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EFFECTS OF ETHANOLON NMDA-MEDIATED INTRACELLULAR FREE Ca^{2+} CONCENTRATION IN DISSOCIATED BRAIN CELLS

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Abstract – Using fluorescent probe fura-2 acetoxymethyl ester, we studied effects of N-Methyl-D-aspartate (NMDA) on free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and interaction of ethanol with NMDA-mediated response in freshly dissociated brain cells from newborn rats. Twenty five micromolar NMDA significantly increased $[Ca^{2+}]_i$ and this increasing effect could be prevented or reversed by the NMDA antagonists Mg^{2+} (1.0 mM) and 2-amino-5-phosphonovalerate (AP5, 100 μM). Ethanol at concentrations from 2.5 to 100 mM inhibited NMDA-mediated calcium current in a concentration-dependent manner. Maximal inhibition of NMDA-mediated calcium current by ethanol was 82% at 50 mM. The ethanol inhibition at 100 mM was not significantly different from the inhibition at 50 mM.

INTRODUCTION

The mechanism of pharmacological action of ethanol in the nervous system has long been a subject of great interest. Recently, evidence has been accumulating that alcohols can affect the function of neurotransmitter-gated ion channels. These membrane proteins are postsynaptic receptors for various excitatory and inhibitory neurotransmitters in the nervous system. The binding of a neurotransmitter to these proteins alters their molecular configuration such that the protein forms a transmembrane pore that permits the flux of particular ions down their concentration gradient. This ion flux in turn generates excitatory or inhibitory postsynaptic potentials in the postsynaptic cell.[1] N-Methyl-D-aspartate (NMDA), an excitatory amino acid analogue of glutamate, activates receptor-operated calcium channels.[2,3] Using Ca^{2+}-sensitive fluorescent probe fura-2 acetoxymethyl ester (fura-2/AM), we determined effects of ethanol on NMDA-mediated calcium channel, particularly on NMDA-mediated increases in free intracellular calcium concentration ($[Ca^{2+}]_i$) in freshly dissociated brain cells from newborn rats.

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MATERIALS AND METHODS

Trypsin, Dulbecco's modified Eagle's medium, and various reagents were purchased from GIBCO (Grand Island, NY, USA). The plasma-derived horse serum purchased from Cocalico Biologicals (Reamstown, PA, USA). Fura-2/AM was purchased from Molecular Probes (Eugene, OR, USA). Deoxyribonuclease I, NMDA, and other reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

Isolation of dissociated brain cells

Brain cells were isolated from pups of newborn (0-1-day-old) Sprague-Dawley females using the method by Raizada *et al.*[4] with some modifications. Nine pups were used for each experiment. Following decapitation, the whole brain was removed and placed in a 35 mm culture dish on ice. The brain stem, cerebellum, and cerebral hemispheres were separated using a scalpel blade. The brain tissue was rinsed with ice-cold balanced salt solution (BSS) containing (in millimolar concentrations) NaCl (137), KCl (5.4), Na_2HPO_4 (0.17), KH_2PO_4 (0.22), glucose (5.5), sucrose (59), and phenol red (0.03) with pH 7.4. Meninges and blood vessels were removed meticulously and the tissue was placed in cold BSS until all dissections were completed.

Following a wash step with BSS, the brains were transferred

to a glass petri dish and minced using scalpel blades. The minced tissue was placed in a trypsinizing flask containing 10 ml of 0.05% trypsin/0.5 mM ethylenediaminetetraacetic acid, disodium salt (EDTA) in BSS. The brain tissue was stirred for 10 min at 37℃ with careful monitoring of pH at 7.4. After 10 min of trypsinization, the undissociated tissue was allowed to settle and the dissociated cells collected and transferred to a trypsinization flask containing 12 ml of warm Dulbecco's modified Eagle's medium (high glucose) plus 10% plasma-derived horse serum plus 5.8 μg/ml of deoxyribonuclease I. After 5 min of gentle stirring at 37℃, the cells were divided equally into two 10 ml centrifuge tubes while taking care to exclude any small tissue fragments. The cells were centrifuged at 300 × g for 5 min. Supernatants were decanted and the cells resuspended in a total of 4 ml of warm HEPES-buffered Hanks (HBH) containing (in millimolar concentrations) HEPES (20), NaCl (137), $CaCl_2$ (1.3), $MgSO_4$ (0.4), $MgCl_2$ (0.5), KCl (5.0), KH_2PO_4 (0.4), Na_2HPO_4 (0.6), $NaHCO_3$ (3.0), and glucose (5.6) with pH 7.4.

Loading dissociated brain cells with fura-2/AM

The cell suspension was further diluted to a total of 8 ml with warm HBH, divided into two 4 ml aliquots, and placed in a Dubnoff shaking water bath for 5 min at 37℃. A final concentration of 5 μM fura-2/AM in dimethylsulfoxide (DMSO) was added to one aliquot and the same volume of DMSO was added to the other aliquot of cells, which served as a control. The cell suspensions were returned to the shaking water bath for 45-60 min. The fura-2/AM-loaded cells and the control cells were transferred to centrifuge tubes and each was diluted with 2 ml of warm HBH. Following a 5 min centrifugation at 300 ×g, the supernatants were decanted and the pellets were resuspended in a volume of warm HBH or Mg^{2+}-free HBH, which resulted in approximately 3.5 $\times 10^6$ cells/2 ml. Because the presence of physiological concentrations of Mg^{2+} inhibits NMDA responses in the absence of depolarization, brain cells in all NMDA experiments were resuspended in HBH without added Mg^{2+}. Cells were used for approximately 60 min after the fura-2/AM loading and final resuspension processes were completed.

Measurement of $[Ca^{2+}]_i$

Suspensions of dissociated cells were kept at room temperature, and each 2 ml was placed in cuvettes and incubated for 2-3 min at 37℃ prior to fluorescence measurement. A magnetic stir bar was placed in each cuvette to keep cells from settling and to allow adequate mixing of drugs. Model RI-D Ratio fluorescence spectrometer (Photon Technology International) with 37℃ water-jacketed cuvette compartment was used for fluorescence determinations. $[Ca^{2+}]i$ was determined by the following equation: Kd of 224 nM: $[Ca^{2+}]_i = K_d \times Sf_2/Sb_2 (R - R_{min}/R_{max} - R)$, where Sf_2 is the signal of the free fura-2 at 380 nm and Sb_2 is the signal of the bound fura-2 at 380 nm; R is the 340/380 nm ratio for a particular sample.

For each experiment, the minimum fluorescence was determined using final concentrations of 0.1% sodium dodecyl sulfate (SDS) and 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) added to a cuvette containing a 2 ml sample of cells. R_{min} was calculated as the ratio of fluorescence intensity at 340/380 nm under the conditions of minimal fura-2/Ca^{2+} binding. The maximum fluorescence was determined in a different sample of cells using final concentrations of 0.1% SDS and 4 mM $CaCl_2$. R_{max} was calculated as the ratio of fluorescence at 340/380 nm under the conditions of excess Ca^{2+}. Corrections were made for extracellular leakage of fura-2 by determining the 340 and 380 nm values of another sample and then adding 40 μM $MnCl_2$ to that sample to quench any extracellular fura-2[5]; the difference in the 340 nm values before and after Mn^{2+} and the difference in the 380 nm values resulted in the Mn^{2+} correction factor for a particular experiment. The 340 and 380 nm values for a sample of unloaded cells (control) were also determined to rectify background or tissue autofluorescence. The correction factors were subtracted from the 340 and 380 nm values per sample prior to determination of $[Ca^{2+}]_i$.

After determination of R_{min}, R_{max}, Mn^{2+}, and autofluorescence values, each 2 ml sample containing approximately 3.5 × 10^6 cells was monitored with dual monochromators (excitation at 340 and 380 nm; emission at 505 nm) for 60 s to determine resting $[Ca^{2+}]_i$. After 60 s either Mg^{2+}-free HBH (control) or ethanol (25-100 mM in Mg^{2+}-free-HBH) was added to the cuvette and incubated for 50 s at which time NMDA was added and fluorescence was monitored for 80-150 s. Drug injection volumes of 20 μl each were added directly into the cuvette. Cytosolic Ca^{2+} values were measured every second and average recorded every 3 s. The net change in free intracellular calcium concentration ($\Delta[Ca^{2+}]_i$) was determined by subtracting NMDA-stimulated increases in $[Ca^{2+}]_i$ after responses had plateaued from pre-NMDA levels; percent increases were calculated by dividing this difference by the pre-NMDA $[Ca^{2+}]_i$ value and multiplying by 100. Analysis of statistical significance of NMDA-stimulated $[Ca^{2+}]_i$ changes, in the presence or absence of ethanol, was determined by paired student's *t*-tests.

The NMDA antagonists, Mg^{2+} (1.0 mM) and AP5 (100 μM in Mg^{2+}-free HBH) were tested for their ability to prevent and reverse NMDA-induced increases in $[Ca^{2+}]_i$. Antagonists were added approximately 60 s before or after 25 μM NMDA addition. $\Delta[Ca^{2+}]_i$ was determined 60 s after NMDA or antagonist addition. Duplicate determinations were made or each condition per experiment.

RESULTS AND DISCUSSION

In the presence of 1.3 mM extracellular Ca^{2+} (in Mg^{2+}-free HBH), the value of intact $[Ca^{2+}]_i$ (resting) in dissociated brain

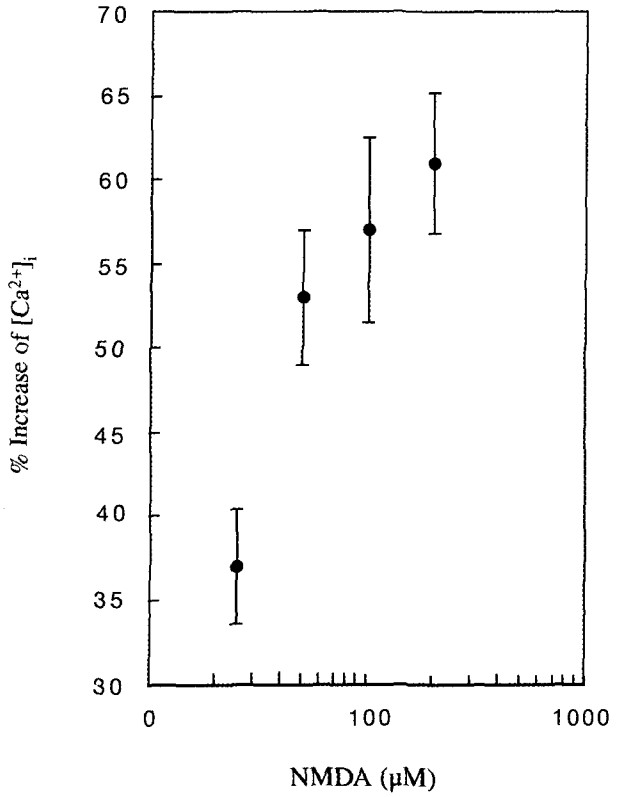

Figure 1. The average percent increase of $[Ca^{2+}]_i$ in brain cells activated by NMDA, as a function of NMDA concentration. Determinations were made approximately 150 s after NMDA addition and fluorescence was monitored for 80 s. Each value represents the mean ± SEM of eight determinations.

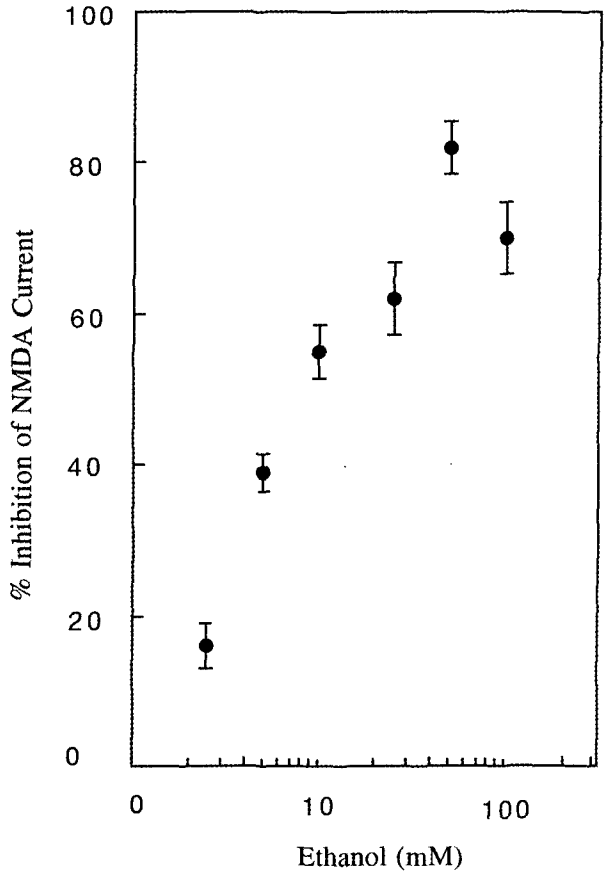

Figure 2. The average percent inhibition of current activated by 50 μM NMDA, as a function of ethanol concentration. Determinations were made approximately 150 s after NMDA addition and fluorescence was monitored for 80 s. Each value represents the mean ± SEM of eight determinations.

cells was 193 ±15.3 nM (n = 8). Several studies[6-8] have shown that intact cytosolic Ca^{2+} levels were from 100 to 200 nM.

Figure 1 illustrates the increasing effect of NMDA on $[Ca^{2+}]_i$ in the brain cells. It also shows that NMDA at concentrations from 25 to 200 μM conferred a concentration-dependent increase of $[Ca^{2+}]_i$ in brain cells. In these cells maximal increase of $[Ca^{2+}]_i$ was 61 ± 4.2% (increased value was approximately 125 nM), which was observed with an NMDA concentration of 200 μM.

Figure 2 illustrates the effect of ethanol (2.5-100 mM) on $[Ca^{2+}]_i$ increased by 50 μM NMDA in dissociated brain cells. In the dissociated brain cells, ethanol conferred a concentration-dependent inhibition of NMDA-mediated current over the concentration range 2.5 to 100 mM (Fig. 2). The inhibition by 50 mM ethanol was 82 ± 3.5% of control, and the ethanol inhibition at 100 mM was not significantly different from the inhibition at 50 mM.

As shown in Table 1, 25 μM NMDA-stimulated increases in $[Ca^{2+}]_i$ could be inhibited or reversed by Mg^{2+}, a noncompetitive NMDA antagonist. Addition of 1.0 mM Mg^{2+} prior to 25 μM NMDA inhibited the expected rise in $[Ca^{2+}]_i$ at 60 s by approximately 79%. Addition of 1.0 mM Mg^{2+} after 60 s NMDA exposures reversed the NMDA-stimulated rise in $[Ca^{2+}]_i$ by approximately 80%. Furthermore, AP5 (100 μM) prevented increases in $[Ca^{2+}]_i$ by approximately 89% when added before 25 μM NMDA. AP5 (100 μM) also reversed NMDA's effects by approximately 66% when added after a 60 s stimulatory period.

Table 1. Effect of Mg^{2+} and AP5 on NMDA-induced $\Delta[Ca^{2+}]_i$

Condition	$\Delta[Ca^{2+}]_i$
NMDA	72.5 ± 4.2
Mg^{2+} + NMDA	15.1 ± 3.1
NMDA + Mg^{2+}	14.3 ± 4.8
NMDA	76.0 ± 6.3
AP5 + NMDA	7.9 ± 1.9
NMDA + AP5	25.6 ± 4.8

NMDA antagonists, Mg^{2+} (1.0 mM) or AP5 (100 μM in Mg-free HBH), were added either 60 s before or after 25 μM NMDA addition. $\Delta[Ca^{2+}]_i$ was determined 60 s after NMDA or antagonist addition. $\Delta[Ca^{2+}]_i$ represent the net increase after subtracting resting (pre-NMDA) levels. Values represent mean nM increases in $\Delta[Ca^{2+}]_i$ ±SEM of 8 experiments.

Similar results to ethanol-induced inhibition of NMDA responses have also been observed in other neural tissue, using a variety of experimental methods. In whole-cell patch-clamp experiments, ethanol produced an inhibition of NMDA-activated current in cultured neurons from the spinal cord and neocortex that was similar to that observed in hippocampal neurons[9]. In slices of the amygdala, ethanol suppressed NMDA receptor-mediated synaptic responses[10]. In extracellular recording experiments, *in vivo*, the intraperitoneal administration of ethanol inhibited NMDA-activated spike firing of neurons in the medial septal nucleus[11] and locus coeruleus.[12] In biochemical experiments, ethanol reduced NMDA-induced intracellular Ca^{2+} elevation in freshly isolated brain cells,[13,14] neurotransmitter release from slices of neocortex[15-17] and striatum,[18] and Ca^{2+} influx and cyclic GMP elevation in cultured cerebella granule cells.[19]

The mechanism involved in the inhibition of NMDA responses by ethanol has not been established. Several possible mechanisms have been tested in whole-cell patch-clamp experiments on cultured hippocampal neurons.[20,21] Membrane potentials between -60 and +60 mV did not significantly affect the percent inhibition of NMDA-activated current by ethanol, and ethanol did not alter the reversal potential of NMDA-activated current. These observations indicate that the effect of ethanol on NMDA channels is not voltage-dependent, and does not result from an alteration of the ion selectivity of the NMDA channel. Other possible mechanisms include alteration of one of the regulatory sites on the NMDA channel. However, the percent inhibition of NMDA-activated current by ethanol did not differ with different concentrations of NMDA (25-100 μM), Mg^{2+} (1.0 mM), and AP5 (100 μM), which indicates that ethanol does not interfere with the regulatory sites on the NMDA channel associated with these agents. It has been suggested that ethanol inhibits NMDA responses by interfering with the glycine modulatory site on the NMDA receptor.[14, 18, 19, 22] Peoples and Weight[21] reported that the percent inhibition of NMDA-activated current by ethanol was not altered by glycine concentrations from 100 nM to 100 μM; the concentration ranges that augment NMDA-activated current. Since glycine concentrations in their experiments were less than 100 nM, this observation indicates that interference with the glycine modulatory site would not explain the inhibition of NMDA-activated current by ethanol in their experiments. This suggests that ethanol may affect the function of NMDA channels by altering gating of the channel.

Weight *et al.*[23] reported on the relationship between the hydrophobicity of different alcohols and their effect on NMDA channels. As the hydrophobicity of the alcohols increased, their potency for inhibiting NMDA-activated current increased. In addition, there was a significant linear relation between these parameters, suggesting that the potency of different alcohols for inhibiting NMDA-activated current increases as a function of increasing hydrophobicity. Similar results have also been reported for alcohol-induced inhibition of NMDA-evoked release of [^{3}H]norepinephrine from cortical slices.[24] As the potency of different alcohols for inhibiting NMDA-activated current increased, their potency for producing intoxication also increased. Moreover, there was a significant linear relation between these parameters, suggesting that the more potent the alcohol is in inhibiting NMDA-activated current, the greater its potency for producing intoxication.

In conclusion, our results demonstrate that ethanol inhibits NMDA-stimulated rises $[Ca^{2+}]_i$ in dissociated brain cells from newborn rats. Ethanol's inhibition of NMDA Ca^{2+} fluxes could be related to the intoxicating effects of ethanol.

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