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Short communication

N-Methyl-D-aspartate-induced enhancement of activator protein-1 DNA binding activity is blocked by ethanol in cerebellar granule cells

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Abstract

The effects of ethanol on *N*-methyl-D-aspartate (NMDA) and non-NMDA receptor agonist-stimulated activator protein-1 (AP-1) DNA binding activity in primary cultures of rat cerebellar granule cells were investigated. The application of intoxicating concentrations of ethanol produced a concentration-dependent inhibition of NMDA-enhanced AP-1 binding with a significant reduction obtained at 50 mM ethanol. The inhibitory actions of ethanol on α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-stimulated AP-1 binding were considerably weaker as compared to the effects seen following administration of NMDA. The AMPA-induced enhancement of AP-1 DNA binding activity was demonstrated both in the absence and presence of cyclothiazide, a drug, which is known to block the desensitization of AMPA receptors. Our data suggest that moderate concentrations of ethanol modulate glutamate-induced alterations of gene expression in brain neurons.

Keywords: Ethanol; NMDA (*N*-methyl-D-aspartate); AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole) propionic acid); AP-1 (activator protein-1) DNA binding; Transcription factor; Granule cell, cerebellar

1. Introduction

It is largely believed that glutamate, the major excitatory neurotransmitter in the brain, plays an important role in the initiation and modulation of neuroadaptive processes in the central nervous system associated with the establishment of long-term changes involved in e.g. learning and memory, alterations in central nervous system excitability and neurodegeneration. Convincing evidence suggests that glutamate produces its effects via an activation of either ionotropic *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainic acid or G-protein-coupled, metabotropic, glutamate receptors. A transient stimulation of NMDA receptors results in a dramatic increase in the production of mRNA of multiple immediate early genes, including c-fos, which is accompanied by a long-lasting activation of AP-1 DNA binding activity in brain neurons (for references, see Morgan and Curran, 1991; Pennypacker et al., 1995) and this action may be related to the long-term changes.

The development of ethanol tolerance and dependence is generally thought to involve a series of long-lasting neuroadaptive modifications of central nervous system functions. However, the molecular mechanisms by which initial biochemical changes induced by acute ethanol exposure is translated into long-term, more persistent changes associated with ethanol tolerance and dependence are currently not known. It has been suggested that more long-lasting central nervous system alterations induced by frequent and/or chronic use of dependence-producing drugs may be due to post-translational modifications of existing proteins and/or alterations in gene expression (e.g. Nestler et al., 1993). Accumulated evidence indicates that brain glutamate receptors may play an important role in the mediation of the neurochemical effects of ethanol. For example, it has been found that intoxicating concentrations of ethanol inhibit Ca^{2+} responses and neurotoxicity induced by NMDA in several types of brain neurons. However, increased NMDA receptor activity has been observed following chronic exposure to ethanol both in vitro and in vivo (for references, see Hoffman, 1995). Furthermore, the development of ethanol tolerance and the expression of ethanol dependence is attenuated following administration of specific NMDA receptor antagonists (for references, see Hoffman, 1995; Karcz-Kubicha and Liljequist, 1995).

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Since glutamate markedly enhances Ca^{2+} responses, production of immediate early genes and AP-1 DNA binding in cerebellar granule cells (e.g. Szekely et al., 1987; Ohtani et al., 1995), and since ethanol blocks NMDA-produced Ca^{2+} fluxes in brain neurons, including cerebellar granule cells (Dildy and Leslie, 1989; Hoffman et al., 1989; Lovinger et al., 1989), we considered it of interest to examine whether glutamate-induced activation of AP-1 DNA binding activity in primary cultures of cerebellar granule cells could be blocked by intoxicating concentrations of ethanol, and if so, this would provide new information regarding which mechanisms might be involved in the altered gene expression initiated by acute and/or chronic exposure to dependence-producing drugs.

2. Materials and methods

All experiments were approved by the Ethical Committee for the use of Animal Subjects at the Karolinska Institute in Stockholm in compliance with the current Swedish guidelines for care and use of experimental animals. Primary cultures of cerebellar granule cells were prepared from 8-day-old Sprague-Dawley rats (B&K Universal, Sollentuna, Sweden) as previously described (Levi et al., 1984; Cebers and Liljequist, 1995). Cells were used on the 8th day in vitro. The effects of the glutamate receptor agonists and/or ethanol on AP-1 DNA binding activity were examined by adding relevant agent(s) into the cell culture media after which the cells were incubated for 2 h. Glutamate receptor antagonists, when used, were added 5 min prior to the application of agonists. Cell extracts were prepared using a slight modification of a previously described protocol (Bakalkin et al., 1993). Briefly, cells were washed with cold phosphate-buffered saline (PBS) and suspended in Digman's buffer C (20 mM HEPES, pH 7.9; 0.42 M NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.4 mM EDTA, 0.5 mM 1,4-dithiothreitol (DTT) supplemented with 0.2% Nonidet P-40 (NP-40) and the following protease inhibitors: 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 0.1 mM aminobenzamidine, 10 μg/ml aprotinin, 1 μg/ml pepstatin A. They were vortexed, incubated on ice for 20 min and then centrifuged for 10 min at 20 000 × *g*. The supernatant was frozen at −80°C until further assayed. Protein concentrations were determined according to Bradford using a Bio-Rad protein assay.

An oligonucleotide, 5′-TAGTGA**TGAGTCA**GCCG-GATCAAG-3′, including the AP-1 motif (bold), was synthesized. Binding reactions were performed on ice for 30 min in 20 μl reaction mixture containing 2–5 μg protein, 10 mM Tris-HCl (pH 7.5), 20 mM KCl, 1 mM EDTA, 7.5% glycerol, 4 mM $MgCl_2$, 1.5 mM DTT, 20 μg bovine serum albumin, 2 μg poly [dI-dC]-[dI-dC] and approximately 0.2 ng of the double-stranded ^{32}P-labeled oligonucleotide probe. The specificity of the binding was determined in the presence of excess amounts of the unlabeled oligonucleotide. DNA-protein complexes were separated from the unbound probe on a native 5% polyacrylamide gel in TGE buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA). After electrophoresis, the gels were fixed, dried and autoradiographed. Autoradiograms were scanned using a Bio-Rad GS-670 imaging densitometer and analyzed using the Bio-Rad Molecular Analyst image analysis software.

The statistical analysis of our data were performed by using one-way analysis of variance (ANOVA) followed by Dunnett's test for comparison of treatment groups to controls and Student-Newman-Keuls test for comparison of different treatment groups. $P < 0.05$ was considered statistically significant.

3. Results

Fig. 1 (upper panel) demonstrates that application of 100 μM NMDA plus 10 μM glycine produced a dramatic increase in the AP-1 DNA binding in rat cerebellar granule cells. The effects of NMDA were blocked by the administration of a competitive, *dl*-(*E*)-2-amino-4-methyl-5-phosphonopentanoate carboxy-ethylester (CGP 39551, 100 μM) or a non-competitive NMDA receptor antagonist, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine hydrogen maleate (MK-801; 10 μM), whereas the competitive AMPA receptor antagonist, 6-nitro-7-sulphamoyl-benzo(*f*)quinoxaline-2-3-dione (NBQX; 20 μM) was without effect. The lower panel of Fig. 1 shows that the non-NMDA receptor agonist AMPA (30 μM) produced a pronounced stimulation of AP-1 DNA binding activity in the presence of 30 μM cyclothiazide, a substance which is known to block agonist-induced desensitization of AMPA receptors. AMPA by itself (up to 100 μM) also enhanced AP-1 binding activity in the absence of cyclothiazide, but this required longer incubation time (4 h) to appear (data not shown). The increase of AP-1 binding produced by AMPA plus cyclothiazide-produced was inhibited by NBQX (20 μM), but not by MK-801 (10 μM), thus indicating that the effects of AMPA were mediated via a specific activation of AMPA receptors. Nifedipine (10 μM), a voltage channel blocking agent, did not block the effects of AMPA alone or AMPA plus cyclothiazide (data not shown).

Data depicted in the upper panel of Fig. 2 demonstrate that the application of increasing concentrations of ethanol, in the presence of 10 μM glycine, produced a concentration-dependent inhibition of NMDA-enhanced AP-1 DNA binding in cerebellar granule cells with a significant reduction starting at 50 mM ethanol. In this context, it should be noted that the addition of glycine enhanced the NMDA-induced response as expected, but that glycine did not alter the inhibitory actions of ethanol on the increased binding activity induced by NMDA (data not shown). In contrast,

clearly weaker inhibitory actions of ethanol on AMPA plus cyclothiazide-induced enhancement of AP-1 binding were noted with a significant reduction seen only at the highest concentration (lower panel of Fig. 2). In this study, we examined the effects of ethanol on the enhancement of AP-1 binding induced by 30 μM AMPA. Similar results

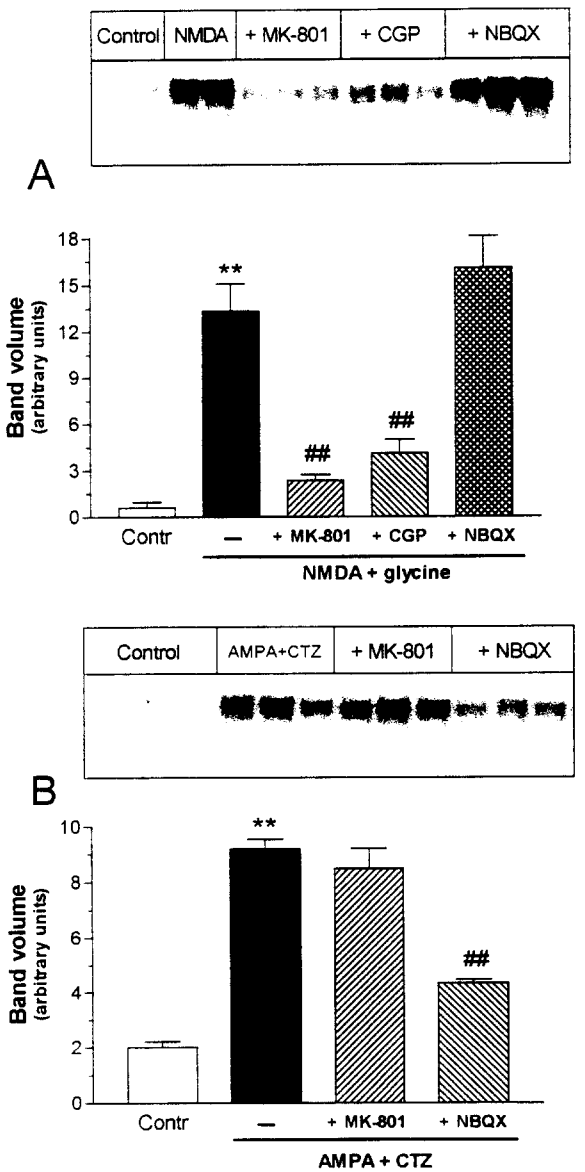

Fig. 1. Upper panel: Enhancement of AP-1 DNA binding induced by NMDA (100 μM; in the presence of 10 μM glycine) in rat cerebellar granule cells. The effects of NMDA were blocked by the non-competitive NMDA receptor antagonist, MK-801 (10 μM) and the competitive NMDA receptor antagonist, CGP 39551 (100 μM), but not by the AMPA receptor antagonist NBQX (20 μM). A representative picture of a scanned autoradiogram is shown in the upper panel. The bars are the means ± S.E.M. of three autoradiograms obtained from separately prepared cultures of granule cells. Lower panel: Enhancement of AP-1 DNA binding induced by AMPA (30 μM) in the presence of cyclothiazide (30 μM). The effect of AMPA plus cyclothiazide was blocked by NBQX (20 μM) but not by MK-801 (10 μM). The significances indicated represent $P < 0.01$, ** as compared to control cultures, ## as compared to cultures exposed only to NMDA or AMPA, respectively.

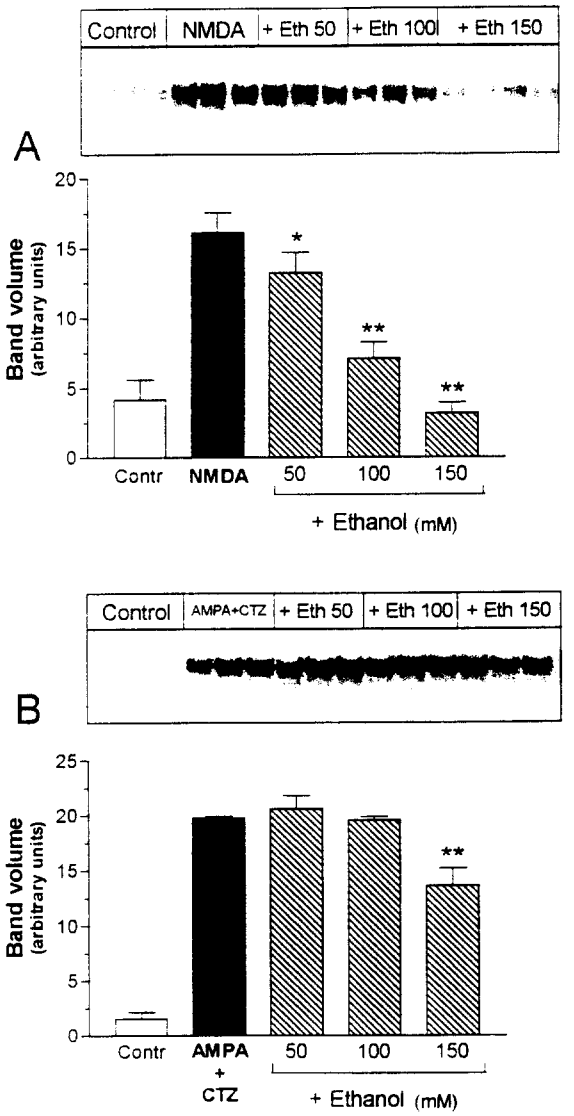

Fig. 2. Upper panel: Effects of increasing concentrations of ethanol on AP-1 DNA binding induced by 100 μM NMDA in the presence of 10 μM glycine in cerebellar granule cells. Lower panel: Effects of increasing concentrations of ethanol on AMPA (30 μM) plus cyclothiazide (30 μM)-induced AP-1 DNA binding in cerebellar granule cells. The means ± S.E.M. of three autoradiograms obtained from separate populations of cultured granule cells are given. The significances indicated represent * $P < 0.05$; ** $P < 0.01$.

were obtained in additional experiments using the maximal enhancement of AP-1 binding induced by 100 μM AMPA.

4. Discussion

The major findings are that: (1) NMDA produced a marked enhancement of AP-1 DNA binding in cerebellar granule cells, which was dose-dependently inhibited by increasing, intoxicating concentrations of ethanol; (2) the combined application of AMPA plus cyclothiazide produced a marked stimulation of AP-1 DNA binding activity which was of the same magnitude; and (3) the enhanced

AP-1 binding produced by NMDA was more sensitive to the inhibitory actions of ethanol than the AMPA-induced enhancement.

Earlier studies have shown that stimulation of NMDA receptors will cause an enhancement of immediate early gene production and change the regulation of transcription (see e.g. Ghosh and Greenberg, 1995). This series of events has been considered to be of essential importance for the regulation of gene expression. The present data show that intoxicating concentrations of ethanol are able to modify glutamate receptor-initiated AP-1 DNA binding activity and therefore perhaps glutamate receptor-activated gene expression. These data are in line with previous findings showing that ethanol is able to reduce glutamate-induced Ca^{2+} fluxes in several types of brain neurons and support the notion that at least some of the pharmacological actions of ethanol are mediated through an interaction with glutamate receptors of the NMDA subtype.

Until recently, the enhancement of functional Ca^{2+} responses produced by glutamate in several types of brain neurons were thought to be almost exclusively mediated via an activation glutamate receptors of the NMDA subtype or through voltage-dependent ion channels. The observations that non-NMDA receptor channels, activated by AMPA and/or kainic acid, also display a considerable permeability to Ca^{2+} have often been explained as due to indirect stimulation of NMDA receptors. However, recently we and others have shown that a pharmacologically induced blockade of AMPA receptor desensitization will reveal Ca^{2+} channels directly activated by AMPA in cerebellar granule cells (Cebers and Liljequist, 1995; Hack and Balázs, 1995). Furthermore, providing AMPA receptor desensitization is blocked, AMPA-induced neuronal necrosis is markedly enhanced in cerebellar granule cells (Hack and Balázs, 1995). AMPA receptors have generally been considered to be less sensitive to ethanol than NMDA receptors. The current findings that there was only a weak inhibition of the AMPA plus cyclothiazide-induced enhancement of AP-1 binding are thus in good agreement with data obtained in earlier studies using other parameters (for references, see Hoffman, 1995).

In summary, ethanol produces a concentration-dependent inhibition of NMDA-stimulated AP-1 DNA binding activity in cerebellar granule cells suggesting that it may be able to modulate glutamate-mediated changes in gene expression. Whether these actions of ethanol are of importance for long-term neuroadaptive changes in brain neurons associated with the development of tolerance to and dependence on ethanol in vivo remains to be clarified further. The results also show that stimulation of AMPA receptor enhances AP-1 DNA binding activity, thus indicating that AMPA receptors may play a significant role in the regulation of glutamate-induced alterations in gene expression.

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References

Bakalkin, G.Y., T. Yakovleva and L. Terenius, 1993, NF-κB-like factors in the murine brain, Mol. Brain Res., 20, 137.

Cebers, G. and S. Liljequist, 1995, Modulation of AMPA/kainate receptors by cyclothiazide increases cytoplasmic free Ca^{2+} and $^{45}Ca^{2+}$ uptake in brain neurons, Eur. J. Pharmac./Mol. Pharmac. Sect. 290, 105.

Dildy, J.E. and S.W. Leslie, 1989, Ethanol inhibits NMDA-induced increases in free intracellular Ca^{2+} in dissociated brain cells, Brain Res. 499, 383.

Ghosh, A. and M.E. Greenberg, 1995, Calcium signaling in neurons: molecular mechanisms and cellular consequences, Science 268, 239.

Hack, N. and R. Balázs, 1995, Properties of AMPA receptors expressed in rat cerebellar granule cell cultures: Ca^{2+} influx studies, J. Neurochem. 65, 1077.

Hoffman, P.L., 1995, The effects of alcohol on excitatory amino acid receptor function, in: Handbook of Experimental Pharmacology, Vol. 114, The Pharmacology of Alcohol Abuse, ed. H. Kranzler (Springer, Heidelberg) p. 75.

Hoffman, P.L., C.S. Rabe, F. Moses and B. Tabakoff, 1989, *N*-methyl-D-aspartate and ethanol: inhibition of calcium flux and cyclic GMP production, J. Neurochem. 52, 1937.

Karcz-Kubicha, M. and S. Liljequist, 1995, Effects of post-ethanol administration of NMDA and non-NMDA receptor antagonists on the development of ethanol tolerance in C57Bl mice, Psychopharmacology 120, 49.

Levi, G., F. Aloisi, M.T. Ciotti and V. Gallo, 1984, Autoradiographic localization and depolarization-induced release of acidic amino acids in differentiating cerebellar granule cell cultures, Brain Res. 290, 77.

Lovinger, D.M., G. White and F.F. Weight, 1989, Ethanol inhibits NMDA-activated ion current in hippocampal neurons, Science 243, 1721.

Morgan, J.I. and T. Curran, 1991, Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes *fos* and *jun*, Annu. Rev. Neurosci. 14, 421.

Nestler, E.J., B.T. Hope and K.L. Widnell, 1993, Drug addiction: a model for the molecular basis of neural plasticity, Neuron 11, 995.

Ohtani, K., H. Sakurai, E. Oh, E. Iwata, T. Tsuchiya and M. Tsuda, 1995, Involvement of protein kinase C in Ca^{2+}-signaling pathways to activation of AP-1 DNA binding activity evoked via NMDA- and voltage-gated Ca^{2+} channels, J. Neurochem. 65, 605.

Pennypacker, K.R., J.-S. Hong and M.K. KcMillian, 1995, Implications of prolonged expression of Fos-related antigens, Trends Pharmacol. Sci. 16, 317.

Szekely, A.M., M.L. Barbacchia and E. Costa, 1987, In primary cultures of cerebellar granule cells the activation of *N*-methyl-D-aspartate-sensitive receptors induces c-*fos* mRNA expression, Neuropharmacology 26, 1770.