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Chronic Exposure of Cerebellar Granule Cells to Ethanol Results in Increased *N*-Methyl-D-aspartate Receptor Function

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SUMMARY

In primary cultures of cerebellar granule cells, activation of the *N*-methyl-D-aspartate (NMDA) receptor leads to Ca^{2+} influx. Previous work showed that this response is selectively inhibited by acute exposure to low concentrations of ethanol. The present results demonstrate that the response to NMDA (measured as an increase in intracellular Ca^{2+} concentration, using fura-2 fluorescence) is significantly enhanced after chronic *in vitro* exposure of the cells to ethanol (100 mM for 2–4 days; 20 mM for 3 or more days). This enhancement is consistent with an increased number of NMDA receptors, with no change in receptor properties. Specifically, there was no change in the EC_{50} values for NMDA and glycine or in the magnitude of inhibition of the NMDA response by competitive or uncompetitive antagonists. There was also no change in the ability of acute ethanol to inhibit the NMDA response after chronic exposure of the cells to ethanol. Furthermore, chronic ethanol exposure did not alter depolarization-dependent increases in intracellular Ca^{2+} observed after exposure of the cells to 30 mM KCl. The data suggest that chronic ethanol exposure produces a selective up-regulation of NMDA receptor function. In the intact animal, such a change may be associated with particular symptoms of ethanol withdrawal, i.e., withdrawal seizures.

Glutamate is believed to be the major excitatory neurotransmitter in the central nervous system, and at least four different glutamate receptor subtypes have been characterized, according to the specificity of their interaction with various ligands (1). One such receptor subtype is the NMDA receptor, which is linked to a cation channel. When the receptor is activated by glutamate or NMDA, the channel opens, resulting in increased permeability to Na^+, K^+, and Ca^{2+}. The function of the NMDA receptor-channel complex can be modulated by a number of agents, including Mg^{2+}, which causes a voltage-dependent block of the channel, and glycine, which binds to a strychnine-insensitive site and acts "allosterically" to enhance NMDA-stimulated channel activation (2–4). The NMDA receptor has been implicated in central nervous system plasticity, as well as in neurotoxicity and epileptiform seizure generation (1).

Studies in our laboratory and those of others have demonstrated that ethanol, when applied acutely at pharmacologically relevant concentrations, inhibits the function of the NMDA receptor. This inhibition has been observed in studies of electrophysiological responses to NMDA in hippocampal cells (5–7), NMDA-induced cyclic GMP production and increases in $[Ca^{2+}]_i$ in cerebellar granule cells (8) and dissociated whole-brain cells (9), and NMDA-stimulated neurotransmitter release in brain slices and synaptosomal preparations (10–12).

These findings raise the possibility that chronic ethanol ingestion could induce an adaptive up-regulation of the NMDA receptor-channel complex in brain, as a response to the initial ethanol-induced inhibition of NMDA receptor function. When mice ingested ethanol chronically, such that they became tolerant to and physically dependent on ethanol (13), an increase in NMDA receptor-channel complexes, as measured by the binding of the uncompetitive NMDA antagonist MK-801, was reported (14). A change in ligand binding, however, does not necessarily reflect a change in the function of the receptor. To assess this function, in the present study we have examined NMDA-stimulated increases in $[Ca^{2+}]_i$ in primary cultures of cerebellar granule cells that were exposed chronically to ethanol *in vitro*.

Materials and Methods

Glycine, NMDA, MK-801, and AP5 were purchased from Research Products Inc. (Natick, MA). Fura-2/acetoxy methyl ester and fura-2 were obtained from Molecular Probes (Eugene, OR). All other products

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohept-5,10-imine hydrogen maleate; AP5, D-2-amino-5-phosphopentanoic acid; VSCC, voltage-sensitive calcium channels; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; ANOVA, analysis of variance; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

were the purest grade available, and unless otherwise stated were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture. Primary cultures of cerebellar granule cells were prepared by a previously described method (8). Briefly, cerebelli were dissected from 8-day-old Sprague-Dawley rats. Cells were dissociated by trypsinization and resuspended in basal Eagle's medium containing 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine, and 100 μg/ml gentamicin (GIBCO, Grand Island, NY). Cell cultures were seeded at 1.6–2 × 10^6 cells/dish, on 1-mm-thick glass coverslips coated with poly-L-lysine, and were maintained at 37° in 10% CO_2. After 18 hr in culture, cytosine arabinofuranoside (10 μM) was added to inhibit replication of nonneuronal cells. Within each preparation, control cells were grown as described above, whereas others were cultured in the presence of ethanol. In the latter case, after 4 days in culture, 100% ethanol (11.7 μl) was added to the culture dish containing the coverslip (volume, 2 ml), to produce a concentration of 100 mM ethanol. The medium was then supplemented daily with 5.9 μl of 100% ethanol, to maintain the proper concentration, as measured by gas chromatography (15). In some experiments, cells were grown in 20 mM ethanol, in which case the medium was also supplemented daily with ethanol (1.2 μl). In both instances, dishes containing the ethanol-exposed cells were maintained within larger dishes containing the appropriate concentration of ethanol.

After a total of 6–8 days in culture (2–4 days in the presence of ethanol), $[Ca^{2+}]_i$ was measured in the cerebellar neurons. Preliminary studies and work in other laboratories (16) showed no significant change in the response of the cerebellar granule cells to NMDA over this time period. Before addition of NMDA, cells were exposed to KCl (30 mM) to remove the Mg^{2+} block (17), and all subsequent exposures to NMDA were carried out in Mg^{2+}-free buffer. In addition, the effect of NMDA was studied in the presence of 10 μM glycine, unless otherwise stated. All cells examined responded to 30 mM KCl or to NMDA plus glycine with an increase in $[Ca^{2+}]_i$.

$[Ca^{2+}]_i$ measurement. Fura-2-/acetoxy methyl ester (10 μM) was added to the culture medium, and cells were incubated for 30 min at 25°, followed by 30 min at 37° to allow fura deesterification. Cells were then washed three times with buffer (10 mM HEPES, 5 mM KCl, 145 mM NaCl, 1 mM $CaCl_2$, 10 mM glucose, 1 mM Na_2HPO_4, pH 7.4) containing 1 mM $MgCl_2$, over a period of 2–3 min. This washing procedure effectively removed ethanol from the cells. Coverslips with adherent cells were then immediately mounted in a Dvorak-Stottler perfusion chamber containing a slotted ring. Chamber volume was 0.1 ml, and solutions were warmed to 37° and perfused at 25 ml/hr. Control and ethanol-exposed cells were tested in each experiment and were perfused with the same solutions of agonists or other compounds, so that comparisons could be made between groups of cells that were exposed to identical conditions during $[Ca^{2+}]_i$ measurements. Cells were viewed from the lower surface of the cell body, using a Nikon TMD microscope with a Fluor 40 oil immersion lens. Emitted fluorescence (emission centered at 505 ± 25 nm) was recorded by an attached photomultiplier. The source of excitation light was a xenon lamp filtered through dual monochrometers (excitation at 345 and 379 nm) (Deltascan system). Data storage was coordinated using an NEC 286 computer with microprocessor and software from Photon Technology Inc. (Monmouth, NJ).

Some experiments were performed using an Attofluor image analysis system (Atto Instruments Inc., Potomac, MD). In this case, excitation light was produced by a mercury lamp filtered through high transmittance band passes centered at 344 or 390 nm, which were moved into the source path of the computer. The time delay between the filters was <0.5 sec. At 10-sec intervals, the emitted fluorescent light was passed through a 420-nm long pass filter and collected by a CCD camera. The digitized image was 10-frame averaged by a 386 computer (Atto Instruments). The average $[Ca^{2+}]_i$ within a 10- × 10-pixel square (11 × 11 μm) that was manually chosen in the soma of a cell was computed off-line for up to 25 cells within a field.

Intracellular calcium concentration was determined according to the method of Grynkiewicz *et al.* (18): $[Ca^{2+}]_i = K(R - R_{min})/(R_{max} - R) \times Sf_2/Sb_2$, where R is the 345/379 wavelength ratio of the sample, R_{min} is the ratio obtained with fura-2 in the absence of Ca^{2+}, R_{max} is the ratio when the dye is saturated with Ca^{2+}, Sf_2/Sb_2 is the ratio of fluorescence values at 379 nm for free and Ca^{2+}-bound dye, and K is the affinity of fura-2 for Ca^{2+} (224 nM) (18). Buffer (19) with 1 μM fura-2 free acid was used for calibration; 2 mM Ca^{2+} was used to determine R_{max} and 2 mM EGTA was used to determine R_{min}. All fluorescence signals in both experimental conditions were corrected for autofluorescence of the neurons.

After the loading of fura-2, spectrofluorometric excitation scans of single cell somata demonstrated that intracellular dye characteristics were similar to those of free fura-2 standards, indicating that the dye was completely deesterified. These scans were identical in control and ethanol-treated cells. When cells loaded with fura-2 were excited by light at 379 nm, the dye was observed to be distributed uniformly throughout the cell and appeared to be free of particulates and clumping.

Cell viability. Cells on the coverslip were perfused with a solution of propidium iodide (3 μg/ml) for 60 sec and then washed with warmed buffer. Nuclei of dead cells were labeled with propidium iodide and were visible at 485-nm excitation, with fluorescence filtered through a 50-nm band pass filter centered at 580 nm. Viability was determined as a percentage of the total number of cells in a field (counted manually).

Statistical analysis. All values are presented as mean ± standard error. The intracellular calcium response is presented as the change in $[Ca^{2+}]_i$ over basal levels (i.e., all values have had basal levels subtracted). Statistical significance was determined by ANOVA and the Tukey-Kramer test, using Systat, or by the unpaired Student's t test, using StatWorks, version 1.2. Differences were considered to be significant at $p \leq 0.05$. EC_{50} values were computed from log-probit analysis of dose-response curves, using the Litchfield and Wilcoxon II Pharmacological Calculation System, version 4.1, and were considered to be significantly different if 95% confidence intervals of the regression lines did not overlap (20).

Results

As previously reported (8), neurons comprised >95% of the population of a typical cerebellar granule cell preparation, as determined by microscopic examination. In control cells, viability was consistently high (94 ± 5%; 10 preparations), and viability did not change significantly when cultures were treated with 100 mM ethanol for 1 day or for up to 5 days (pooled value for 1–5 days, 87 ± 4%; 10 preparations). The basal concentration of Ca^{2+} in the soma of control cells was calculated to be 148 ± 20 nM (28 cells). This value is similar to that reported in other work (21) and was not significantly altered in cells exposed chronically to 100 mM ethanol for 1–5 days (119 ± 26 nM; 14 cells).

NMDA, in the presence of glycine, produced an increase in $[Ca^{2+}]_i$ that was dependent on the presence of extracellular Ca^{2+} (i.e., NMDA increased Ca^{2+} influx into the cells) (Fig. 1). Cells were able to respond repeatedly to NMDA with an increase in $[Ca^{2+}]_i$ when they were washed with Mg^{2+}-free buffer for 3 min or more between agonist exposures.

As shown in Fig. 2, NMDA produced a concentration-dependent increase in $[Ca^{2+}]_i$. The EC_{50} value for NMDA in control cells was 10.6 μM. Chronic exposure of cells to ethanol produced an enhanced response to all concentrations of NMDA greater than 5 μM (Fig. 2). However, the EC_{50} for NMDA was not significantly altered in the ethanol-exposed cells (13.0 μM). The data shown in Fig. 2 represent pooled values from cells exposed to 100 mM ethanol for 2–4 days, because the magnitude

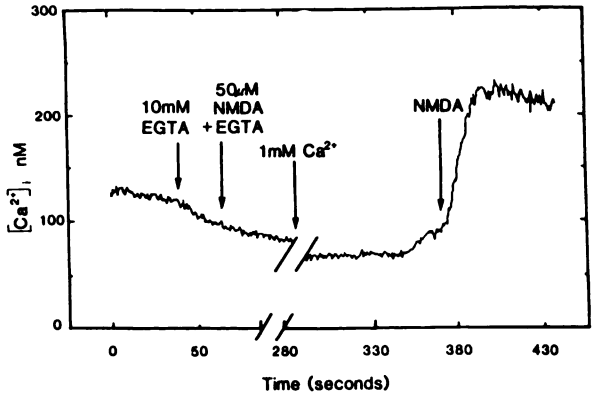

Fig. 1. Role of extracellular Ca^{2+} in the NMDA-stimulated increase in $[Ca^{2+}]_i$ in cerebellar granule cells. Primary cultures of rat cerebellar granule cells were prepared and loaded with fura-2 as described in the text. Data represent $\Delta[Ca^{2+}]_i$ in a single cell, which was initially perfused with Mg^{2+}-free buffer containing 10 mM EGTA and 1 mM Ca^{2+} for 20 sec, and then exposed to 50 μM NMDA plus 10 μM glycine in the presence of EGTA. The cell was then perfused for 180 sec with Mg^{2+}-free buffer containing 1 mM $CaCl_2$, followed by NMDA and glycine in the presence of Ca^{2+}. All perfusion solutions were kept at 37°. Fluorescence was monitored with a dual-monochrometer-photomultiplier system, as described in the text. Corrections were made for autofluorescence, and $[Ca^{2+}]_i$ was determined by the method of Grynkiewicz *et al.* (18).

of the change in response to NMDA did not vary significantly over this time period [$\Delta[Ca^{2+}]_i$ in response to 50 μM NMDA (in nM): determined on the Deltascan system, control, 185 ± 14 (24 cells); 2-day ethanol exposure, 313 ± 65 (seven cells); 4-day ethanol exposure, 404 ± 38 (eight cells); determined on the Attofluor system, control, 72 ± 9 (49 cells); 2-day ethanol exposure, 337 ± 57 (19 cells); 4-day ethanol exposure, 229 ± 56 (18 cells); for both sets of data, control values are significantly different from values after 2 or 4 days of ethanol exposure (ANOVA, $p < 0.05$); values after 2 days of ethanol exposure are not significantly different from those after 4 days of exposure]. Similarly, in all other experiments described below, data obtained from these durations of ethanol exposure (2–4 days for 100 mM ethanol) were pooled.

When cells were chronically exposed to 20 mM ethanol for 3–4 days, the NMDA (100 μM)-stimulated increase in $[Ca^{2+}]_i$ was also found to be significantly elevated above the control response [$\Delta[Ca^{2+}]_i$ (in nM): determined on the Deltascan system, control, 381 ± 60 (13 cells); 3–4-day ethanol exposure, 698 ± 89 (six cells); $p < 0.02$, Student's t test].

Glycine enchanced the NMDA-elicited Ca^{2+} influx in a concentration-dependent manner. Notably, in the absence of added glycine, NMDA produced only a barely detectable increase in $[Ca^{2+}]_i$ (Fig. 3). The glycine EC_{50} was not significantly changed in the ethanol-exposed cells (control, $EC_{50} = 0.18$ μM; ethanol-exposed, $EC_{50} = 0.19$ μM). However, similar to the NMDA dose-response curve, the response to various concentrations of glycine, in the presence of 50 μM NMDA, was increased (Fig. 3).

In control cells, the response induced by NMDA was inhibited by AP5 (a competitive NMDA antagonist) (1) but was not significantly reduced by nifedipine, a dihydropyridine voltage-sensitive Ca^{2+} channel antagonist (21) (Fig. 4A). These data indicate that NMDA, under our conditions, does not promote Ca^{2+} flux through L-type VSCC. Inhibition of the NMDA response by AP5 was not altered in cells exposed chronically to 100 mM ethanol (Fig. 4A). Furthermore, as in the control cells, nifedipine had no significant effect on the NMDA response in the chronically ethanol-treated cells (Fig. 4A). We also examined the effect of an uncompetitive NMDA antagonist (MK-801) in control and ethanol-exposed cells and found that chronic ethanol exposure did not alter the percentage of inhibition produced by any concentration of MK-801 or the IC_{50} for MK-801 (20.7 nM for control cells and 21.6 nM for ethanol-exposed cells) (Fig. 4B). Mg^{2+} also attenuated the response to NMDA in a dose-dependent manner, as expected (2) (Fig. 4C). Inhibition was slightly greater in the ethanol-exposed cells than in control cells, but this difference only reached statistical significance at 1 mM Mg^{2+}.

Ethanol, when applied acutely, was a potent inhibitor of the response to NMDA in control cells (Fig. 5). These results are similar to our previous work, in which NMDA-stimulated $^{45}Ca^{2+}$ flux in cerebellar granule cells was measured (8). Chronic treatment of the cells with ethanol did not alter the ability of ethanol, acutely, to inhibit the NMDA-stimulated response. As shown in Fig. 5, the percentage of inhibition produced by

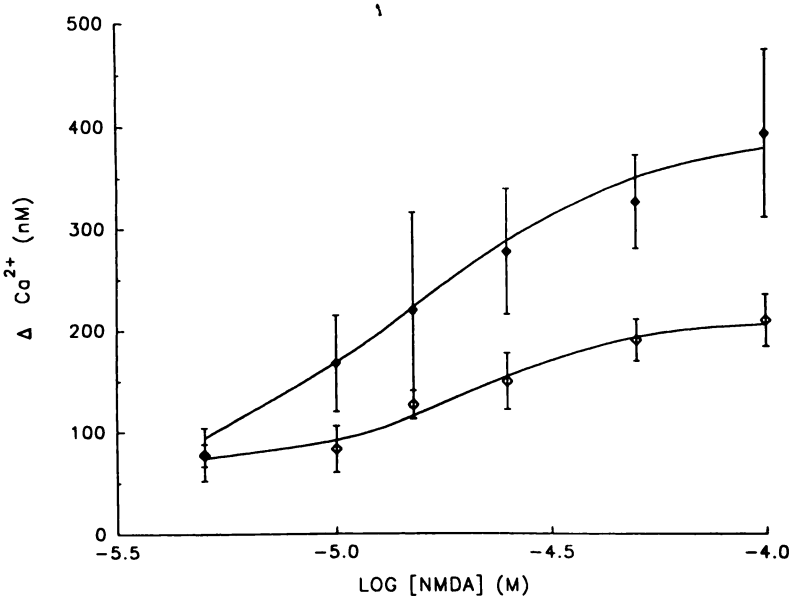

Fig. 2. Effect of chronic ethanol exposure on NMDA-stimulated increases in $[Ca^{2+}]_i$ in cerebellar granule cells. Cells were perfused with the indicated concentration of NMDA plus 10 μM glycine, after being cultured in the absence (◇) or presence (◆) of 100 mM ethanol for 2–4 days (data for 2–4 days of ethanol exposure have been pooled). Values are the mean ± standard error from three to seven determinations in at least three separate experiments. ANOVA revealed a significant effect of NMDA concentration ($F = 10.8$, $p < 0.000$) and ethanol treatment ($F = 12.5$, $p < 0.001$), with a nonsignificant concentration × treatment interaction ($F = 0.80$, $p < 0.55$).

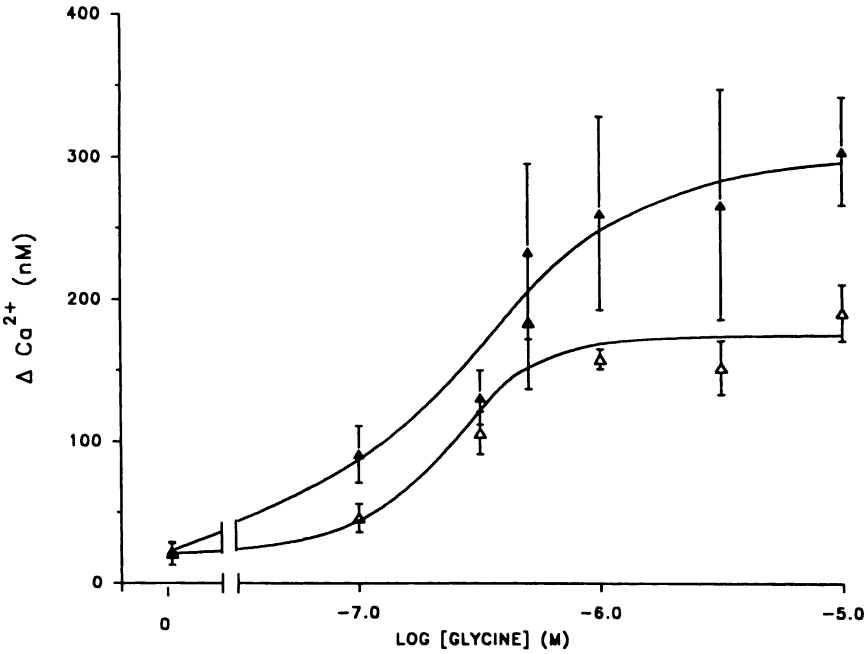

Fig. 3. Effect of glycine on NMDA-stimulated increases in $[Ca^{2+}]_i$ in control and ethanol-exposed cerebellar granule cells. Cells were perfused with NMDA (50 μM) and the indicated concentrations of glycine, after being cultured in the absence (Δ) or presence (▲) of 100 mM ethanol for 2–4 days. Values represent pooled data for 2–4 days of ethanol exposure and are the mean ± standard error of five to 10 determinations in at least three separate preparations. ANOVA revealed a significant effect of glycine concentration ($F = 9.6$, $p < 0.000$) and ethanol treatment ($F = 10.5$, $p < 0.002$), with a nonsignificant concentration × treatment interaction ($F = 0.8$, $p = 0.56$).

various concentrations of ethanol was the same in control and ethanol-exposed cells.

The cerebellar granule cells responded to depolarization by 30 mM KCl with an increase in $[Ca^{2+}]_i$, presumably via activation of VSCC (22). The response to KCl was attenuated by the L channel blocker nifedipine and was not affected by AP5 (Fig. 6). In contrast to NMDA-stimulated Ca^{2+} influx, the response to 30 mM KCl was not affected by the acute application of ethanol [$\Delta[Ca^{2+}]_i$: control, 346 ± 46 nM (16 cells); plus 100 mM ethanol, 343 ± 45 nM (16 cells)]. Furthermore, the magnitude of the KCl-induced increase in $[Ca^{2+}]_i$ and the sensitivity to nifedipine or AP5 (Fig. 6), as well as the rate of increase in $[Ca^{2+}]_i$ after stimulation with 30 mM KCl (data not shown), were not affected by chronic incubation of the cells with ethanol.

Discussion

The results of this study demonstrate that 2 or more days of exposure of cultured cerebellar granule cells to 100 mM ethanol results in significant enhancement of the functional response to NMDA (i.e., an increase in $[Ca^{2+}]_i$). Rats exposed chronically to ethanol *in vivo* can achieve blood ethanol levels in the range of 100 mM (23). Three or more days of exposure of cells to 20 mM ethanol, a concentration easily reached *in vivo* even in humans (24), also resulted in a significantly increased response to 100 μM NMDA. This observation indicates that the threshold concentration of ethanol necessary to produce a chronic effect on NMDA receptor function is quite low. It was previously reported, in studies of opiate receptor number in NG 108–15 cells (25), that low (25–50 mM) concentrations of ethanol produced a similar change as a higher (200 mM) concentration, but only after a longer period of exposure. The same phenomenon may occur in the system investigated here, but a more detailed time-course study is necessary.

The characteristics of the NMDA receptor complex do not appear to be altered by chronic ethanol exposure, because the EC_{50} for NMDA was unchanged and there was no significant difference between control and ethanol-treated cells in the sensitivity to inhibition of the NMDA response by MK-801 or AP5. Although there appeared to be a slight increase in sensitivity of the response to Mg^{2+} in the ethanol-treated cells, the physiological significance of this change is questionable, because it was observed at only one of the concentrations of Mg^{2+} tested. However, it is of interest that the sensitivity of NMDA responses to Mg^{2+} was reported to be increased, albeit to a much greater extent than observed here, in the hippocampus of adult rats whose mothers ingested ethanol chronically during gestation (26).

The increased response to NMDA in the ethanol-exposed cells may represent a functional correlate of the increase in MK-801 binding and, in preliminary studies, in NMDA-specific glutamate binding (27) that was previously observed in various brain areas of mice that had ingested ethanol chronically (14, 28). Thus, the increased MK-801 binding in the brain and the increased response to NMDA in cultured cells may result from an increase in the total number of NMDA receptor-channel complexes.

Glycine, binding to the strychnine-insensitive site on the NMDA receptor-channel complex, has been postulated to be a coagonist with NMDA for activation of the ion channel (4). Our data support this hypothesis, because, in our rapidly perfused system, little or no response to NMDA was observed unless glycine was added to the perfusing buffer. The acute inhibitory effect of ethanol on NMDA-stimulated responses can, in some cases, be antagonized by high concentrations of glycine, suggesting a specific site of action of ethanol at the NMDA receptor-channel complex (9, 11, 29, 30). Therefore, it is possible that adaptation to chronic ethanol exposure might involve a change at this site. However, in the chronically ethanol-treated cells, there was no change in the glycine EC_{50}, consistent with a lack of change in the interaction of glycine with its binding site. The increased maximum response to glycine (in the presence of NMDA) is most easily explained as a result of an increase in the number of NMDA receptor complexes.

Although these results are consistent with our previous data

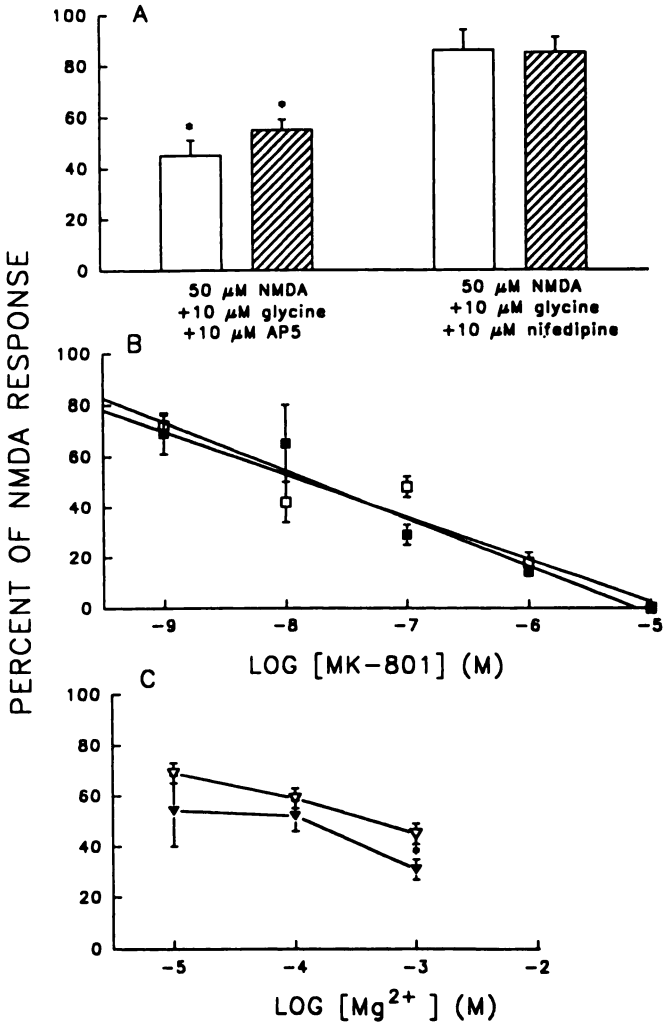

Fig. 4. A, Effect of the NMDA antagonist AP5 and the dihydropyridine Ca^{2+} channel antagonist nifedipine on NMDA-stimulated increases in $[Ca^{2+}]_i$ in control and ethanol-exposed cerebellar granule cells. Cultures were prepared and fura-2 fluorescence was measured as described in the text. Cells were cultured in the absence (□) or presence (▨) of 100 mM ethanol (data from 2–4 days of ethanol exposure are pooled), and responses to NMDA (50 μM) plus glycine (10 μM) were determined. Values represent the mean ± standard error from three to five determinations in at least three separate preparations and are plotted as percentage of the response to NMDA plus glycine alone: $\Delta[Ca^{2+}]_i$ (in nM): control, 119 ± 26 (six cells); ethanol-treated, 245 ± 58 (eight cells). *, $p < 0.05$, compared with response in appropriately treated cells in the absence of AP5 (Student's *t* test). B, Inhibition by MK-801 of the NMDA-stimulated increase in $[Ca^{2+}]_i$ in control (□) and ethanol-exposed (■) cerebellar granule cells. Cell cultures were prepared and fluorescence was measured as described in the text. Data obtained with cells exposed to ethanol for 2–4 days are pooled. The results are plotted as percentage of the response to 50 μM NMDA plus 10 μM glycine alone: $\Delta[Ca^{2+}]_i$ (in nM): control, 71 ± 12 (nine cells); ethanol-treated, 156 ± 31 (14 cells). Values represent mean ± standard error from four to 11 determinations in three separate preparations. C, Mg^{2+} inhibition of NMDA-stimulated increases in $[Ca^{2+}]_i$ in cerebellar granule cells cultured in the absence (▽) or presence (▼) of 100 mM ethanol. Data from 2–4 days of ethanol exposure are pooled. Results are plotted as percentage of the response to 50 μM NMDA plus 10 μM glycine, in the absence of Mg^{2+}: $\Delta[Ca^{2+}]_i$ (in nM): control, 52 ± 4 (36 cells); ethanol-treated, 72 ± 6 (38 cells). Values represent mean ± standard error from four to 30 determinations in three separate preparations. *, $p < 0.05$, compared with cells cultured in the presence of ethanol (Student's *t* test).

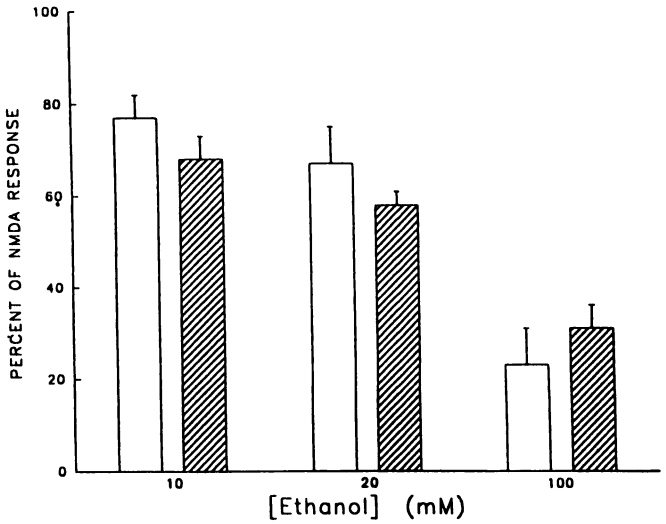

Fig. 5. Acute inhibition by ethanol of NMDA-induced increases in $[Ca^{2+}]_i$ in control (□) and ethanol-exposed (▨) cerebellar granule cells. Cerebellar granule cells were cultured and $[Ca^{2+}]_i$ was quantitated as described in the text. Cells were treated chronically with 100 mM ethanol for 2–4 days, washed, and then perfused with buffer containing 50 μM NMDA plus 10 μM glycine, in the absence or presence of the indicated concentrations of ethanol. Data from 2–4 days of ethanol exposure are pooled, and values represent mean ± standard error from three to 30 determinations in three separate preparations. Results are plotted as percentage of the response to NMDA plus glycine alone: $\Delta[Ca^{2+}]_i$ (in nM): control, 63 ± 4 (38 cells); ethanol-treated, 176 ± 36 (40 cells).

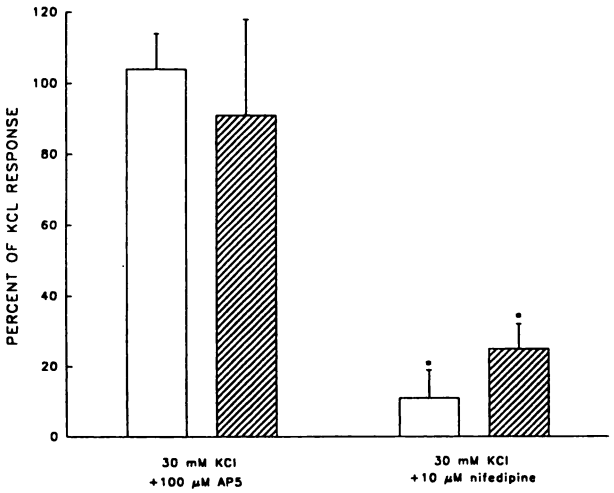

Fig. 6. KCl-stimulated increase in $[Ca^{2+}]_i$ in control (□) and ethanol-exposed (▨) cerebellar granule cells. Cells were cultured and $[Ca^{2+}]_i$ was determined as described in the text; data from cells exposed to 100 mM ethanol for 2–4 days are pooled. Values represent mean ± standard error from three to five determinations in at least five separate preparations and are plotted as percentage of response to KCl alone: $\Delta[Ca^{2+}]_i$ (in nM): control, 201 ± 30 (28 cells); ethanol-treated, 209 ± 36 (14 cells). *, $p < 0.05$, compared with response in appropriately treated cells in the absence of nifedipine (Student's *t* test).

showing an increase in MK-801 binding sites in brains of ethanol-fed mice (14, 28), there is an apparent discrepancy between the present results and those obtained in a recent study of NMDA receptor function. In the latter work, no change was found in NMDA-stimulated release of neurotransmitters from brain slice preparations of rats fed ethanol in a liquid diet for 3 weeks (17). However, it was not determined whether the animals were tolerant to or physically dependent on ethanol,

and no ligand binding studies were performed, making it difficult to compare the results of these studies with the previous results in mice (14, 28). A further obstacle to comparison among studies is the fact that the release of catecholamines may be several steps removed from the more direct effect of NMDA on $[Ca^{2+}]_i$ (i.e., motility elements and exocytosis are involved) (10–12).

Ethanol, when applied acutely, produced a concentration-dependent inhibition of the NMDA-stimulated increase in $[Ca^{2+}]_i$, which, on a percentage basis, was not altered after chronic exposure of the cells to ethanol. This finding is in agreement with the results of Brown *et al.* (17), who found that ethanol inhibited NMDA-stimulated neurotransmitter efflux equally in brain slices from control rats and those treated chronically with ethanol, and with a preliminary report of an electrophysiological study of rat hippocampal slices (31). However, in the present study, the "base-line" response to NMDA was increased in the chronically ethanol-treated cells, apparently as a result of an increased number of receptors. The fact that there was no change in percentage of inhibition produced acutely by ethanol suggests that "tolerance" to ethanol did not occur at the level of individual receptors. However, because more receptors were present, overall NMDA-stimulated $[Ca^{2+}]_i$ remained higher in the chronically ethanol-treated cells than in the control cells after acute exposure to ethanol. This difference could be interpreted as an attenuation of the inhibitory effect of acute ethanol in the chronically ethanol-exposed cells, i.e., as a type of tolerance in the whole system. One may speculate, for example, that in cells treated chronically with ethanol the NMDA-stimulated $[Ca^{2+}]_i$ in the presence of acute ethanol might be comparable to the NMDA-stimulated $[Ca^{2+}]_i$ in control cells in the absence of acute ethanol.

On the other hand, an increased response to NMDA seems to be more clearly implicated in ethanol dependence and/or the seizures precipitated by ethanol withdrawal in the intact animal. The role of the NMDA receptor, and NMDA-induced Ca^{2+} influx, in the generation of seizure activity is well characterized (1). Furthermore, the severity of handling-induced or audiogenic ethanol-withdrawal seizures in mice and rats, respectively, was attenuated by administration of MK-801 (14, 32), and handling-induced seizures were exacerbated by the administration of NMDA (14). More recent studies have shown that administration of competitive NMDA receptor antagonists also reduces the severity of ethanol-withdrawal seizures in mice (33). Although the NMDA receptors in the cerebellar granule cells used in this study are most likely not involved in ethanol-withdrawal seizures in adult animals, these receptors may well serve as a model for NMDA receptors in brain areas (e.g., hippocampus) that are believed to contribute to ethanol-withdrawal and other seizures.

The increase in response to NMDA observed after chronic ethanol exposure could reflect an adaptive response to the initial ethanol-induced inhibition of receptor function. However, it is not clear that prolonged inhibition of NMDA receptor function invariably leads to such an adaptive increase. Although chronic infusion of rats with phencyclidine, which acts as an antagonist at the same site as MK-801 (1), appeared to produce an increase in NMDA receptor-coupled channels in brain (34), chronic *in vivo* MK-801 administration had less consistent effects on ligand binding and produced a decrease in the electrophysiological response to NMDA (35, 36). Therefore, further studies will be necessary to determine whether the change in NMDA receptor function observed in the present work is a neuroadaptive response to ethanol.

A similar situation applies to VSCC. Ethanol can inhibit Ca^{2+} uptake through dihydropyridine-sensitive VSCC (L channels) (37, 38), and there is an increase in dihydropyridine binding in brain (39) and cultured cells (38), as well as an increase in depolarization-dependent $^{45}Ca^{2+}$ flux (40), after chronic ethanol treatment. However, chronic exposure of cells or animals to specific L channel antagonists did not result in an "up-regulation" of VSCC (41, 42). These data suggest that the change in VSCC after chronic ethanol exposure is not a simple adaptation to inhibition of VSCC function. In the cerebellar granule cells, we observed neither acute inhibition by ethanol of the response to 30 mM KCl nor an increase in the depolarization-dependent rise in $[Ca^{2+}]_i$ after chronic ethanol exposure. These data support the hypothesis that the response of VSCC to ethanol may depend on complex intracellular mechanisms (43) and, therefore, may vary in different cell types.

Overall, our results suggest that chronic ethanol exposure can produce a selective up-regulation of the function of the NMDA receptor-channel complex in cerebellar granule cells. Whether this change occurs via increased synthesis of some or all receptor proteins or via another mechanism (such as post-translational modification) (43) needs further investigation. The increased NMDA receptor function seen in the cells may be analogous to the increased glutamate and MK-801 binding observed in chronically ethanol-fed animals (14, 27, 28) and, as such, it may be a contributing factor, along with changes in other systems such as the γ-aminobutyric $acid_A$ receptor-channel complex (44, 45) and VSCC (46), to the occurrence of physical dependence on ethanol and/or ethanol-withdrawal seizures.

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