycine were not significantly depressed by MCPG ($7.5 \pm 3.5\%$ inhibition, $n = 4$) (Fig. 6*B*).

DISCUSSION

We have shown that commercial preparations of drugs used as agonists and antagonists of mGluRs also have direct effects on native and recombinant NMDA receptors. In the case when a single compound caused both inhibition and NMDA receptor activation, this is best explained by a combination of direct inhibition of NMDA receptors by the drug and NMDA receptor activation due to the contaminating glutamate, aspartate, and glycine. Given that these drugs are generally used at concentrations where the inhibitory effects are dominant, further purification of these compounds to remove the contaminant amino acids would be expected to increase their antagonist properties.

It is conceivable that some of the inhibitory effects seen could be due to the interaction of the mGluR compounds with a previously uncharacterized receptor endogenous to the expression systems that we used. We think this is a remote possibility given the similarity of the effects seen in both HEK293 cells and oocytes and also the fact that both these cell types have been extensively screened for such endogenous responses by others and ourselves without success. MCPG, which appears to act solely as an antagonist, appears not to interact directly with the two agonist binding sites, leaving its mode of action unclear. The level of amino acid contamination in these drugs was unexpectedly high. However, it is beyond the scope of this study to define the extent of contamination between batches and it is sufficient here just to note that this problem potentially exists with other batches and sources of the drugs. In addition, the relative concentrations at which antagonist and agonist effects of these compounds were observed makes their antagonism of NMDA receptors a more important issue when using these drugs to study mGluR/NMDA receptor interactions.

Our results have implications both for the interpretation of studies on mGluR effects on NMDA receptors and on the practical issue of identifying truly selective mGluR ligands. There has been much disagreement in the literature regarding the involvement of mGluRs in NMDA receptor-dependent

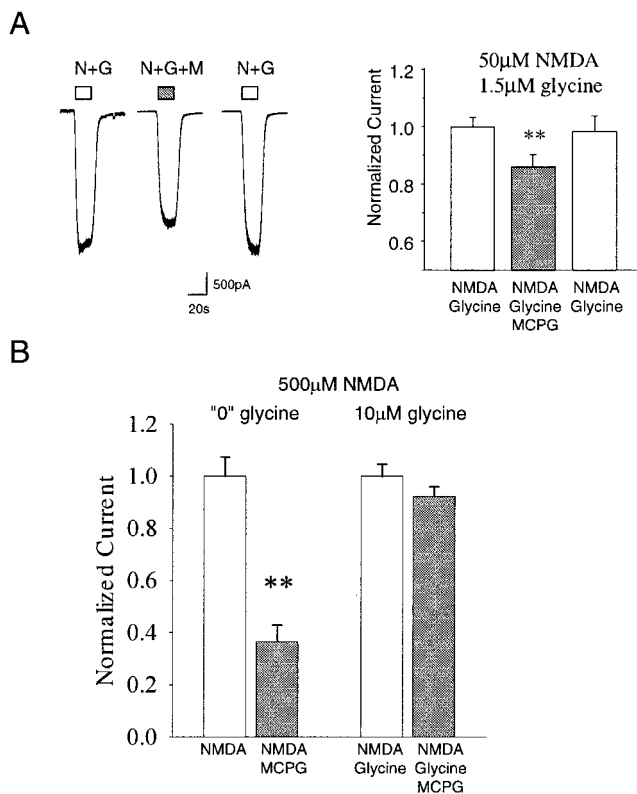


FIG. 6. Effect of MCPG on native NMDA receptors. (*A*) Whole-cell responses from cultured hippocampal neurons induced by application of NMDA (N; 50 μM) and glycine (G; 1.5 μM) or NMDA, glycine, and (S)-MCPG (M; 500 μM). The bar chart shows normalized responses from six cells similarly treated. (**, $P < 0.01$; one-sample Student's *t* test.) (*B*) Inhibition by MCPG (500 μM) of whole-cell responses to NMDA (500 μM) in the presence of zero added glycine or 10 μM glycine ($n = 4$ in both cases).

long-term potentiation (25). Several studies have shown that the mGluR antagonist, MCPG, can block high frequency stimulation-induced long-term potentiation at the Schaffer collateral-CA1 synapse (7, 26–30). Several other groups have reported that they cannot block long-term potentiation under apparently identical conditions (31–35). The lack of truly selective pharmacology of these mGluR compounds could in part help explain problems in using these drugs to study synaptic plasticity. Our results show that the effects of MCPG on NMDA receptor function are highly dependent on the concentrations of NMDA and glycine. By extrapolation, it is very likely that differences in levels of glycine at the synapse could also drastically alter the significance of the effects of MCPG on synaptic NMDA receptors. We observed that at a saturating concentration of NMDA and glycine, native NMDA receptors were not blocked by MCPG, suggesting a way of circumventing this lack of drug selectivity.

Similar problems exist in the literature regarding the effects of mGluR activation on excitotoxicity. Depending on the mGluR agonists, the method of inducing excitotoxicity, and the type of preparation used, one can observe exacerbation, protection, or no effect of mGluR compounds on neuronal damage induced by excitatory amino acids (5, 36). Some of these results might similarly be affected by direct interactions of these mGluR ligands with NMDA receptors. It is therefore clear that more selective mGluR compounds or in some cases compounds with reduced amino acid contamination, which have no demonstrable effect on NMDA receptors, will need to be developed before the role of mGluRs in many neuronal processes can be defined.

We thank P. Jeffrey Conn, Raymond J. Dingeldine, and Gary Westbrook for critically reviewing the manuscript. In addition we thank Geoff Swanson, Alasdair Gibb, and Cheryl Rogers for discussion and comments; David Colquhoun and Ioanna Vais for providing single-channel analysis software; Kim McAllister and Jane Sullivan for providing cultured neurons; and Quynh-Chi Phan for preparation of oocytes and HEK293 cells. This research was supported by an International Travelling Research Fellowship from The Wellcome Trust (A.C.), a National Institutes of Health Training Grant (R.W.G.) and grants from the National Institutes of Health and the McKnight Foundation (S.F.H.).

- Sladeczek, F., Pin, J. P., Récasens, M., Bockaert, J. & Weiss, S. (1985) *Nature (London)* **317**, 717–719.
- Pin, J. P. & Duvoisin, R. (1995) *Neuropharmacology* **34**, 1–26.
- Conn, P. J. & Pin, J. P. (1997) *Annu. Rev. Pharmacol. Toxicol.* **37**, 205–237.
- Reidel, G. (1996) *Trends Neurosci.* **19**, 219–224.
- Nicoletti, F., Bruno, V., Copani, A., Casabona, G. & Knöpfel, T. (1996) *Trends Neurosci.* **19**, 267–271.
- Wang, Y., Rowan, M. J. & Anwyl, R. (1995) *Neurosci. Lett.* **197**, 207–210.
- Bashir, Z. I., Bortolotto, Z. A., Davies, C. H., Berretta, N., Irving, A. J., Seal, A. J., Henley, J. M., Jane, D. E., Watkins, J. C. & Collingridge, G. L. (1993) *Nature (London)* **363**, 347–350.
- Riedel, G., Casabona, G. & Reymann, K. G. (1995) *J. Neurosci.* **15**, 87–98.
- Richter-Levin, G., Errington, M. L., Maegawa, H. & Bliss, T. V. P. (1994) *Neuropharmacology* **33**, 853–857.
- Koh, J. Y., Palmer, E. & Cotman, C. W. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 9431–9435.
- Buisson, A., Yu, S. P. & Choi, D. W. (1996) *Eur. J. Pharmacol.* **8**, 138–143.
- Pizzi, M., Galli, P., Consolandi, O., Arrighi, V., Memo, M. & Spano, P. F. (1996) *Mol. Neuropharmacol.* **49**, 586–594.
- Ambrosini, A., Bresciani, L., Fracchia, S., Brunello, N. & Racagni, G. (1995) *Mol. Pharmacol.* **47**, 1057–1064.
- Gereau, R. W. & Heinemann, S. F. (1998) *Neuron* **20**, 143–151.
- Hollmann, M., Boulter, J., Maron, C., Beasley, L., Sullivan, J., Pecht, G. & Heinemann, S. (1993) *Neuron* **10**, 943–954.
- Colquhoun, D. & Sigworth, F. J. (1995) in *Single Channel Recording*, eds. Sakmann, B. & Neher, E. (Plenum, New York), pp. 483–587.
- Bekkers, J. M. & Stevens, C. F. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 7834–7838.
- Swanson, G. T., Gereau, R. W., IV, Green, T. & Heinemann, S. F. (1997) *Neuron* **19**, 913–926.
- Höfner, G. & Wanner, K. T. (1997) *Neurosci. Lett.* **226**, 79–82.
- Laurie, D. J. & Seeburg, P. H. (1994) *Eur. J. Pharmacol.* **268**, 335–345.
- Kutsuwada, T., Kashiwabuchi, N., Mori, H., Sakimura, K., Kushiya, E., Araki, K., Meguro, H., Masaki, H., Kumanishi, T., Arakawa, M., *et al.* (1992) *Nature (London)* **358**, 36–41.
- Evans, R. H., Francis, A. A., Jones, A. W., Smith, D. A. & Watkins, J. C. (1982) *Br. J. Pharmacol.* **75**, 65–75.
- Stern, P., Behe, P., Schoepfer, R. & Colquhoun, D. (1992) *Proc. R. Soc. London B* **250**, 271–277.
- Cheung, Y. C. & Prusoff, W. H. (1973) *Biochem. Pharmacol.* **22**, 3099–3102.
- Nakanishi, S. (1994) *Neuron* **13**, 1031–1037.
- Bashir, Z. I. & Collingridge, G. L. (1994) *Exp. Brain Res.* **100**, 437–443.
- Izumi, Y. & Zorumski, C. F. (1994) *Neurosci. Lett.* **176**, 89–94.
- Little, Z., Grover, L. & Teyler, T. J. (1995) *Neurosci. Lett.* **201**, 73–76.
- Sergueeva, O. A., Fedorov, N. B. & Reymann, K. G. (1993) *Neuropharmacology* **32**, 933–935.
- Breakwell, N. A., Rowan, M. J. & Anwyl, R. (1998) *J. Neurophysiol.* **79**, 1270–1276.
- Manzoni, O. J., Weisskopf, M. G. & Nicoll, N. (1994) *Eur. J. Neurosci.* **6**, 1050–1054.
- Chinestra, P., Anikszajn, L., Diabra, D. & Ben-Ari, Y. (1993) *J. Neurophysiol.* **70**, 2684–2689.
- Brown, R. E., Rabe, H. & Reymann, K. G. (1994) *Neurosci. Lett.* **170**, 17–21.
- Selig, D. K., Lee, H.-K., Bear, M. F. & Malenka, R. C. (1995) *J. Neurophysiol.* **74**, 1075–1082.
- Thomas, M. J. & O'Dell, T. J. (1995) *Brain Res.* **695**, 45–52.
- Knöpfel, T., Kuhn, R. & Allgeier, H. (1995) *J. Med. Chem.* **38**, 1417–1426.