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# Sustained Exposure to 1-Aminocyclopropanecarboxylic acid, a Glycine Partial Agonist, Alters *N*-Methyl-D-Aspartate Receptor Function and Subunit Composition

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## SUMMARY

Partial agonists at the strychnine-insensitive glycine sites coupled to *N*-methyl-D-aspartate (NMDA) receptors reduce both glutamate-induced neurotoxicity *in vitro* and ischemia-induced neurodegeneration *in vivo*. Paradoxically, sustained exposure of cultured cerebellar granule cell neurons to glycinergic ligands, including glycine and the glycine partial agonists (±)-3-amino-1-hydroxy-2-pyrrolidone, 1-aminocyclopropanecarboxylic acid (ACPC), and D-cycloserine, attenuates the neuroprotective effects of (±)-3-amino-1-hydroxy-2-pyrrolidone and ACPC. In the present study, we investigated the mechanisms responsible for this attenuated neuroprotection. Three NMDA receptor-mediated responses were examined after sustained exposure to ACPC: glutamate-induced neurotoxicity, NMDA-stimulated increases in cGMP levels, and NMDA-stimulated increases in $[Ca^{+2}]_i$. Consistent with previous findings, coincubation with ACPC blocked glutamate-induced neurotoxicity, whereas sustained (24 hr) exposure to ACPC attenuated its protective effects. Moreover, sustained exposure to ACPC caused an apparent ~2-fold increase in the potency of both glutamate to act as a neurotoxin and NMDA to stimulate cGMP formation. Sustained exposure to ACPC also increased NMDA-stimulated $[Ca^{+2}]_i$ ~3-fold compared with control granule cell cultures but did not affect basal $[Ca^{+2}]_i$. This apparent increase in glutamate sensitivity may be attributable to a change in NMDA receptor subunit composition as sustained ACPC exposure resulted in a ~2.5-fold increase in NMDA receptor 2C RNA levels, without concomitant changes in the amounts of RNA encoding the NMDA receptor 2A, 2B, or 1 subunit. This is the first demonstration that sustained exposure to a glycinergic ligand can alter the expression of RNAs encoding NMDA receptor subunits. Because glycinergic ligands are potential clinical candidates, these results may have important implications for the treatment of neurodegenerative disorders.

During the past decade, converging lines of evidence have linked the abnormal release or leak of glutamate and other endogenous activators of glutamate receptors to the neuronal degeneration associated with a wide range of pathologies (1–4). Thus, there is considerable interest in developing therapeutic strategies that blunt or block glutamatergic transmission. Among the ionotropic family of glutamate receptors, NMDA receptors have been prominent targets for therapeutic intervention (5–7).

NMDA receptors are unique among ligand-gated ion channels in an absolute requirement for the coordinate occupation of two recognition sites (glutamate and strychnine-insensitive glycine sites) for channel opening (8, 9). Based on this requirement, it was hypothesized that if glycine is present at (or near) saturating concentrations *in situ*, then partial agonists at strychnine-insensitive glycine sites could function as NMDA antagonists (10, 11). Consistent with this hypothesis, the high affinity glycine partial agonist ACPC (12, 13) reduces glutamate-induced neurotoxicity in primary cultures (14, 15) and protects vulnerable neurons against ischemic damage *in vivo* (15–18). In these *in vivo* models, ACPC was neuroprotective when administered either at the time of ischemic insult or for 1 week before insult (15–18). However, the neuroprotection produced by acute and chronic administration of ACPC appears to be mediated through fundamentally different mechanisms. Thus, in the chronic treatment regimens where the last dose of ACPC was administered 24 hr before ischemia, blood and brain levels of ACPC were undetectable at the time of insult (16, 19, 20).

In studies using primary neuronal cultures to model the *in vivo* effects of chronic treatment with ACPC, it was observed that sustained (24 hr) exposure of cerebellar granule cells to glycinergic ligands (including glycine and the glycine partial agonists ACPC, HA-966, and D-cycloserine) resulted in an attenuation of the neuroprotective effects of ACPC and HA-966 (14). Using ACPC as a model glycinergic ligand, we

**ABBREVIATIONS:** NMDA, *N*-methyl-D-aspartate; NMDAR, *N*-methyl-D-aspartate receptor; ACPC, 1-aminocyclopropanecarboxylic acid; HA-966, (±)-3-amino-1-hydroxy-2-pyrrolidone; $[Ca^{+2}]_i$, intracellular calcium concentration; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.



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examined the potential mechanisms responsible for its attenuated neuroprotective effects in cerebellar granule cell neurons after sustained (24 hr) exposure. We report that sustained exposure to ACPC results in (a) a ~2-fold increase in the potency of glutamate to induce cell death, (b) a ~2-fold increase in the potency of NMDA to elevate cGMP levels, (c) a ~3-fold increase in NMDA-stimulated increases in $[Ca^{+2}]_i$, and (d) a ~2.5-fold increase in the levels of RNA encoding the NMDAR-2C subunit without a concomitant change in RNA levels for NMDAR-1, -2A, or -2B subunits. We postulate that the apparent increase in NMDAR function after sustained exposure to glycinergic ligands is mediated through a change in NMDAR subunit composition.

## Experimental Procedures

**Materials.** [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol) was purchased from ICN Biomedicals (Costa Mesa, CA). Fetal bovine serum was purchased from Intergen Company (Purchase, NY) and heat-inactivated for 30 min (55°). Basal medium with Eagle's salts was obtained from GIBCO-BRL (Grand Island, NY). Glutamate, NMDA, and glycine were purchased from Sigma Chemical Co (St. Louis, MO), and ACPC was obtained from Research Organics (Cleveland, OH). Plasmids containing cDNA for $\epsilon$1 (the mouse homologue of the rat NMDAR-2A subunit), $\epsilon$2 (the mouse homologue of the rat NMDAR-2B subunit), and $\epsilon$3 (the mouse homologue of the rat NMDAR-2C subunit) were the generous gifts of Dr. M. Mishina, Tokyo University, Tokyo, Japan. A plasmid containing the entire coding region of the rat NMDAR-1A subunit was donated by Drs. G. Wong and Y. Sei (National Institute for Diabetes and Digestive and Kidney Diseases).

**Cell culture.** Primary granule cell neurons were isolated from cerebella of 6–8-day-old Sprague-Dawley rat pups (Taconic Farms, Germantown, NY) as previously described (21). Cells were plated in basal Eagle's medium with Earle's salts (containing 2 mM glutamine and supplemented with 25 mM KCl, 0.1 mg gentamicin/ml, and 10% fetal bovine serum) on poly-D-lysine-coated culture dishes at a density of $4 \times 10^6$ cells (3.5 ml medium) per 35-mm dish (6-well plates) or $1.4 \times 10^6$ cells (in 1.25 ml medium) per 10-mm dish (12-well plate). Cells were maintained at 37° and 5% $CO_2$/95% air in a humidified incubator. Cytosine arabinoside (10–15 $\mu$M) was added to inhibit the growth of nonneuronal cells.

**Neurotoxicity assays.** Cells were grown in culture for 8 days. On the day of testing, conditioned medium was removed from control cells and reserved for subsequent use. The cells were rinsed twice with $Mg^{+2}$-free Locke's solution (154 mM NaCl, 2.3 mM $CaCl_2$, 8.6 mM HEPES, pH 7.4) and incubated with $Mg^{+2}$-free Locke's solution (1.5 ml/35-mm well or 0.5 ml/10-mm well, respectively) for 25 min at 37°. The Locke's solution was replaced with fresh Locke's solution (with or without ACPC), and the cells were incubated for an additional 15 min before glutamate was added. The Locke's solution was removed after 30-min glutamate exposure; the cells were rinsed twice with Hank's buffered salt solution; conditioned medium was added (1 ml/35-mm well or 0.5 ml/10-mm well, respectively); and the cells were returned to the incubator. The percentage of surviving cells was determined 18–24 hr later by trypan blue exclusion. Trypan blue solution was added to a final concentration of 0.1%, and the medium was removed 4 min later. The cells were rinsed with phosphate-buffered saline and fixed with 1% $CaCl_2$/1% formaldehyde for at least 10 min before counting. Cells with normal granule cell morphology that excluded trypan blue were counted as live cells. Cells that retained trypan blue and/or had condensed morphology were counted as dead cells. At least 150 cells were counted per well, and three wells were analyzed per treatment in each experiment. Cells were counted from coded plates, and the codes (specifying treatments) were broken after the cells had been counted. For cells receiving sustained ACPC treatment, ACPC was added directly to the medium 24 hr before neurotoxicity testing (i.e., after 7 days in culture). It should be noted that the ACPC added to the medium for sustained treatment was removed by the washing procedure before neurotoxicity testing and that ACPC was not present during glutamate exposure unless explicitly stated.

**Measurement of cGMP.** Cells were grown in culture for 8 days. On the day of testing, medium was removed, and the cells were rinsed twice with $Mg^{+2}$-free Locke's solution. Cells were treated with $Mg^{+2}$-free Locke's solution (1 ml/35-mm well or 0.5 ml/10-mm well, respectively) containing test agents (e.g., glycine or ACPC) and left at room temperature for 15 min. NMDA was then added, followed 2 min later by rapid aspiration of the media and the addition of 0.5 ml of 0.4 M $HClO_4$. Cells were scraped into the $HClO_4$ and disrupted by sonication. An aliquot of the acid sonicate was reserved for protein determination. The remaining acid sonicate was neutralized with 0.4 M KOH and centrifuged at 14,000 rpm for 15 min, and the supernatant was analyzed for cGMP levels by radioimmunoassay (Biomedical Technologies, Stoughton, MA). Protein was measured as described by Bradford (22) using immunoglobulin as a standard. cGMP levels were assayed in cells from three wells per treatment in each experiment. Sustained exposure to ACPC was accomplished as described for neurotoxicity testing. As previously noted, ACPC added to the medium for sustained treatment was removed by the washing procedure, and ACPC was not present during NMDA treatment unless explicitly stated.

**Measurement of $[Ca^{2+}]_i$.** Cerebellar granule cells were plated on poly-D-lysine-coated glass coverslips at a density of $1–2 \times 10^5$ cells/12-mm diameter coverslip and grown in 0.5 ml medium for 8 days. Before loading with Fura-2, the medium was removed and replaced with 0.5 ml of $Mg^{+2}$-free Locke's solution. Fura-2 acetoxymethyl ester (F-1201, Molecular Probes, Eugene, OR) in was added to each coverslip to yield a final concentration of 5 $\mu$M. The cells were loaded in the dark at 37° for at least 30 min. After loading, the coverslips were gently washed twice with 1 ml of Locke's solution and then transferred to a recording chamber (Biophysica Technologies, Baltimore, MD) containing 1 ml of Locke's solution. Fura-2 fluorescence was measured by dual excitation wavelength ratio microfluorescence at excitation wavelengths of 340 and 380 nm with emission monitored at 510 nm using an inverted microscope (Nikon Diaphot) at 400× magnification. Data were acquired and processed using Image-1/FL software (Universal Imaging Corp., West Chester, PA). Fluorescence measurements were taken from 65 cells of 100–150 cells observed in each field. Baseline fluorescence readings were taken for ~10 min. Data acquisition was then suspended for ~30 sec while NMDA (5 $\mu$M) was added to the medium and data acquisition resumed for 20 min. $[Ca^{+2}]_i$ was determined by *in vitro* calibration curves using buffered solutions of known $Ca^{+2}$. Data were expressed as the percentage increase in $[Ca^{+2}]_i$ elicited by NMDA compared with baseline $[Ca^{+2}]_i$. Data from 109 control cells (25–58 cells from each of three coverslips) and 124 ACPC-pretreated cells (62 cells from each of two coverslips) were further analyzed by frequency distribution and fit by a gaussian curve (GraphPad Prism software, version 1.0, GraphPad Software, San Diego, CA). Sustained exposure of cells to ACPC was accomplished as described for neurotoxicity testing. As previously noted, the ACPC added to the medium for sustained exposure was removed by the washing procedure and was not present during NMDA exposure.

**Measurement of NMDAR RNA.** Total cellular RNA was isolated from primary granule cells by the acid guanidinium thiocyanate/phenol/chloroform method of Chomczynski and Sacchi (23). RNA concentration was estimated by absorbance spectrometry. Specific RNAs for four NMDAR subunits were measured by hybridization to the following cDNA fragments that were random primer-labeled with [$^{32}$P]dCTP: (a) 1518-bp *Pst*I fragment of NMDAR-1A cDNA for NMDAR-1 RNA, (b) 1228-bp *Pst*I fragment of $\epsilon$1 cDNA for NMDAR-2A RNA, (c) 747-bp *Apa*I fragment of $\epsilon$2 cDNA for NMDAR-2B RNA, and (d) 1008-bp *Not*I/*Hin*dIII fragment of $\epsilon$3 cDNA for NMDAR-2C RNA. These probes were chosen from the

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3′-ends of the cDNAs where there is little subunit homology. The specificity of each cDNA probe was verified in two ways. (i) First, to ensure that there was no cross-reactivity among the four NMDAR subunits, each probe was tested by slot-blot analysis of 0.01, 0.1, and 1 μg of plasmids containing each of the full-length cDNA probes. Each probe exhibited >1000-fold specificity for plasmids containing the corresponding full-length cDNA. (ii) Each probe was also used for Northern blot analysis of total cellular RNA from rat cerebral cortex and cerebellum. Each probe recognized RNA species of appropriate size and distribution between the two brain areas (e.g., NR1 was abundant in both cerebral cortex and cerebellum, NR2A and NR2B were more abundant in cerebral cortex than cerebellum, and NR2C was much more abundant in cerebellum). NMDAR RNAs were quantified by slot-blot analysis using the same membrane and hybridization and wash conditions that were used for Northern blot analysis. Five micrograms of total cellular RNA were applied to Nytran membrane (Schleicher and Schuell, Keene, NH) using a slot-blot manifold (Schleicher and Schuell) and immobilized by UV-irradiation (UV Stratalinker 1800, Stratagene Cloning Systems, La Jolla, CA). cDNA probes that had been radiolabeled with [$^{32}$P]dCTP to a specific activity of 1–2 × $10^9$ cpm/μg DNA were hybridized to the slot-blotted RNA samples in aqueous buffer (1% bovine serum albumin, 1 mM EDTA, 0.5 M $NaPO_4$, pH 7.4, 7% sodium dodecyl sulfate [24]) overnight at 65°. After washing (to stringency of 0.1× SSC/0.1% sodium dodecyl sulfate at 55°), the membranes were exposed to X-ray film for 1–3 days at −70°. The radioactivity that hybridized to the slot-blotted RNA was quantified by densitometric analysis of autoradiograms with conversion to calibrated optical density units using Image-1 software (Universal Imaging Corp.). The linearity of the slot-blot procedure was verified using RNA isolated from rat cerebral cortex and cerebellum.

**Statistical analysis.** For each study, data from several granule cell preparations were analyzed by analysis of variance (Systat 5.0 software, Systat, Evanston, IL) followed by least-significant difference tests for planned comparisons.

## Results

**ACPC reduces NMDA-stimulated cGMP levels.** A 2-min exposure to NMDA increased cGMP levels in a concentration-dependent manner, an action that could be potentiated by the addition of glycine and blocked by ACPC (Fig. 1). In these experiments, a submaximal concentration of NMDA (15 μM) increased cGMP levels 3-fold, and this was further enhanced to 5-fold by the addition of a saturating concentration of glycine (10 μM). ACPC reduced by 50% the 3-fold increase in cGMP levels produced by NMDA (15 μM), although this attenuation failed to reach statistical significance at this concentration of NMDA. Similarly, the 6.5-fold increase in cGMP level produced by 25 μM NMDA was increased 39% by glycine and reduced 31% by ACPC. The maximally effective concentration of NMDA (50 μM) was neither potentiated by glycine nor attenuated by ACPC (Fig. 1).

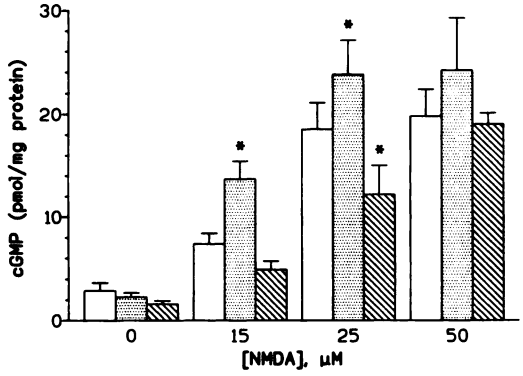


**Fig. 1.** Effects of glycine and ACPC on NMDA-stimulated cGMP levels in cultured cerebellar granule cell neurons. Cell culture conditions are described in Experimental Procedures. Cells were treated for 2 min with NMDA alone (*open bars*) or in the presence of 10 μM glycine (*shaded bars*) or 1 mM ACPC (*striped bars*), and cGMP levels were assayed as described in Experimental Procedures. Values represent mean ± standard error of four to seven experiments. *, $p < 0.05$ vs NMDA alone.

**Sustained exposure to ACPC potentiates glutamate-induced toxicity, NMDA-stimulated cGMP levels, and NMDA-stimulated $[Ca^{+2}]_i$.** Consistent with previous reports (14, 19), the neuroprotection produced by acute exposure to a maximally effective concentration of ACPC (1 mM) was significantly reduced by a 24-hr incubation before glutamate challenge (Fig. 2A). In these experiments, glutamate (10 μM) killed 63% of control cells, and concurrent application of ACPC reduced this toxicity by 50%. After sustained exposure to ACPC, glutamate (10 μM) killed 75% of the cells, and concurrent application of ACPC did not significantly reduce this toxicity. Concomitant with the reduced neuroprotection by ACPC, an apparent increase in the neurotoxic potency of glutamate was manifested after sustained exposure to ACPC

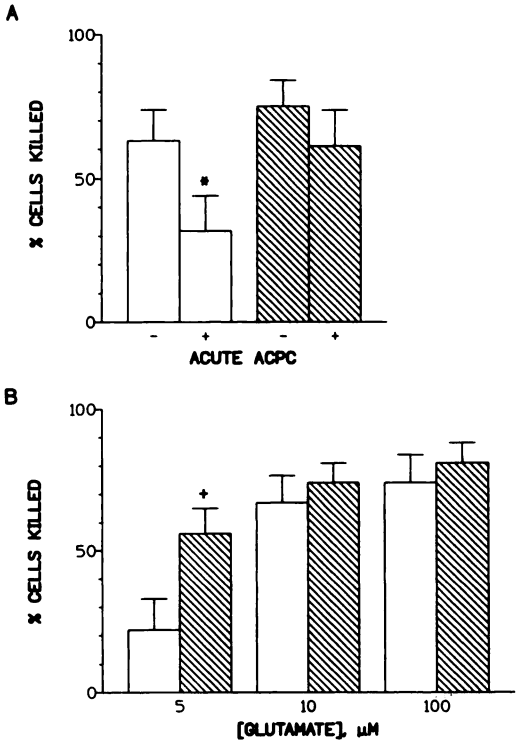


**Fig. 2.** Sustained treatment with ACPC attenuates the neuroprotective effect of ACPC (A) and potentiates glutamate-induced toxicity (B). Cells were treated for 24 hr in the absence (open bars) or presence of 1 mM ACPC (*striped bars*) before testing for glutamate-induced neurotoxicity. A, *Open bars*: acute exposure to ACPC reduces glutamate (10 μM)-induced toxicity; *striped bars*: the neuroprotective effect of ACPC is attenuated after sustained exposure. B, Glutamate toxicity in control (*open bars*) and ACPC-pretreated (*striped bars*) cells. Data are expressed as percent cells killed, calculated as {[(% cells dead after treatment with glutamate ± ACPC) − (% cells dead under control conditions)]/[100 − (% cells dead under control conditions)]} × 100%. Values represent mean ± standard error of four to six experiments. Basal cell death was 16 ± 3.5%, and 24-hr exposure to ACPC had no effect (16 ± 3.1%). *, $p < 0.05$ vs no acute ACPC; +, $p < 0.05$ vs no 24-hr ACPC treatment.

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(Fig. 2B). The neurotoxicity produced by a submaximal concentration of glutamate (5 μM) was increased 3-fold (from 22% in control cells to 67%) by 24-hr pretreatment with ACPC; the neurotoxicity produced by higher concentrations of glutamate was unaffected. Similar results were observed in cultures exposed to ACPC for 48 hr (data not presented).

Sustained exposure to ACPC resulted in a similar potentiation of NMDA-stimulated cGMP levels (Fig. 3A). A concentration of NMDA (25 μM) that elevated cGMP levels 5-fold in control cells produced a 9-fold increase in cells exposed to ACPC for 24 hr. Similarly, in response to 50 μM NMDA, cGMP levels were increased 9- and 15-fold in control and ACPC pretreated cells, respectively. The addition of a saturating concentration of glycine (10 μM) abolished the apparent difference in the potency of NMDA between control and ACPC-pretreated cells (Fig. 3B). Moreover, although glycine increased the absolute levels of cGMP in response to 50 μM NMDA in control cells (16 ± 0.9 versus 31 ± 5.3 pmol/mg protein, *open bars*, Fig. 3, A and B), cGMP levels in ACPC-pretreated cells exposed to 50 μM NMDA were unaffected by glycine (26 ± 3.8 versus 32 ± 4.4 pmol/mg protein, *hatched bars*, Fig. 3, A and B).

Sustained exposure to ACPC also potentiated NMDA-evoked increases in $[Ca^{+2}]_i$ but did not significantly affect resting levels of $[Ca^{+2}]_i$ (Fig. 4A). Thus, NMDA (5 μM) increased $[Ca^{+2}]_i$ 2.7-fold in control cells compared with the 9.4-fold increase in cells treated for 24 hr with ACPC. The concentration of NMDA used was near threshold in the majority of control neurons, with $[Ca^{+2}]_i$ elevated ≤2-fold in 55% of cells and ≤5-fold in 95% of the cells (Fig. 4B). In contrast, NMDA increased $[Ca^{+2}]_i$ >5-fold in 87% of cells and >8-fold in 50% of cells that had been treated for 24 hr with ACPC (Fig. 4B).

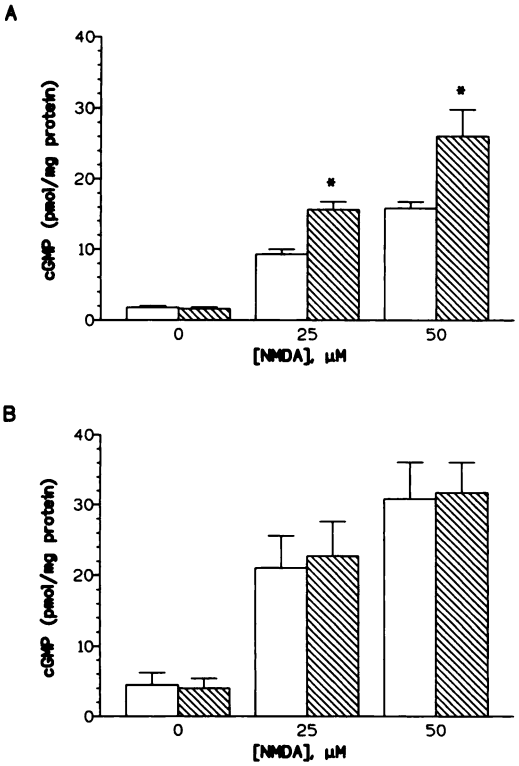


**Fig. 3.** Effect of sustained exposure to ACPC on NMDA-stimulated increases in cGMP. A, NMDA-stimulated cGMP formation in control (*open bars*) and ACPC pretreated cells (*striped bars*). B, NMDA-stimulated cGMP formation in the presence of glycine (10 μM). Cell culture conditions are described in Experimental Procedures. Control cells (*open bars*) and cells exposed to 1 mM ACPC (*striped bars*) for 24 hr were assayed for NMDA-stimulated cGMP levels (as described in Experimental Procedures). Values represent mean ± standard error of three or four experiments. *, $p < 0.05$ vs no 24-hr ACPC treatment.

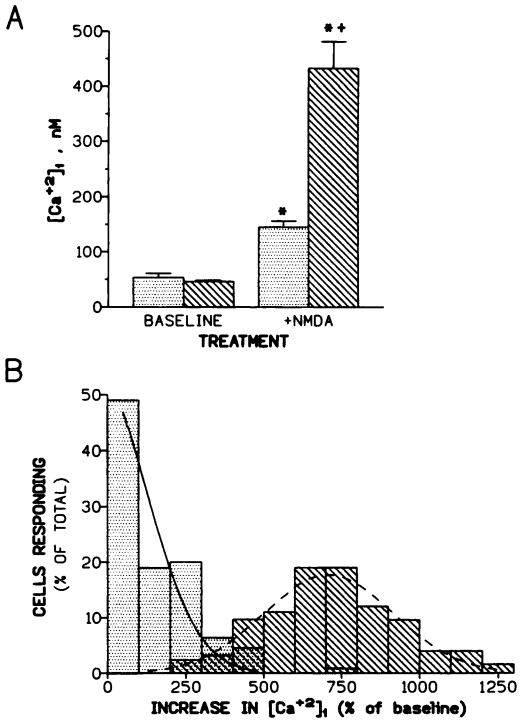


**Fig. 4.** Sustained treatment with ACPC potentiates NMDA-stimulated increases in $[Ca^{+2}]_i$. A, Data from cells that had been pretreated for 24 hr in the absence (*shaded bars*) or presence of 1 mM ACPC (*striped bars*). Values represent mean ± standard error for 39 control cells (three coverslips) and 26 cells (two coverslips) exposed to ACPC for 24 hr. Baseline values were obtained 1 min before the application of 5 μM NMDA, and levels were monitored for 5 min after NMDA application as described in Experimental Procedures. B, Frequency distributions and gaussian curves of increases in $[Ca^{+2}]_i$ in response to 5 μM NMDA. Data are expressed as a percentage of baseline for 109 control cells (25–58 from each of three slips; *shaded bars*) and 124 cells (62 from each of two slips; *striped bars*) that had been treated for 24 hr with 1 mM ACPC. These data were replicated once with similar results (data not presented). *, $p < 0.001$ vs base-line; +, $p < 0.001$ vs no 24-hr ACPC treatment.

**Sustained exposure to ACPC selectively increases the expression of NMDAR-2C subunit RNA.** Treatment of granule cells for 24 hr with ACPC increased levels of the RNA encoding the 2C subunit of the NMDAR by 245% relative to control neurons (Fig. 5). In contrast, the levels of RNA encoding the NMDAR-1, -2A, and -2B subunits were not significantly altered by this treatment.

## Discussion

Sustained exposure of cultured cerebellar granule cells to glycinergic ligands has been reported to attenuate the protective effects of the glycine partial agonists ACPC and HA-966 against glutamate-induced neurotoxicity (14, 19). In the present study, the potential molecular mechanisms responsible for this phenomenon were examined using ACPC as a model glycinergic ligand. ACPC not only blocks glutamate-induced toxicity *in vitro* (14, 15, 19) but also reduces ischemic

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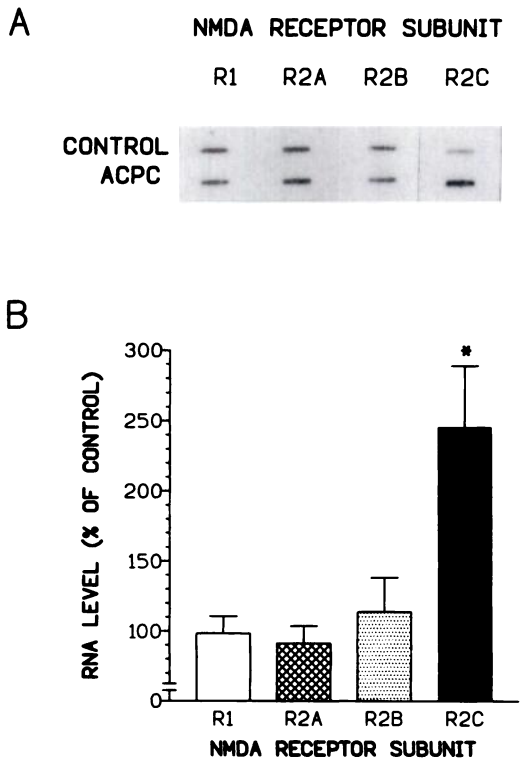


**Fig. 5.** Effects of sustained treatment with ACPC on NMDAR subunit RNA levels. Cells were treated for 24 hr with or without 1 mM ACPC. Total cellular RNA was isolated, and RNA specific for individual NMDAR subunits was quantified by slot-blot hybridization as described in Experimental Procedures. A, Autoradiograms of slot-blot analyses of RNA from control and ACPC-treated cells from a representative study. The autoradiogram for NMDA-R1 was exposed for 1 day at −70°; autoradiograms for NMDAR-2A–2C were exposed for 3 days. B, RNA for each subunit in cells that had been treated with ACPC was expressed as a percentage of that in control cells in each study. Values represent mean ± standard error of four or five experiments. *, $p < 0.005$ vs no 24-hr ACPC treatment.

brain damage in animal models after both acute (15, 17) and chronic (16–18) administration and is currently in Phase I clinical trials (25).

Boje *et al.* (14) suggested that the attenuation of the effects of ACPC and HA-966 after sustained exposure to glycinergic ligands was attributable to a homologous desensitization of the NMDAR as the neuroprotective effects of relatively high concentrations of both a competitive NMDA antagonist (2-amino-5-phosphonopentanoic acid) and a use-dependent channel blocker [(+)-dizocilpine maleate] were not significantly altered by this exposure (14). However, the present study reveals that in addition to an attenuation of the neuroprotective effects of ACPC after sustained exposure (Fig. 2A), there is an apparent 2-fold increase in the neurotoxic potency of glutamate (Fig. 2B) that would not have been apparent using the experimental design used by Boje *et al.* (14). A parallel increase in the apparent potency of NMDA to increase cGMP levels was also observed after sustained ACPC exposure (Fig. 3A). Because this latter measure is a more proximal event in receptor-effector coupling than glutamate-induced neurotoxicity, it provides evidence, albeit indirect, that sustained treatment with ACPC results in altered NMDAR function rather than affecting an event (e.g., direct activation of a proteolytic enzyme) further downstream in the excitotoxic cascade (26). The 9-fold elevation in $[Ca^{+2}]_i$ in response to a submaximal concentration of NMDA (5 μM) (compared with a 3-fold elevation in control neurons) provides perhaps the most compelling evidence that sustained exposure to ACPC alters NMDAR function (Fig. 4).

Although sustained exposure to ACPC produces an apparent increase in the potency of glutamate (Fig. 2) and NMDA (Figs. 3 and 4), the results presented here cannot exclude the possibility that these effects are mediated through a change in glycine recognition sites. Both electrophysiological (27) and neurochemical (28) investigations have demonstrated that glycine can increase the potency of NMDA. Because glycine is present at subsaturating concentrations in the medium (as evidenced by the ability of exogenous glycine to augment the increases in cGMP elicited by submaximally effective concentrations of NMDA; Fig. 1), an increase in the affinity of glycine could account for the apparent increase in potency of glutamate-site ligands. The observation (Fig. 3B) that saturating concentrations of glycine eliminated the differences between control and ACPC-treated cells by increasing the responsiveness of control cells to NMDA is consistent with this explanation. Based on changes in the expression of RNA encoding NMDAR subunits (see below), the effects of sustained exposure to ACPC may best be explained by increases in the potencies of both coagonists.

Increased (2.5-fold) expression of RNA encoding the NMDAR-2C subunit without concomitant changes in RNA levels of the NMDAR-1 and -2A, or -2B subunits was observed after sustained exposure to ACPC. Although it will ultimately be necessary to determine whether this increase in RNA reflects a coordinate increase in expression of the NMDAR-2C subunit protein, an increase in the proportion of NMDA receptors expressing the NMDAR-2C subunit provides an explanation for the increased sensitivity to glutamate and/or glycine as well as the attenuated neuroprotective effects of ACPC. Thus, glycine and glutamate are significantly more potent in activating recombinant NMDA receptors constituted with NMDAR-1 and -2C subunits than when constituted with NMDAR-1, -2A, or -2B subunits (29, 30). For example, Kemp *et al.* (30) reported potencies ~25- and ~6.5-fold higher for glycine and glutamate, respectively, in *Xenopus* oocytes expressing NMDAR-1 and -2C subunits compared with receptors constituted with NMDAR-1 and -2A subunits. Moreover, although the affinity of ACPC has not been examined in heteromeric recombinant systems, there is evidence to suggest that ACPC has a low affinity for wild-type receptors expressing NMDAR2-C compared with other subunits. Thus, based on *in situ* hybridization studies, RNA encoding the NMDAR-2C subunit is localized to the cerebellar granule cell layer in adult brain (29, 31), and ACPC has been reported to have a >20-fold lower affinity to inhibit [$^3$H]glycine binding in cerebellar compared with hippocampal membranes (32). Based on these observations, it is hypothesized that the combination of a reduced affinity for ACPC coupled with increased affinities for glutamate (and NMDA) and glycine could substantially contribute to the attenuated neuroprotection by glycine partial agonists after sustained exposure. Nevertheless, the possibility that sustained exposure of granule cell cultures to ACPC may alter some other, as-yet-unidentified cellular process(es) that may also play a role in the enhanced sensitivity to NMDAR activation cannot be dismissed.

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A series of experiments designed to establish a base-line for the effects of acute exposure to ACPC provided some additional insights into the mechanisms contributing to its attenuated neuroprotection after sustained exposure. Thus, the demonstration that ACPC is neuroprotective against low to moderate concentrations of glutamate (Fig. 2A) but is ineffective against maximal concentrations of glutamate (15) indicates that the increased potency of glutamate observed after sustained exposure to ACPC contributes to the attenuated neuroprotection. Moreover, because concentration-effect curves established that 1 mM is a maximally neuroprotective concentration of ACPC (14), the apparent efficacy of ACPC appears dependent upon glutamate concentration. This was corroborated by studies of NMDA-stimulated cGMP levels, where exogenous glycine potentiated the effects of low concentrations of NMDA (15 μM), whereas ACPC reduced cGMP levels. Under these conditions, the efficacy of ACPC relative to glycine was 0.3 (Fig. 1). At the highest concentrations of NMDA used in this study (50 μM), cGMP levels were neither potentiated by exogenous glycine nor reduced by ACPC (Fig. 1). These findings are consonant with neurochemical studies (12) demonstrating that the efficacy of ACPC to increase [$^3$H](+)-dizocilpine maleate binding in rat forebrain membranes was ~0.6 in the nominal absence of glutamate but increased to ~0.9 in the presence of glutamate. Similarly, electrophysiological studies of *Xenopus* oocytes expressing NMDA receptors from rat brain RNA (performed in the presence of saturating concentrations of glutamate or NMDA) yielded an efficacy of ACPC of 0.8–0.9 (13, 33). Other neurochemical measures with variable amounts of glutamate or NMDA result in efficacies ranging from 0.4 to 0.6 (34, 35). Based on these observations, it is hypothesized that ACPC could mimic the effects of NMDA antagonists under low glutamate conditions but would be ineffective as an NMDA antagonist at high glutamate concentrations. This hypothesis is consistent with the demonstration that ACPC blocks the convulsant and lethal effects of moderate doses of NMDA (10, 36) but was ineffective against higher doses (37). The observation that ACPC can also mimic other pharmacological properties of competitive NMDA antagonists and use dependent channel blockers *in vivo* (15, 38–43) coupled with the ability of glycine to reverse these effects (38, 39) suggests that glutamate and glycine concentrations are within the range that permits ACPC to act as a low-to-moderate efficacy glycine partial agonist under most physiological and pathophysiological conditions.

The results of the present study demonstrate that sustained exposure of cerebellar granule cell neurons to ACPC increases the levels of RNA encoding the NMDAR-2C subunit concomitant with increased sensitivity to glutamatergic ligands (Figs. 2B, 3A, and 4) and attenuated neuroprotective effects of glycine-site partial agonists/antagonists (Fig. 2A [14, 19]). The very high affinities of glutamate and glycine in recombinant systems expressing the NMDAR-2C subunit (29, 30) offers a plausible explanation for both functional effects. However, these findings indicate that primary cultures of cerebellar granule cell neurons do not adequately model the *in vivo* situation as chronic treatment with ACPC is neuroprotective in both global and focal ischemias (16–18). In cultured cerebellar granule cells, the enhancement of NMDAR-mediated neurotoxicity by sustained exposure (24–48 hr) to ACPC is in dramatic contrast to the neuroprotective effect of ACPC applied concurrently with glutamate-site agonists. This is not to say that longer exposure (e.g., 2 weeks) might not produce a different effect on NMDAR function. However, it seems more likely that chronic administration of ACPC *in vivo* may result in compensatory responses that occur in specific brain areas. The expression of these alterations (e.g., in NMDA receptors and/or other functions) requires an interaction among cell types and synaptic communications with a more complex organization than is present in primary cerebellar granule cell cultures. Consequently, these findings in cerebellar granule cell cultures offer a "proof of concept" that sustained exposure to ACPC can effect region-specific changes in the expression of specific NMDAR subunits, which may not be confined to or may not even occur in cerebellar granule cells *in vivo* but may result in decreased susceptibility to ischemia. Future experiments will be necessary to test other glycinergic ligands for the generality of this effect; however, because ACPC is currently in clinical trials (25), such findings could have important therapeutic implications for the treatment of neurodegenerative disorders linked to excessive activation of glutamate receptors.

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