The Mechanism for Prejunctional Enhancement of Neuromuscular Transmission by Ethanol in the Mouse

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ABSTRACT

Ethanol has been shown to have both presynaptic and postsynaptic effects on synaptic transmission. However, the mechanisms by which ethanol affects evoked neurotransmitter release have not been studied at the mouse neuromuscular junction, a synapse at which binomial analysis of neurotransmitter release and measurements of prejunctional ionic currents can be made. Ethanol (400 mM) increased neurotransmitter release independently of both the cAMP and phorbol ester/Munc13 signaling pathways. Binomial analysis of neurotransmitter release revealed that ethanol increases the average probability of secretion without an effect on the immediately available store of the neurotransmitter. Application of ethanol also resulted in an inhibition of potassium currents in the motor nerve endings. These results suggest that the potentiating effects of ethanol on neurotransmitter release at the skeletal neuromuscular junction are mediated by an inhibition of the delayed rectifier potassium current, thus increasing both calcium entry into the nerve ending and the probability of neurotransmitter release. Identifying the mechanism through which ethanol enhances neurotransmitter release at the neuromuscular junction may be useful in determining the processes underlying the enhancement of neurotransmitter release at other synapses.

Introduction

Ethanol has been shown to exert its effects on synaptic transmission at a variety of loci in the mammalian nervous system (see, e.g., Liu and Hunt, 1999). Although there is a wealth of information on the effects of ethanol on specific proteins in the postsynaptic membrane, the mechanisms by which ethanol affects neurotransmitter release remain relatively unexplored. At the neuromuscular junction, ethanol has been reported to increase the release of the neurotransmitter acetylcholine (ACh) in both rat (Gage, 1965) and amphibian (Inoue and Frank, 1967) preparations over a wide range of concentrations (from 15 mM to 1 M ethanol). In addition, concentrations of ethanol as low as 40 mM have been reported to stimulate ACh release in the mammalian hippocampus (Henn et al., 1998). Similarly, the release of the inhibitory neurotransmitter GABA has been shown to be increased by ethanol at clinically relevant concentrations in cerebellar Purkinje neurons (Mameli et al., 2008). These results are suggestive of a commonality in the underlying process responsible for the increased neurotransmitter release produced by ethanol for these distinct neurotransmitter systems. However, the precise mechanisms governing these effects of ethanol on neurotransmitter secretion are unknown.

We previously found that, at the mouse neuromuscular junction, concentrations of ethanol ranging from 12 to 100 mM affected neuromuscular transmission by postjunctional actions but had no effect on neurotransmitter output (Searl and Silinsky, 2010). However, preliminary data suggested that higher concentrations of ethanol (>100 mM) increased evoked ACh release at mouse motor nerve endings. Investigation of the effects of these higher concentrations of ethanol on neurotransmitter release may serve both to resolve the contradictory record regarding the actions of ethanol at the skeletal neuromuscular junction and to help identify and eliminate potential mechanisms for ethanol action in the process of neurotransmitter release.

A wide variety of previously identified targets for ethanol action in neurons have been reported (Diamond and Gordon, 1997), including important signal transduction cascades present in nerve endings. For example, the adenylyl cyclase/cAMP pathway, which is known to exert modulatory actions on neurotransmitter release at many synapses (for a review, see Seino and Shibasaki, 2005), has been implicated in the actions of ethanol (Maas et al., 2005; Kelm et al., 2008). Activation of this pathway stimulates ACh release at the skeletal neuromuscular junction through both protein kinase

ABBREVIATIONS: ACh, acetylcholine; PKA, protein kinase A; EPP, end-plate potential; MEPP, miniature end-plate potential; EPC, end-plate current; MEPC, miniature end-plate current; PDBu, phorbol dibutyrate.
A (PKA)-dependent and PKA-independent mechanisms (Searl and Silinsky, 2008). In addition, the Munc13/syntaxin pathway, which is activated by diacylglycerol or phorbol esters, has been suggested to be an important target for ethanol (Fehr et al., 2005). Phorbol esters increase ACh release via an increase in the immediately available store of neurotransmitter using this pathway at the neuromuscular junction (Searl and Silinsky, 2003, 2008) and at other synapses (for a review, see Silinsky and Searl, 2003). It is also possible that the increases in neurotransmitter release produced by ethanol are mediated through direct interactions between ethanol and presynaptic membrane proteins, such as voltage-gated K+ channels and Ca2+ channels (Covarrubias et al., 1995; Brodie et al., 1999). Such effects on membrane ionic currents would lead to increases in the probability of release of preformed ACh quanta rather than changes in the availability of ACh. The aim of this study is to determine the mechanisms underlying the effects of ethanol on neurotransmitter release at the mouse neuromuscular junction.

Materials and Methods

Mice (B6129F2/J, 20–30 g in weight; The Jackson Laboratory, Bar Harbor, ME) were humanely anesthetized with 5% isoflurane for 3 to 5 min until they were unresponsive to touch, followed by cervical dislocation and exsanguination. This method is in accordance with guidelines laid down by our institutional animal welfare committee and the National Institutes of Health. Nerve-muscle preparations (phrenic nerve-hemidiaphragms) were then dissected and pinned in a recording chamber. Solutions were delivered by superfusion with a peristaltic pump and removed by vacuum suction. Electrophysiological recordings were made at room temperature (22–24°C) with an Axoclamp 2A amplifier (Molecular Devices, Sunnyvale, CA). Signals were fed into a personal computer using a Digidata 1200 A/D converter (Molecular Devices). Responses were recorded and analyzed using CDR, WCP, and SCAN programs (developed by J. Dempster, University of Strathclyde, Glasgow, Scotland).

For all experimental treatments, recordings were made from single end plates with each individual fiber serving as its own control. The data were further analyzed using Microsoft Excel, Corel Quattro Pro, and Sigma Plot and Sigma Stat software packages (SPSS Inc., Chicago, IL).

Electrophysiological Recording Methods. The majority of experiments used single-electrode intracellular voltage recordings for synaptic potentials, in which evoked responses were measured as end-plate potentials (EPPs) and spontaneous events were measured as miniature EPPs (MEPPs), or conventional two-electrode voltage-clamp techniques for synaptic currents, in which end-plate currents (EPCs) and miniature EPCs (MEPCs) were measured. In experiments in which intracellular recordings were made, microelectrodes were filled with 3 M KCl with resistances of 3 to 10 MΩ. Perineural recordings were made using electrodes filled with 1 M NaCl. Electrodes were placed just under the perineural sheath under visual control. Although not strictly membrane currents, these perineural electrophysiological recordings reflect local circuit currents produced by conductance changes that occur at the nerve endings. For convenience, these waveforms thus will be termed prejunctional currents. The prejunctional currents produce voltage changes in the extracellular space and thus are calibrated in millivolts (for further justification and details, see Silinsky, 2004). The motor nerve was stimulated at frequencies of 5 Hz for recording EPPs in the low Ca2+/high Mg2+ solutions and 0.5 Hz for recording the perineural waveforms. When EPPs or EPCs were measured at normal levels of ACh release, the frequency of stimulation was 0.05 Hz to eliminate the effects of endogenous adenosine derivatives released together with ACh (Redman and Silinsky, 1994).

Physiological Salt Solutions and Drugs. Control physiological saline solution (pH 7.2–7.4) consisting of 137 mM NaCl, 5 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, and 11 mM dextrose was used unless otherwise stated. For the low Ca2+/high Mg2+ solution, we used 0.35 mM CaCl2 and 3.5 mM MgCl2, except in experiments where the temperature of the superfusing physiological solution in the tissue chamber was raised to 32–36°C. In these experiments, we used 0.45 mM CaCl2 and 3.0 mM MgCl2 as a result of the reduction in quantal output at these temperatures (Hubbard et al., 1971). In experiments in strontium solutions, we used 1 mM SrCl2 and 2 mM MgCl2. Drugs were obtained from Sigma-Aldrich (St. Louis, MO).

Binomial Analysis of the Statistical Parameters of Neurotransmitter Release. Binomial analysis of EPPs in Sr2+ solutions was similar to that in previously published studies (Searl and Silinsky, 2008). A brief description follows:

\[ m = np, \]

where \( m \) is the mean number of quanta released, \( n \) is the number of quanta available for release, and \( p \) is the probability of the quanta being released. In the binomial distribution, the variance \( \text{var} \) is related to the average probability of release by the following equation:

\[ p = 1 - \frac{\text{var}}{m}. \]

In brief, the EPPs and MEPPs were collected with the EPPs corrected for nonlinear summation according to the method of McLachlan and Martin (1981). Binomial analysis of EPPs requires the use of a binomial model that incorporates the size and variance of the individual quantal amplitudes into the distribution (Miyamoto, 1975; Robinson, 1976; McLachlan, 1978). The value of \( p \) was determined from the following equation:

\[ p = 1 - \left( \frac{\text{var}^{2}_{\text{EPP}} + \text{var}^{2}_{\text{MEPP}}}{\text{EPP-MEPP}} \right). \]

where EPP is the mean EPP amplitude, \( \text{var}^{2}_{\text{EPP}} \) is the variance of EPP amplitudes, \( \text{var}^{2}_{\text{MEPP}} \) is the variance of MEPP amplitudes, and MEPP is the mean amplitude of the MEPPs (Miyamoto, 1975; McLachlan, 1978). Finally, \( n \) was determined by rearranging eq. 1:

\[ n = m/p, \]

where

\[ m = \frac{(\text{EPP})}{(\text{MEPP})}. \]

The phrenic nerve was stimulated at a rate of 0.2 Hz in these experiments to avoid anomalous results attributable to variance in \( n \) (Searl and Silinsky, 2002, 2003).

Statistical Methods. Comparisons were made by either parametric statistics (e.g., a Student’s paired t test) or nonparametric statistics (Mann-Whitney rank sum test; see Glantz, 1992). For more than two groups, an analysis of variance for normally distributed data was followed by multiple comparisons using the Bonferroni inequality (see Glantz, 1992; page 93). For the purpose of discussion of the results, differences between groups were considered significant when \( P < 0.05 \). Unless otherwise stated, \( n \) represents the number of single experiments carried out at single end plates on individual preparations. Data are presented as the mean ± 1 S.E.M.

Results

Application of 400 mM ethanol resulted in an increase in neurotransmitter release compared with the control. Figure 1 compares the evoked synaptic responses (EPPs) recorded under control conditions (Fig. 1, a and b) and in the presence of 400 mM ethanol (Fig. 1, c and d) in low Ca2+/high Mg2+ solutions. In the control condition, both superimposed
records [responses to 10 stimuli (Fig. 1a)] and EPPs in response to 750 consecutive stimuli. In these experiments, EPPs were recorded in a low Ca$^{2+}$/high Mg$^{2+}$ solution. Asterisk, the retouched stimulus artifact.

Fig. 1. The effects of 400 mM ethanol on the electrophysiological correlates of evoked neuromuscular transmission. Traces a and b show the control situation; c and d show the situation after the addition of 400 mM ethanol (for further discussion, see Results). Traces a and c show superimposed responses to 10 stimuli. Traces b and d show amplitudes of responses to 750 consecutive stimuli. In these experiments, EPPs were recorded in a low Ca$^{2+}$/high Mg$^{2+}$ solution. Asterisk, the retouched stimulus artifact.

Presynaptic modulators of secretion that stimulate the cAMP or Munc13/syntaxin signaling cascades have been shown to produce increases in spontaneous ACh release at skeletal neuromuscular junctions (Hirsh and Silinsky, 2002; Searl and Silinsky, 2008), and does so through phosphorylation via PKA and activation of specific presynaptic proteins that are

The concentration dependence of ethanol effects on EPP amplitudes (a), MEPP amplitudes (b), and the mean number of quanta released by a nerve impulse (c). Note that ethanol produces dose-dependent increases in nerve-evoked EPP and MEPP amplitudes. An increase in the evoked release of ACh is observed at 400 mM ethanol (c). Error bars represent the S.E.M.
not phosphorylated by PKA (Searl and Silinsky, 2008). In these experiments, forskolin was used to stimulate adenylate cyclase and hence produce both PKA-dependent and PKA-independent actions of cAMP (Botelho et al., 1988; Ozaki et al., 2000; Seino and Shibasaki, 2005). As shown in Fig. 3, the application of forskolin, while increasing evoked ACh release on its own, failed to occlude the enhancement of neurotransmitter release by ethanol. Specifically, in the presence of forskolin, ethanol increased evoked quantal release to $217.1 \pm 20.2\%$ of forskolin alone compared with the increase seen in the absence of forskolin, which was $165.8 \pm 18.9\%$ of control ($n = 5$ preparations for these experiments).

Another potential prejunctional target is the phorbol ester signaling system. There is good evidence that at the skeletal neuromuscular junction the effects of phorbol esters increase neurotransmitter release through actions on Munc13/syntaxin. The interactions initiated by phorbol esters increase the priming of the secretory apparatus with cholinergic synaptic vesicles independently of protein kinase C (Searl and Silinsky, 1998, 2003; Betz et al., 1998; Silinsky and Searl, 2003). Indeed, both syntaxin itself as well as syntaxin-binding partners have been implicated as potential targets for regulating behavioral responses to ethanol (Fehr et al., 2005). As shown in Fig. 4, an application of 100 nM phorbol dibutyrate (PDBu) increased ACh release to $190.0 \pm 24.8\%$ of the control, but it failed to occlude the actions of ethanol on ACh release. Specifically, in the presence of PDBu, 400 mM ethanol increased evoked quantal release to $219.0 \pm 29.9\%$ compared with phorbol alone, whereas in the absence of PDBu, EPPs were increased to $204.9 \pm 15.4\%$ of the control.

These results suggest that the effects of ethanol on neurotransmitter release at the mouse neuromuscular junction are independent of both adenyl cyclase and phorbol ester/Munc13 pathways. They also raise the possibility that the effects of ethanol are targeted more directly to membrane events associated with prejunctional ionic channels or the neurotransmitter release machinery.

We previously found that an application of binomial analysis to neurotransmitter release in Sr$^{2+}$ solutions can aid in identifying whether the transmitter release machinery or events associated with membrane ionic currents are responsible for changes in neurotransmitter release at the frog neuromuscular junction (Searl and Silinsky, 2008). It thus seemed possible that using similar procedures might be useful in identifying the site of action of ethanol at the mouse neuromuscular junction. In particular, these experiments were made to determine whether the increases in $m$ (the mean number of ACh quanta released) produced by ethanol are due to increases in the binomial parameter $n$ (the immediately available store of ACh quanta) or $p$ (the average probability of release, where $m = np$).

First, using 1 mM Sr$^{2+}$ and 2 mM Mg$^{2+}$ solutions, we tested the effects of phorbol esters on the binomial parameters of release. As described above, it has been shown that phorbol esters increase ACh release by activation of the presynaptic protein Munc13, which in turn interacts with syntaxin (Betz et al., 1998) to increase the immediately available store of ACh quanta; this is reflected by increases in the binomial parameter $n$ (Searl and Silinsky, 2003, 2008). As
shown in Fig. 5a, the increase in ACh release produced by phorbol esters at mouse motor nerve endings is also reflected as an increase in \( n \), without any changes in the probability of release (see the Fig. 5 legend for further details of all the binomial experiments).

The effects of increasing Sr\(^{2+} \) concentrations on the binomial parameters of secretion were then tested. Increasing Sr\(^{2+} \) concentration, which produces an increase in Sr\(^{2+} \)/H\(^{11001} \) entry via P-type Ca\(^{2+} \)/H\(^{11001} \) channels (Xu and Atchison, 1996; Silinsky, 2004), would be expected to increase \( p \) selectively. Indeed, increasing [Sr\(^{2+} \)] from 1 to 1.5 mM (Fig. 5b) resulted in an increase in \( m \) that was associated with an increase in the binomial parameter \( p \) without a significant change in \( n \). The results demonstrate that, in contrast to the interdependence of the binomial parameters in Ca\(^{2+} \)/H\(^{11001} \) solutions (Searl and Silinsky, 2003), binomial analysis of transmitter release in Sr\(^{2+} \) solutions (under these conditions) provides independent measures of the parameters of ACh release, \( n \) and \( p \) (Searl and Silinsky, 2008). Hence, the effect of ethanol on the binomial parameters of release in Sr\(^{2+} \) solutions was then investigated. As shown in Fig. 5c, the increase in release produced by 400 mM ethanol was confined to an effect on the probability of release (as reflected by the binomial parameter \( p \)).

The most likely mechanism for increasing the binomial parameter \( p \) is through increases in the entry of divalent ions into the nerve terminal, either through effects on the Ca\(^{2+} \)-
channels themselves or through inhibition of the nerve terminal K⁺ channels, leading to enhanced calcium entry (see, e.g., see figure 1 of Silinsky, 2004). The effects of ethanol on the motor nerve terminal currents were thus examined using the perineural recording method. A typical experimental result is shown in Fig. 6. In this experiment, the absolute magnitude of the potassium component of the perineural waveform (K⁺, Fig. 6a) was decreased from −1.89 ± 0.03 mV to −1.27 ± 0.04 mV in the presence of 400 mM ethanol (P < 0.001, n = 17 stimuli, averaged responses). This effect began within 15 s of superfusion with ethanol and was rapidly reversed, with recovery of the potassium component beginning within 15 s after the cessation of ethanol treatment (1.88 ± 0.02, n = 17 stimuli; Fig. 6c). In contrast to the effects of ethanol on potassium currents, the sodium component of the perineural waveform (Na⁺, Fig. 6a) was unchanged by ethanol in this experiment (−1.28 ± 0.02 mV in the control, −1.22 ± 0.05 mV in ethanol; P = 0.12) and four other experiments. In these five experiments, 400 mM ethanol produced reversible, highly significant decreases in the K⁺ component of the perineural waveform without a change in the Na⁺ component (for further details, see the Fig. 6 legend). It is generally believed that the component inhibited by ethanol reflects the voltage-activated K⁺ channel known as the delayed rectifier (Anderson et al., 1988), and inhibition of this current produces a large increase in the Ca²⁺ current that mediates evoked ACh release (see Silinsky, 2004).

Discussion

There are many potential mechanisms for modulating and enhancing neurotransmitter release, including those involved in regulating the behavior of nerve terminal voltage-gated ionic channels in addition to actions on signal transduction pathways downstream of ionic channels. Because both cAMP-regulated systems (Moore et al., 1998; Lai et al., 2007) and those systems regulated by phorbol esters (Fehr et al., 2005) have been implicated as potential targets for ethanol, we decided to investigate the potential involvement of ethanol in these pathways. However, we found that neither pretreatment with the adenylyl cyclase activator forskolin nor with phorbol dibutyrate had any measurable effect on the degree by which neurotransmitter release was potentiated by ethanol, suggesting that neither system is involved in the acute effects of ethanol on neurotransmitter release at the skeletal neuromuscular junction.

Given the rapidity of the effect of ethanol and its reversibility, two approaches were used to determine whether changes in prejunctional ionic currents or the immediately available store of ACh were directly affected by ethanol. Previous findings showed that at the frog neuromuscular junction, the application of binomial statistics in the presence of Sr²⁺ ions could provide insights into the prejunctional mechanisms underlying the modulation of neurotransmitter release. Applying this technique to the mouse neuromuscular junction in this study (Fig. 5), we found that phorbol esters (which act through Munc13/syntaxin to increase the number of primed vesicles) increased EPP quantal contents (m) through effects on the binomial parameter n, a parameter that reflects the number of primed vesicles available for release. By contrast, increasing m by raising Sr²⁺ concentrations caused an increase in ACh release through an effect on the parameter p, an effect consistent with an increased probability of release produced by increased alkaline earth cation entry into the nerve ending. Thus using these recording conditions, we could distinguish between differing mechanisms of action. Using this approach, we found that the increase in neurotransmitter release produced by 400 mM ethanol was entirely through an action on p, with no effect on the parameter n. A complementary approach to confirm and amplify the binomial experiments was then used, namely, measuring prejunctional ionic currents. The results showed that the component of the perineural waveform associated with the prejunctional K⁺ current was inhibited to an extent that would cause an increase in Ca²⁺ entry into the nerve ending (see Silinsky, 2004). This effect is consistent with the increase in the parameter p.

In earlier reports, the effects of ethanol on neurotransmitter release at the skeletal neuromuscular junction have been described as occurring at much lower concentrations (Gage, 1965; Inoue and Frank, 1967) than found in our study. One partial explanation for this might be the postjunctional interactions between ethanol and neuromuscular blockers that we recently described (Searl and Silinsky, 2010). Specifically, the spurious appearance of a prejunctional effect of ethanol is likely to occur if the effects of ethanol on EPPs in the presence of a reversible postjunctional blocking agent are compared with the effects of ethanol on MEPPs in the absence of a blockade. We should note that in this regard, the effects found in this study occurred in conditions both of low neurotransmitter output as well as at more physiological levels of release. In these latter studies, at normal levels of ACh release, we used α-bungarotoxin to allow recording end-plate responses rather than the more typical reversible nonpolarizing neuromuscular blockers, which produce the complex interactions with ethanol (see Searl and Silinsky, 2010).

It should also be noted that despite the combination of both facilitatory postjunctional and prejunctional mechanisms, ethanol at high concentrations is a known muscle relaxant. It
is likely that this muscle relaxant effect is due to the actions of ethanol on the muscle, in particular, through inhibiting muscle sodium channel activation (Inoue and Frank, 1967). In this regard, we note that we found it possible to record large magnitude EPPs (greater than 10 mV) in the presence of 400 mM ethanol, which normally would result in the production of action potentials and muscle contraction in control preparations.

Inhibitory effects of ethanol on potassium currents have been reported previously (Covarrubias et al., 1995; Brodie and Appel, 1998). It is generally believed that the K⁺ current inhibited in our studies of mouse motor nerve endings (see, e.g., Fig. 5) is the delayed rectifier (Anderson et al., 1988; Silinsky, 2004). Indeed, when the effects of ethanol were studied on potassium channels expressed in Xenopus oocytes, the delayed rectifier type of K⁺ channel appears to be inhibited by concentrations of ethanol similar to those that we used herein (see Fig. 1 in Anantharam et al., 1992). In addition, Appel et al. (2003) have shown that excitation by ethanol of dopaminergic ventral tegmental neurons results from inhibition of a delayed rectifier K⁺ current. Our results on the effects of ethanol on nerve terminal K⁺ currents are thus consistent with the published results on expressed K⁺ channels and those on cell bodies in the tegmentum. It should also be recognized, however, that other factors, including the precise genetic composition of the potassium channel (Covarrubias et al., 1995), the exact locale of the specific potassium channel, and even the specific composition of a region of the carboxyl terminus (Anantharam et al., 1992), may potentially affect the potency of ethanol as an inhibitor of K⁺ currents.

In summary, our results suggest that ethanol increases evoked ACh release at the neuromuscular junction by reducing the potassium current that normally repolarizes the nerve ending, enhancing Ca²⁺ entry into the nerve ending. This effect is reflected as an increase in the probability of ACh release without a change in the presynaptic store of releasable neurotransmitter.

References


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