Suppression of L-Type Voltage-Gated Calcium Channel-Dependent Synaptic Plasticity by Ethanol: Analysis of Miniature Synaptic Currents and Dendritic Calcium Transients

ADAM W. HENDRICSON, MARK P. THOMAS, MELANIE J. LIPPMANN, and RICHARD A. MORRISETT

The Division of Pharmacology and Toxicology, The College of Pharmacy, The University of Texas, Austin, Texas

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ABSTRACT

Intoxicating concentrations of ethanol inhibit N-methyl-D-aspartate (NMDA) receptor-dependent long-term potentiation, an interaction thought to underlie a major component of the central nervous system actions of ethanol. Another form of synaptic potentiation involving activation of L-type dihydropyridine-sensitive voltage-gated calcium channels (VGCCs) has been described, but very little information concerning ethanol effects on VGCC-dependent synaptic potentiation is available. Here, we assessed ethanol effects on VGCC-dependent synaptic potentiation using whole cell patch-clamp recordings of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated miniature excitatory post synaptic currents (mEPSCs) in area CA1 of the rat hippocampus. No potentiation was observed in artificial cerebrospinal fluid containing 2 to 3 mM Ca2⁺, but marked potentiation of mEPSCs was consistently observed in 4 mM Ca2⁺ and with patch pipettes containing an ATP-regenerating system. This potentiation was insensitive to the NMDA receptor antagonist DL-2-amino-5-phosphonovaleric acid, whereas it was completely blocked by bath application of ethanol (25–75 mM), which had no effect on baseline mEPSC amplitude or frequency. The synaptic potentiation involved enhancement of both presynaptic and postsynaptic components because significant increases in both the frequency and amplitude of AMPA mEPSCs were observed. Ethanol inhibition of VGCC-dependent synaptic potentiation seemed to occur at the induction step because both the increases in mEPSC frequency and amplitude were affected. To address that question more directly, we used fluorescent imaging of synaptically evoked dendritic calcium events, which displayed a similarly marked ethanol sensitivity. Thus, ethanol modulates fast excitatory synaptic transmission by inhibiting the induction of an NMDA receptor-independent form of synaptic potentiation observed at excitatory synapses on central neurons.

Efforts directed toward unraveling the synaptic basis for alcohol-related brain disorders have centered upon direct ethanol effects on the major excitatory and inhibitory ligand-gated ion channels (LGICs) operated by GABA_A and NMDA receptors (NMDARs). Indeed, the direct actions of ethanol on NMDARs impact the functional properties of neurons and profoundly alter the short-term processing and long-term storage of information in neural networks (Durand and Carlen, 1984; Lovinger et al., 1989, 1990; Blitzer et al., 1990; Morrisett and Swartzwelder, 1993; Thomas et al., 1998; Thomas and Morrisett, 2000; Malde et al., 2002).

Although these efforts to understand ethanol-LGIC interactions have focused upon direct effects, it is likely that ethanol may also exert indirect effects on synaptic transmission via interactions with pathways of synaptic plasticity. To this end, we have demonstrated that ethanol inhibits an NMDAR-independent form of LTP induced by bath application of the potassium channel antagonist TEA (Zhang and Morrisett, 1993). This study was prompted by several studies in which induction of LTP seemed to use Ca2⁺ entry through L-type voltage-gated calcium channels (L-VGCCs). Grover and Teyler (1990, 1992) initially described a form of LTP induced by activation of hippocampal afferents. Likewise, potentiation induced by a brief application of TEA is insen-

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1 These authors contributed equally to this work.

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ABBREVIATIONS: LGIC, ligand-gated ion channel; NMDA, N-methyl-D-aspartate; NMDAR, N-methyl-D-aspartate receptor; LTP, long-term potentiation; TEA, tetraethylammonium chloride; L-VGCC, dihydropyridine-sensitive voltage-gated calcium channel; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; DIP, depolarization-induced potentiation; mEPSC, miniature excitatory postsynaptic current; ACSF, artificial cerebrospinal fluid; DL-APV, DL-2-amino-5-phosphonovaleric acid; IEI, interevent interval; AM, acetoxymethyl ester; HiCa, high calcium; LoCa, low calcium.
itive to NMDAR antagonists (Aniksztejn and Ben-Ari, 1990, 1991; Huang and Malenka, 1993; Hanse and Gustafsson, 1994; Huber et al., 1995). The linking feature of these forms of synaptic potentiation is that their induction mechanisms require activation of L-VGCCs (Huang and Malenka, 1993; Hanse and Gustafsson, 1994; Huber et al., 1995). Therefore, strong activation of L-VGCCs, independent of NMDAR activation, is sufficient to elicit a level of Ca^{2+} influx that can induce increases in AMPA receptor function underlying the potentiated response.

More extensive analysis of L-VGCC-dependent synaptic potentiation has been provided in reports using whole-cell voltage-clamp recordings of hippocampal neurons involving prolonged postsynaptic depolarizing pulses (termed depolarization-induced potentiation, or DIP; Kullmann et al., 1992; Wyllie et al., 1994; Wyllie and Nicoll, 1994). This potentiation was independent of synaptic activation of afferents and was inhibited by the L-VGCC antagonist nifedipine (Kullmann et al., 1992). Via analysis of AMPA receptor-mediated miniature EPSCs (mEPSCs), Wyllie et al. (1994) reported evidence of pre- and postsynaptic components of enhancement after DIP-induction protocols. Similarities between the previously-discussed VGCC-dependent forms of synaptic potentiation induced indirectly (such as by application of TEA) and potentiation induced by direct VGCC activation with depolarizing trains suggest that the synaptic potentiation reported in these studies likely occurs via similar, if not identical, mechanisms.

Synaptic potentiation mediated by L-VGCC activation has been implicated in aspects of fear conditioning in the lateral amygdala that are distinct from those mediates by NMDAR-dependent synaptic potentiation (Bauer et al., 2002). In amygdala slices, these investigators showed that high-frequency activation of thalamic inputs paired with postsynaptic depolarization induced a nondecrementing, L-VGCC-dependent form of synaptic potentiation. Conversely, low-frequency theta burst-type stimulation of distinct cortical afferents also elicited a conventional associative NMDAR-LTP. Most importantly, this study also demonstrated distinct roles for synaptic potentiation mediated by these pathways (i.e., VGCC-dependent versus NMDAR-dependent synaptic potentiation) in the formation as well as the maintenance of fear conditioning to auditory stimuli in behaving animals. These results provide some important insights into the relative roles of NMDA and L-type channels in the excitatory synaptic plasticity that underlies a classical form of associative behavioral conditioning.

In relation to alcoholism and alcohol-related brain disorders, ethanol inhibition of NMDAR-LTP is well documented. Conversely, our previous study involving VGCC-dependent synaptic potentiation (Zhang and Morrisett, 1993). In light of the above-mentioned evidence for complex behavioral alterations mediated by L-VGCCs in amygdala, it is highly likely that ethanol-induced alterations in different forms of plasticity play distinct roles in the development of ethanol dependence as well as in the induction of withdrawal hyperexcitability (and other manifestations of ethanol neurotoxicity).

To achieve the mechanism of ethanol inhibition of VGCC-dependent synaptic potentiation, we used methods reported by Kullmann et al. (1992) and Wyllie et al. (1994). We tested the effect of ethanol on the induction of DIP in hippocampal CA1 pyramidal neurons and used mEPSC analysis to determine whether alterations of DIP by ethanol were expressed as changes at pre- or postsynaptic sites. We hypothesize that the interaction of ethanol with synaptic potentiation mediated by VGCCs represents an important and previously undescribed pathway by which this drug affects information processing in the central nervous system.

Materials and Methods

Slice Preparation. Hippocampal slices were prepared using techniques described previously (Morrisett and Swartzwelder, 1993; Thomas et al., 1998). All experiments were carried out in accordance with National Institutes of Health guidelines. Sprague-Dawley rat pups of both sexes (12–20 days old) were anesthetized with halothane, decapitated, and the brain was rapidly removed and placed in ice-cold oxygenated artificial cerebrospinal fluid (ACSF) for 3 to 4 min. The hippocampi were dissected bilaterally and 500-μm-thick transverse slices were cut using a tissue chopper (Stoelting, Wood Dale, IL). The slices were equilibrated for at least 1 h before recording in ACSF containing 120 mM NaCl, 3.3 mM KCl, 1.23 mM NaHPO₄, 25 mM NaHCO₃, 1.8 mM CaCl₂, 2.4 mM MgSO₄, and 10 mM dextrose, pH 7.4, 280 to 290 mOsM (all chemicals obtained from Sigma-Aldrich, St. Louis, MO). The ACSF was gassed with 95% O₂, 5% CO₂ and maintained at 32°C. Slices were transferred to a submerged style recording chamber and perfused continuously with ACSF at approximately 1.5 ml/min. Recording ACSF contained 0.9 mM MgSO₄ and from 2 to 4 mM CaCl₂, as noted under Results.

Patch-Clamp Recording. Whole cell patch-clamp recordings were made at 32 ± 1°C from CA1 pyramidal cells as described previously (Morrisett and Swartzwelder, 1993). Recording electrodes (tip resistances of 1–2 MΩ) were made from thin-walled borosilicate glass (TW150F-4; WPI, Sarasota, FL) and filled with 115 mM CsMeSO₄, 12 mM NaCl, 0.2 mM EGTA, 10 mM HEPES, 2 mM Mg-ATP, 0.3 mM Tris-GTP, 20 mM phosphocreatine, and 50 μM creatine kinase, 270 to 280 mOsM, pH 7.2 to 7.3 with CsOH. In some experiments, the ATP-regenerating system (phosphocreatine/creatine kinase) was not included, and the CsMeSO₄ concentration was raised to 135 mM.

Miniature EPSCs were recorded in 1 μM tetrodotoxin, 25 μM picrotoxin, and 50 μM (DL)-APV at a holding potential of −80 mV. Currents were amplified using either 2300A (Dagan, Minneapolis, MN) or 200B (Axon Instruments, Union City, CA) patch-clamp amplifiers, filtered at 1 kHz, and recorded via digital tape recorder (Sony DAT model 75 ES) and then digitized using either a Lab Master DMA interface (Scientific Solutions, Mentos, OH) and Fetchex software (Axon Instruments) under a DOS environment, or using a Digidata 1200B interface (Axon Instruments) with pClamp version 8.02 software (Axon Instruments) under a Windows XP environment. Access resistance and cell input resistance were monitored throughout the experiment; no series compensation was used to maximize the signal-to-noise ratio, in accordance with a previous study (Wyllie et al., 1994). Access resistance for most experiments ranged from 10 to 20 MΩ and never exceeded 40 MΩ. Recordings during which access resistance changed more than 20% at anytime during the experiment, or changed in a manner that could explain the experimentally induced changes in mEPSC amplitude, were not included in data analyses.

Experimental Protocol and Data Analysis. After a 10- to 15-min baseline recording period after break-in to whole cell mode, a series of 3-s depolarizing pulses from a holding potential of −80 mV to +20 mV was applied every 6 s for 2 min for a total of 20 pulses, as described previously (Kullmann et al., 1992; Wyllie et al., 1994). Miniature EPSCs were then recorded for at least 30 min after the pulse train. For offline analysis, mEPSCs were detected and analyzed using a Windows-based analysis program (Mini Analysis; Syn-
aptosoft, Inc., Decatur, GA). Events were detected by choosing a threshold current amplitude (ranging from 5 to 9 pA) for each recording, which was then held constant for the entire experiment. Variation in this threshold from cell to cell did not result in differences in mean mEPSC amplitude between groups (see Results). Noise spikes were eliminated by including an additional area threshold criterion (30 to 40 fCoulombs) such that events below this area threshold were not accepted. The mean mEPSC amplitude and interevent interval (IEI) were calculated for 1-min epochs during the entire course of the experiment to determine where peak changes occurred. Individual event amplitude and IEI were also plotted as cumulative occurrence histograms for a period of 3 to 5 min surrounding the peak change in mean amplitude and mean IEI during the 30-min post-train period for comparison with a similar time period during the baseline recording just before cell depolarization. These distributions (baseline period versus peak period) for event amplitude and IEI were compared using the Kolmogorov-Smirnov test embedded in the analysis software. Mean peak values for mEPSC amplitude and IEI derived from these same epochs for each cell were used for comparison between groups using Student’s t test. Results were considered significantly different at p < 0.05. Calculations of extracellular free Ca\(^{2+}\) concentration in recording solutions were performed using MINEQL+ (version 4.5) analytical chemistry software (Environmental Research Software, Hallowell, ME).

**Calcium Imaging.** Slices were prepared exactly as described for electrophysiological experiments, and all imaging was performed in the presence of (DL)-APV. Slices were incubated for 60 min with the membrane permeant label, Fura-2-AM (15 μM; Molecular Probes, Eugene, OR) in 0.02% pluronic acid/ACSF at 32°C. Slices were then transferred into a standard recording chamber mounted in the light path of a BX-50WI microscope (Olympus, Tokyo, Japan) fitted with a Fura-2 fluorescence cube set (Omega Optical, Brattleboro, VT), which lacked an excitation filter. Excitation at 340 and 380 nm was provided by a monochromator under computer command and delivered via liquid light guide (Cairn Instruments, London, UK). Images were acquired via a Quantix digital camera (Photometrics, Tucson, AZ) using Axon Imaging Workbench software (version 2.214). Images were collected in ping-pong mode and typically were 200 to 500 ms in duration. Ratioed pairs were collected at 2 to 4 Hz. Linearity of Ca\(^{2+}\) signals was verified by calibration with Fura-2-coated beads (Molecular Probes). Signals were recorded from multiple regions of interest (3–5) in distal dendritic areas of stratum radiatum chosen to avoid more proximal dendritic areas of punctate staining indicative of labeled interneurons. Signals were elicited by standard synaptic stimulation using a tungsten electrode placed within stratum radiatum and placed lateral to the regions of interest used for analysis (200–400-ms trains of 100-Hz stimuli at 100–400 μA). Signals were detected at 510 ± 20 nM and are presented as percentage of ΔF/ΔF.

**Results**

**VGCC Plasticity.** DIP was reliably produced when the CaCl\(_2\) concentration in the recording ACSF was increased from 2 to 4 mM (yielding an increase in the calculated extra- cellular free Ca\(^{2+}\) from 1.29 to 2.58 mM), and an ATP-regenerating system was included in the recording pipette. All experiments were performed in the presence of the NMDAR antagonist (DL)-APV (50 μM). Figure 1 shows typical responses of a cell to depolarizing trains delivered under the above-mentioned conditions. After the application of depolarizing pulses, a marked increase in the amplitude of events was readily apparent, as well as an increase in the frequency of events (Fig. 1A). For this cell, the mean mEPSC amplitude was 14.3 ± 0.4 pA during the control period and peaked at 23.7 ± 1.0 pA after cell depolarization. The mean frequency of mEPSCs was 2.2 ± 0.1 Hz during the control period and peaked at 5.8 ± 0.3 Hz after depolarization. Thus, the application of depolarizing pulses resulted in a 166% increase in mEPSC amplitude and 264% increase in mEPSC frequency for this cell.

The potentiation of mEPSCs is illustrated graphically in

Fig. 1. Depolarization-induced potentiation is reliably induced in elevated extracellular calcium. A, miniature EPSCs recorded from a CA1 pyramidal cell before and after a train of depolarizing pulses. For these experiments, the recording ACSF contained 4 mM Ca\(^{2+}\) and the recording pipette contained an ATP-regenerating system (see Materials and Methods). Note the increase in frequency and amplitude of events after the depolarizing train under these recording conditions. B, cumulative frequency histogram of mEPSC event amplitudes for epochs before (open circles) and after (filled circles) the depolarizing train for the cell shown in A (number of events are shown in parentheses). C, cumulative frequency histogram of mEPSC interevent intervals for the same epochs as in B.
the cumulative histograms for event amplitude (Fig. 1B) and IEI (Fig. 1C). The distributions for both mEPSC amplitude and IEI after cell depolarization differed significantly from baseline (Kolmogorov–Smirnov test; \( p < 0.05 \)). The mean peak change in mEPSC amplitude for seven cells in this group (designated the high calcium, or HiCa group) was 187 ± 11% of baseline and ranged from 148 to 237%. Comparison of cumulative amplitude distributions showed that for seven of seven cells, the event amplitude distribution at the peak of potentiation after depolarization was significantly different from the baseline distribution (Kolmogorov–Smirnov test; \( p < 0.05 \)). The mean peak change in frequency was 197 ± 19% of baseline, ranged from 112 to 264%, and is presented in a cumulative graph (see Fig. 5) for comparison of data from all the recording conditions in this study. Comparison of IEI cumulative distributions showed that for six of seven cells, the mEPSC IEI distribution at the peak change in frequency after depolarization was significantly different from the baseline distribution (Kolmogorov–Smirnov test; \( p < 0.05 \)).

Initial examination of the factors required to elicit DIP in our laboratory included analysis of the effects of a train of prolonged depolarizing pulses in ASCF containing a standard concentration of Ca\(^{2+}\) (2 mM) and with no ATP-regenerating system included in the recording pipette. Under these conditions, a series of depolarizing steps to +20 mV from a holding potential of −80 mV induced no significant change in mEPSC characteristics. In a typical cell, the mean mEPSC amplitude was 13.9 ± 0.7 pA during the control period and 14.2 ± 0.3 pA after application of depolarizing pulses, whereas the mean event frequency was 1.43 ± 0.1 Hz before depolarization, and 1.43 ± 0.02 Hz after. Similar results were obtained for two other cells recorded in ASCF containing 2 mM Ca\(^{2+}\) and four cells recorded in ASCF containing 3 mM Ca\(^{2+}\). These six experiments combined were designated the low Ca\(^{2+}\) (LoCa) group and used for comparison with subsequent experimental groups. The mean mEPSC amplitude and frequency measured after depolarization were 102 ± 4 and 102 ± 7% of baseline (predepolarization) values, respectively, as depicted in the averaged data graph for comparison of all experimental groups (Fig. 5). Cumulative histograms showing the distribution of mEPSC amplitudes and IEIs before and after the depolarizing steps demonstrate that no significant change in mEPSC characteristics occurred in cells conditioned in 2 mM Ca\(^{2+}\) (Kolmogorov–Smirnov test; \( p > 0.05 \); data not shown).

Figure 2 shows the time course of the potentiation of mEPSC amplitude (Fig. 2A) and frequency (Fig. 2B) induced by delivery of depolarizing steps in the presence of elevated Ca\(^{2+}\) (4 mM) and an ATP-regenerating intracellular solution (recordings made under these conditions were designated the HiCa group). In this representative cell from the HiCa group, the peak enhancement of mEPSC amplitude occurred 8 min after the end of the depolarization protocol initiated at \( t = 10 \) min (Fig. 2A), whereas mEPSC frequency increased to a maximum within a minute (Fig. 2B). Both mEPSC amplitude and frequency returned to near baseline values by 35 to 40 min postdepolarization. For the seven experiments in the HiCa group, the peak enhancement of mEPSC amplitude occurred from 1 to 15 min after cell depolarization (mean 8 min), and the time of peak enhancement of mEPSC frequency ranged from 1 to 11 min after depolarization (mean 6 min). The potentiation of mEPSCs normally returned to control levels within 30 to 40 min after the depolarization pulses, a result in agreement with those from Nicoll’s laboratory. Importantly, these investigators have shown that this form of potentiation becomes sustained (nondecremental) if the recording is performed under conditions of phosphatase inhibition (Wyllie and Nicoll, 1994).

The potentiation of mEPSC amplitude was not associated with a change in the time course of individual events. Figure 2C shows representative mEPSC waveforms averaged from 10 events during the baseline period and during the peak increase after depolarization for the cell shown in Fig. 2, A and B. The mean mEPSC amplitude was 9.5 pA during the control period and 18.9 pA after cell depolarization. However, scaling the postdepolarization waveform to the same peak amplitude as the predepolarization waveform demonstrates
that no change in the kinetics of the mEPSCs occurred after potentiation. Analysis of the averaged events from this representative cell revealed decay time constants of 2.59 ± 0.18 ms for pretrain mEPSCs, whereas post-train mEPSCs had 2.56 ± 0.18-ms decay time constants (n = 7 cells).

The potentiation induced by prolonged depolarizing pulses has been shown to be dependent upon the activation of L-VGCCs (Kullmann et al., 1992). To confirm that the potentiation induced in rat hippocampal neurons in our hands was similarly dependent on activation of L-VGCCs, we performed experiments with nifedipine (20 μM) in the bathing solution (Fig. 3). No significant difference in mean event amplitude (15.7 ± 0.5 versus 12.8 ± 1.0 pA for HiCa group; p > 0.05) or frequency (3.0 ± 1.1 versus 2.8 ± 0.3 Hz for HiCa group; p > 0.05) was observed during the baseline period for the nifedipine group compared with the HiCa group, indicating that the L-VGCC antagonist had no effect on baseline mEPSC characteristics. Antagonism of L-VGCCs had a profound effect on the induction of DIP, however. As observed in representative raw traces (Fig. 3A) and the cumulative histograms (Fig. 3, B and C), no significant change was observed in mEPSC amplitude or frequency after cell depolarization in the presence of nifedipine. In four experiments performed under this condition, the mean mEPSC amplitude and frequency after depolarization was 110 ± 13 and 111 ± 16% of control levels, respectively (Fig. 5).

Ethanol Inhibits VGCC-Dependent Synaptic Potentiation in a Concentration-Dependent Manner. Because ethanol is well known to inhibit voltage-gated calcium channels, we hypothesized that ethanol would inhibit this novel form of VGCC-dependent synaptic potentiation. To test this hypothesis, experiments were performed in the presence of ethanol (25–75 mM; ethanol was present in the bathing solution throughout the entire experiment). No significant difference was observed in the properties of mEPSCs recorded in the presence of ethanol (25–75 mM) versus cells from the HiCa group; the mean mEPSC amplitude for cells recorded in ethanol (75 mM) was 11.8 ± 2.5 pA (n = 5) during the baseline period versus 12.8 ± 1.0 pA (n = 8) for cells in the HiCa group (p > 0.05), whereas the mean mEPSC frequency for the ethanol group (75 mM) was 3.3 ± 0.3 versus 2.8 ± 0.3 Hz for the HiCa group (p > 0.05).

Ethanol (50 and 75 mM) displayed a concentration-dependent and highly efficacious inhibitory effect on the induction of DIP. Data from a representative cell in which depolarizing steps were delivered in the presence of 75 mM ethanol are depicted in Fig. 4. The mean mEPSC amplitude was 8.9 ± 0.3 pA during the baseline period and 10.5 ± 0.3 pA after cell depolarization, or 118% of baseline. The mean mEPSC frequency was 3.8 ± 0.4 Hz during the baseline period and peaked at 4.5 ± 0.3 Hz after depolarization, also an increase to 118% of baseline.

The magnitude of mEPSC amplitude and frequency potentiation after conditioning for all cells tested in this study is summarized in Fig. 5. This figure depicts the raw data from all cells (open squares) and the averaged mEPSC parameters (solid squares) observed after DIP-induction delivered in each condition [LoCa, HiCa, nifedipine, and ethanol (25, 50, and 75 mM)]. As described above, the LoCa group and the nifedipine groups did not exhibit any potentiation after the DIP-induction protocol. Importantly, ethanol displayed a distinct concentration-dependent inhibition of DIP of mEPSCs across a range pharmacologically relevant levels. A high concentration of ethanol (75 mM) completely disrupted DIP. The mean change in mEPSC amplitude at this concentration of ethanol was 108 ± 4% of baseline (n = 5), very significantly less than the HiCa group (Student’s t test; p < 0.05), whereas the mean change in mEPSC frequency for the ethanol group

![Fig. 3. Depolarization-induced potentiation requires activation of L-type voltage-gated calcium channels.](image-url)
was 91 ± 8% of control mEPSC frequency, a result that also differed significantly from the HiCa group (n = 8; Student’s t test; p < 0.05). At a lower concentration of ethanol (50 mM), the suppression of DIP was less marked but remained significant relative to the HiCa group (mEPSC amplitude enhancement, 146%; mEPSC frequency enhancement, 140%).

Fig. 5. Cumulative results show ethanol inhibition of L-VGCC dependent depolarization-induced potentiation. Cumulative data depicting mean peak mEPSC amplitude (A) and mean mEPSC frequency (B) after cell depolarization for individual experiments in each group (open symbols) and overall means for each group (filled symbols). Means are expressed as percentage of baseline (predepolarization) values [HiCa, 4 mM Ca²⁺ group (n = 8); LoCa, 2–3 mM Ca²⁺ group (n = 7); EtOH, 75 mM ethanol group (n = 5); NIFED, 20 μM nifedipine group (n = 4)].

Fig. 4. Ethanol inhibits the induction of depolarization-induced potentiation. A, mEPSCs recorded from a CA1 pyramidal cell before and after a train of depolarizing pulses, in the presence of 75 mM ethanol. B, cumulative frequency histogram of mEPSC event amplitudes for epochs before (open circles) and after (filled circles) the depolarizing train for the cell shown in A (number of events are shown in parentheses). C, cumulative frequency histogram of mEPSC interevent intervals for the epochs in B.
At the lowest concentration tested (25 mM, slightly greater than legal intoxication), the degree of DIP was not significantly reduced (although there was a nonsignificant trend toward reduced mEPSC amplitude enhancement relative to the HiCa group).

Comparisons of event distributions during the baseline period and after depolarization showed some variability for all cells but seemed greater in cells recorded in ethanol. For example, in three of five cells from the 75 mM ethanol group, the event amplitude distribution after depolarization was significantly different from the baseline distribution (Kolmogorov-Smirnov test; \( p < 0.05 \)). Two of these cells showed an increase in mean mEPSC amplitude (117 and 118% of baseline), whereas one cell showed a decrease in mean amplitude (97% of baseline) after depolarization. Similarly, in three of five cells, the event frequency distribution after depolarization was significantly different from baseline (Kolmogorov-Smirnov test; \( p < 0.05 \)). Only one of these cells showed an increase in mean frequency (118% of baseline) after depolarization, whereas two cells showed a decrease (76 and 81% of baseline). Nevertheless, these changes are minimal compared with the changes in mEPSC amplitude and frequency histograms in cells from the HiCa group. Therefore, ethanol markedly, and at certain concentrations (75 mM), completely inhibited VGCC-dependent potentiation of glutamatergic synaptic transmission without affecting basal transmission.

**Calcium Imaging.** Our laboratory and those of several others have demonstrated ethanol inhibition of NMDA receptor-dependent LTP. This observation, which raises the question of the pharmacological site of action of ethanol in the disruption of DIP, given that the present experiments, as well as the previously described studies from Nicoll’s laboratory, was conducted in the presence of the NMDAR receptor antagonist APV. To address this question, we sought to determine directly whether ethanol inhibits \( \text{Ca}^{2+} \) influx in CA1 dendrites in hippocampal slices prepared under the same conditions as those used for mEPSC recordings. To maximize our likelihood of eliciting a strong \( \text{Ca}^{2+} \) signal in the dendritic region and to investigate whether synaptic stimulation (rather than direct neuronal depolarization) could induce VGCC activation, we sought to induce \( \text{Ca}^{2+} \) responses via stimulation of Schaeffer collateral fibers using a standard monopolar stimulating electrode. Figure 6A depicts findings from \( \text{Ca}^{2+} \) imaging experiments in which standard slices were labeled with the membrane-permeant \( \text{Ca}^{2+} \) indicator Fura 2-AM. As in all previously described experiments, imaging was performed in the presence of the NMDA receptor antagonist (DL)-APV. Brief, high-frequency trains (200–500 ms, 100 Hz) reliably elicited dendritic \( \text{Ca}^{2+} \) responses that rapidly (within 20 s) returned to baseline levels. Ethanol (75 mM) profoundly and reversibly attenuated these \( \text{Ca}^{2+} \) signals to approximately 50% of the peak percentage of \( \delta \). These \( \text{Ca}^{2+} \) signals were elicited by L-VGCCs because they were also strongly inhibited by the L-VGCC antagonist verapamil (20 \( \mu \)M). The cumulative data summarizing the dendritic \( \text{Ca}^{2+} \) signals indicates that approximately one-half of the \( \text{Ca}^{2+} \) response was significantly inhibited when synaptic stimulation was delivered in ethanol or verapamil. Importantly, there was no additive inhibitory effect when ethanol and verapamil were combined, a finding that is pharmacologically consistent with a common site of action. Finally, all responses returned at least partially (≥50%) to baseline upon washout of either drug (Fig. 6B).

**Discussion**

**VGCCs and Synaptic Potentiation Mechanisms.** In this report, we provide new evidence concerning depressant effects of ethanol on synaptic information processing involving L-VGCC-dependent potentiation of mEPSCs. The DIP described here seems to be identical to the potentiation originally described in guinea pig hippocampus (Wyllie et al., 1994) in that the presently described phenomena was similarly dependent upon the presence of elevated extracellular \( \text{Ca}^{2+} \) and an intracellular ATP-regenerating system. Similarly reminiscent of previous studies, the potentiation described here is transient, with frequency and amplitude of mEPSCs returning to control levels within 35 to 40 min after the application of depolarizing pulses (Fig. 2), and sensitive to L-VGCC antagonists.

The physiological relevance of VGCC-dependent synaptic potentiation may seem at first glance to be diminished by its decremental nature. However, it is important to note that this transient potentiation can be converted to a stable, non-decrementing form under conditions of phosphatase inhibition (Wyllie and Nicoll, 1994), or, by extension, a physiological condition under which phosphatase activity may be
reduced such as that proposed by Maldve et al. (2002). The potentiation described here involves an increase in both the amplitude and frequency of mEPSCs. This finding indicates that DIP induction may involve changes at both pre- and postsynaptic sites at affected synapses, or, alternately, the activation of silent synapses via the activity-dependent membrane insertion of L-GHCs stored in subynaptic deposits (Shi et al., 1999). Finally, the potentiation described here and elsewhere does not require activation of NMDARs because it occurs in the presence of the NMDAR antagonist (DL)-APV. The sum total of the present electrophysiological data strongly supports an induction mechanism for hippocampal DIP, which centrally involves voltage-dependent Ca^{2+} influx.

L-VGCCs are expressed in the soma and proximal dendrites of hippocampal pyramidal neurons (Westenbroek et al., 1990) and support Ca^{2+} influx after dendritic depolarization (Regehr et al., 1989; Jaffe et al., 1992; Miyakawa et al., 1992). Increasing evidence demonstrates a role for strong activation of L-VGCCs in the promotion of excitatory synapses. For instance, LTP can be induced in hippocampal neurons by “pairing” back-propagated dendritic action potentials with trains of EPSPs (Magee and Johnston, 1997; Markram et al., 1997). In these experiments, high-frequency synaptic stimulation alone was subthreshold for LTP induction, whereas LTP could be induced when this stimulus was paired with action potentials in the postsynaptic neuron. Two aspects of these experiments are particularly salient. First, the potentiation observed using this pairing protocol can occur by activation of a single presynaptic neuron (Markram et al., 1997). Second, in the study by Magee and Johnston (1997), pairing-induced potentiation was blocked by nifedipine, indicating a critical role for L-VGCCs. These experiments suggest that LTP occurring in vivo may require the activation of L-VGCCs, in addition to NMDAR activation (Magee and Johnston, 1997).

Recently, an elegant study by Bauer et al. (2002) provided behavioral correlates for two pharmacologically distinct forms of synaptic potentiation in the lateral amygdala. Therein, pairing of presynaptic stimulation with spike-inducing postsynaptic depolarizing steps elicited VGCC-dependent synaptic potentiation, whereas tetanic stimulation inducing sustained subthreshold postsynaptic depolarization induced NMDA receptor-dependent LTP. Likewise, these distinct forms of synaptic potentiation were shown to play complementary roles in supporting fear conditioning to auditory stimulation in behaving animals. This fascinating finding is relevant to the study of ethanol abuse on an elemental level. In recent years, interest in the role of the amygdala in certain aspects of ethanol abuse has increased; indeed the National Institute on Alcohol Abuse and Alcoholism-Integrative Initiative on Alcoholism research consortium known as INIA-West is focused upon this structure in excessive ethanol intake. Therefore, the present finding that a form of synaptic potentiation (i.e., VGCC-mediated) that contributes substantially to fear conditioning in the amygdala is profoundly ethanol-sensitive at concentrations commonly associated with binge-level intoxication may have broad implications for understanding the role of excessive ethanol consumption in modulation of emotional processing by the mesocorticolimbic system.

**VGCC-Dependent Synaptic Effects of Ethanol.** We have demonstrated that acute application of ethanol across a range of pharmacologically relevant concentrations strongly inhibited the induction of DIP in hippocampal neurons (Figs. 4 and 5). An obvious candidate for the locus of inhibition is a direct action on L-VGCCs because this channel type is inhibited by ethanol in several preparations (Carlen et al., 1991; Mullikin-Kilpatrick and Treistman, 1994; Wang et al., 1994). To directly investigate this possibility, we examined ethanol effects on synaptically-evoked Ca^{2+} transients. Our findings are consistent with the concept that ethanol directly interacts with L-VGCCs to inhibit DIP because these Ca^{2+} signals were suppressed by ethanol and verapamil in a nonadditive manner, indicative of a common site of action. Furthermore, the use of electrical activation of synaptic inputs lends an additional level of physiological significance to the manner in which VGCC-dependent synaptic potentiation may indeed be induced (although it should be noted that the extent of VGCC-activating membrane depolarization may have differed between experiments using voltage steps and those using synaptic stimulation). Although our data do not directly address the mechanism of ethanol action at VGCCs, they indicate a strong sensitivity of VGCC mediated processes to ethanol, especially at concentrations of about 50 mM, frequently attained by intoxicated individuals. Most importantly, they provide evidence for a unique and indirect impact of ethanol on synaptic processing due to alterations in VGCC function.

Considerable published evidence exists that links L-VGCCs and the molecular mechanisms that underlie both adaptation to chronic ethanol exposure and withdrawal hyperexcitability. For instance, L-VGCC density is increased during chronic exposure, and hyperexcitability after ethanol withdrawal in vivo is inhibited by L-VGCC blockers (Dolin et al., 1987; Wu et al., 1987; Whittington and Little, 1991; Whittington et al., 1995, Gerstir et al., 1998). Whittington and Little (1989) also reported that dihydropyridines block hyperexcitability in hippocampal slices prepared from chronic ethanol-treated animals, whereas control slices were insensitive to these antagonists, a finding later reproduced in cultured Purkinje neurons (Gruol and Parsons, 1994).

In light of the above-mentioned studies, the present findings suggest that the inhibition of VGCC-dependent synaptic potentiation by ethanol may have implications for the pathophysiology of ethanol withdrawal. Indeed, the type of network overstimulation characteristic of the hippocampal circuitry during withdrawal hyperexcitability might be envisioned as an ideal induction stimulus for DIP, especially in light of the aforementioned studies in both hippocampus and amygdala in which pairing of pre- and postsynaptic stimuli proved efficacious in eliciting this phenomena. It follows that increases in VGCC number and function resulting from chronic ethanol exposure may facilitate the induction of DIP in this manner. In fact, it is possible that the experimental elevation of extracellular Ca^{2+} that the present study entailed would not be necessary for DIP induction in a setting of increases in VGCC activity induced by chronic ethanol exposure (alternately, this requirement of extracellular Ca^{2+} elevation may be a variation from conditions required for DIP induction in behaving animals imposed by in vitro recording). If, then, synaptic strengthening mediated by DIP is a phenomena that augments ethanol withdrawal hyperexcitability (or perhaps plays a role in the progression from electrophysiological hyperexcitability to frank electro-
graphic seizures), it follows that the attenuation of DIP by ethanol may play a role in the similar attenuation of the neurological symptoms of ethanol withdrawal by reinstatement of ethanol consumption.

The literature describing the effects of chronic ethanol exposure on VGCCs prompts the delineation between out-right Ca$^{2+}$ spiking behavior that may occur due to withdrawal from chronic exposure and the present results pertaining to modulation of information processing at the synaptic level. Therefore, an important research effort might involve identification of the relative role(s) of the alterations in VGCC number and function that are responsible for chronic ethanol effects or withdrawal hyperexcitability. Additionally, strong evidence exists for functional upregulation of NMDARs due to chronic ethanol exposure, which suggests an important role for these receptors in postwithdrawal hyperexcitability (Grant et al., 1990; Thomas et al., 1998; Thomas and Morrisett, 2000). It is likely that NMDAR and VGCC mechanisms interact in an additive or synergistic manner in mediating mechanisms of postwithdrawal hyperexcitability, although the relative role of these changes remains to be determined.

In summary, we have shown that ethanol inhibits a form of potentiation at hippocampal excitatory synapses that is dependent on activation of L-VGCCs and independent of NMDAR receptor activation. These experiments suggest that, in addition to its well established direct effects on NMDARs, ethanol may exert higher order effects on central synapses by altering the mechanisms of plasticity governing activity-dependent changes in excitatory synaptic transmission via an inhibitory action on VGCCs. The determination of the relative contributions of ethanol effects on NMDARs versus ethanol effects on VGCCs to this modification is an important step toward increasing our understanding of many alcohol-related brain disorders. Additionally, the present results represent an important recapitulation of elegant early studies describing ethanol inhibition of Ca$^{2+}$ influx (Harris and Hood, 1980; Leslie et al., 1983) and provide concrete evidence of the significance of this interaction with regard to ethanol effects on information processing at central synapses.

References


Address correspondence to: Dr. Richard Morrisett, The University of Texas at Austin, PHAR-Pharm 1, University Station, A191S, Austin, TX 78712-0125. E-mail: rmorrisr@mail.utexas.edu.