The mechanism for pre-junctional enhancement of neuromuscular transmission by ethanol in the mouse.

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Running Title: The enhancement of ACh release by ethanol at motor nerves

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Non standard abbreviations: Phorbol dibutyrate (PDBu)
Abstract

Ethanol has been shown to have both pre-synaptic and post-synaptic 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 either the cAMP or 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 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 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). While there is a wealth of information on the effects of ethanol on specific proteins in the post-synaptic 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 as high as 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 12-100 mM affected neuromuscular transmission by post-junctional 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 the release of neurotransmitter may both serve to resolve the contradictory record regarding the actions of ethanol at the skeletal neuromuscular junction and additionally, help identify and eliminate potential mechanisms for ethanol action in the synaptic machinery governing neurotransmitter release.

There are a wide variety of previously identified targets for ethanol action in neurons (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 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 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 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 pre-synaptic 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 pre-formed ACh quanta rather than changes in the availability of ACh. The aim of this paper is to determine the mechanisms underlying the effects of ethanol on neurotransmitter release at the mouse neuromuscular junction.

**Methods**

Mice (B6129F2J, 20-30 g in weight), were humanely anaesthetized with 5% isoflurane for 3-5 minutes, until 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 Inc., 1311 Orleans Drive Sunnyvale, CA 94089-1136 USA). Signals were fed into a personal computer using a Digidata 1200 A/D converter (Molecular Devices Inc.). Responses were recorded and analysed using CDR, WCP and SCAN programs (Strathclyde University Software; John Dempster).

For all experimental treatments, recordings were made from single endplates 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 Illinois).

**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 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 3-10 MΩ. Perineural recordings were made using electrodes filled with 1 M NaCl. Electrodes were placed just under the perineural sheath under visual control. While 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 will thus be termed pre-junctional currents. The pre-junctional currents, produce voltage changes in the extracellular space and are thus calibrated in mV (for further justification and details, see Silinsky, 2004). The motor nerve was stimulated at frequencies of 5 Hz for recording of EPPs in the low Ca²⁺ high Mg²⁺ studies 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 to 7.4) consisting of (mM) NaCl, 137; KCl, 5; CaCl₂, 2; MgCl₂, 2; HEPES, 10; dextrose, 11 was used unless otherwise stated. For low Ca²⁺ / high Mg²⁺ solution; CaCl₂ was 0.35 mM and MgCl₂ was 3.5 mM except in experiments where the temperature of the superfusing physiological solution in the tissue chamber was raised to 32-36 °C. In these experiments, the CaCl₂ was 0.45 mM and MgCl₂ was 3.0 mM due to the reduction in quantal output at these temperatures (Hubbard et al., 1971). In experiments in strontium solutions SrCl₂ was 1 mM and MgCl₂ was 2 mM. Drugs were obtained from Sigma, St Louis, USA.

Binomial analysis of the statistical parameters of neurotransmitter release. Binomial analysis of EPPs in Sr²⁺ solutions was similar to that previously published (Searl and Silinsky, 2008). A brief description follows;

\[ m = n \, p \]  \hspace{1cm} (1')

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;

\[ p = 1 - \frac{(\text{var})}{m} \]  \hspace{1cm} (2)

Briefly, the EPPs and MEPPs were collected with the EPPs corrected for non-linear summation according to the method of McLachlan and Martin (1981).
Binomial analysis of EPPs requires the use of a binomial model which 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:

$$ p = 1 - \frac{S^2_{EPP}}{EPP \times MEPP} + \frac{\sigma^2_{MEPP}}{MEPP^2} $$

(3)

Where $EPP$ is the mean EPP amplitude, $S^2_{EPP}$ is the variance of EPP amplitudes, $\sigma^2_{MEPP}$ 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 eqn 2:

$$ n = \frac{m}{p} $$

(4)

where

$$ m = \frac{(EPP)}{(MEPP)} $$

(5)
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 non-parametric 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 means ± 1 S.E.M.

**Results**

Application of 400 mM ethanol resulted in an increase in neurotransmitter release compared with control. Figure 1 compares the evoked synaptic responses (EPPs) recorded under control conditions (a and b) and in the presence of 400 mM ethanol (c and d) in low Ca$^{2+}$/ high Mg$^{2+}$ solutions. In the control condition, both the superimposed records (responses to 10 stimuli in (a) and EPPs in response to 750 stimuli (b) reveal a larger number of failures and smaller size than EPPs made in the presence of 400 mM ethanol (c and d). The change in kinetics of the EPP and the increase in amplitude of the individual quantal responses are due to post-junctional effects (see Searl and Silinsky, 2010). The
reduced failure rate shown in Fig 1 suggests a pre-synaptic increase in ACh release in ethanol (for review see Silinsky, 1985).

These pre-synaptic effects of ethanol were confirmed by making direct measurements of the mean number of ACh quanta evoked by a nerve impulse \((m)\), from the ratio of the amplitudes of EPPs to MEPPs. It has been previously found that 100 mM ethanol had no effect on evoked ACh release at the mouse neuromuscular junction (Searl and Silinsky, 2010). However, as shown in figure 2, higher concentrations of ethanol produced large increases in the amount of transmitter release. Figure 2 shows concentration-response curves for ethanol on EPP amplitudes (a), MEPP amplitudes (b) and evoked quantal release (c). Note that 400 mM ethanol produced a highly significant increase in evoked ACh release to 175.3 ± 14.1 % of control \((P=0.001, n=5)\). Similar potentiating effects of 400 mM ethanol were also seen at higher temperatures. Specifically, in low calcium/high magnesium experiments where the preparation chamber’s bathing solution was heated to 32 - 36°C, average evoked quantal release was increased from 2.14 ± 0.88 quanta to 4.27 ± 1.065 quanta \((n=5; P=0.022)\); an increase of 199.5 % of control.

These experiments were made at low levels of neurotransmitter output using low Ca\(^{2+}\)/ high Mg\(^{2+}\) solutions such that measurements of both EPPs and MEPPs could be made simultaneously. In order to determine if these changes also occurred at normal levels of transmitter release, the effect of 400 mM ethanol on EPCs recorded in normal calcium solutions following treatment with the
irreversible neuromuscular blocking agent α-bungarotoxin was investigated. Unlike some competitive neuromuscular blockers, nicotinic receptor blockade by α-bungarotoxin is not modulated by ethanol (Searl and Silinsky, 2010). Under these conditions, when corrections for the increase in post-junctional sensitivity are made from the effects seen on MEPC amplitudes, evoked ACh release as determined from the EPC amplitudes were increased to 154.0 ± 11.27 % (n=5) compared with control. It thus appears that 400 mM ethanol increases ACh release both at low and normal levels of neurotransmitter secretion.

In addition to the effects of ethanol on evoked release, ethanol has been reported to produce increases in spontaneous ACh release reflected as increases in the frequency of occurrence of MEPPs at the mouse neuromuscular junction (Linder et al., 1984). In our experiments, application of 400 mM ethanol produced similar increases in MEPP frequency. Specifically, the mean frequency in control was 0.89 ± 0.21 MEPPs/sec and in ethanol 2.9 ± 0.66 MEPPs/sec, an increase of 333 ± 33% (P=0.014, n=5 preparations).

Pre-synaptic modulators of secretion that stimulate the cAMP or Munc13/syntaxin signaling cascades have been shown to produce increases in both evoked and spontaneous ACh release (Hirsh and Silinsky, 2002; Shapira et al., 1987; Searl and Silinsky, 2008), effects similar to those described above for ethanol. Also, these signal transduction pathways have been implicated in the actions of ethanol (Moore et al., 1998; Lai et al., 2007). If ethanol exerts its effects on neurotransmitter release through these pathways it might be expected...
that pre-activation of these pathways would occlude the action of ethanol. In these experiments, low Ca\(^{2+}\) / high Mg\(^{2+}\) solutions were used to allow the direct measurement of the number of ACh quanta released.

Activation of the cAMP signaling cascade can enhance evoked and spontaneous neurotransmitter release at skeletal neuromuscular junctions (Hirsh and Silinsky, 2002; Searl and Silinsky, 2008) and does so through phosphorylation via protein kinase A (PKA) and through by activation of specific pre-synaptic proteins that are 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 figure 3, 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 ± 20.2 % of forskolin alone compared to the increase seen in the absence of forskolin which was 165.8 ± 18.9 % of control (n=5 preparations for these experiments).

Another potential pre-junctional target is the phorbol ester signaling system. There is good evidence that at the skeletal neuromuscular junction the effects of phorbol esters to increase neurotransmitter release is through actions on Munc 13/syntaxin. The interactions initiated by phorbol esters increase the priming of the secretory apparatus with cholinergic synaptic vesicles independently of
protein kinase C (Searl & 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 figure 4, application of 100 nM Phorbol dibutyrate (PDBu) increased ACh release to 190.0 ± 24.8 % of control, but 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 ± 29.9 % compared with phorbol alone whereas in the absence of PDBu EPPs were increased to 204.9 ± 15.4 % of control.

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

We previously found that 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 employing similar procedures might be useful in identifying the site of action of ethanol at the mouse neuromuscular junction. In particular, these experiments were made in order to determine if 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, whereby $m=np$).

First, using 1 mM Sr$^{2+}$ and 2 mM Mg$^{2+}$ solutions, the effects of phorbol esters were tested 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 figure 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 Figure 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$^{2+}$ entry via P-type Ca$^{2+}$ channels (Xu and Atchison, 1996; Silinsky, 2004) would be expected to increase $p$ selectively. Indeed, increasing [Sr$^{2+}$] from 1 mM to 1.5 mM (figure 5b) resulted in an increase in $m$ which was associated with an increase in the binomial parameter $p$ without significant change in $n$.

The results demonstrate that, in contrast to the interdependence of the binomial parameters in calcium Ca$^{2+}$ 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 \( \text{Sr}^{2+} \) solutions was then investigated. As shown in figure 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 \( \text{Ca}^{2+} \) channels themselves or through inhibition of the nerve-terminal \( \text{K}^{+} \) channels, leading to enhanced calcium entry (see e.g. Silinsky 2004, Figure 1). 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 Figure 6. In this experiment, the absolute magnitude of the potassium component of the perineural waveform (Figure 6a, \( K^+ \)) was decreased from \(-1.89 \pm 0.03 \text{ mV}\) to \(-1.27 \pm 0.04 \text{ mV}\) in the presence of 400 mM ethanol \((P<<0.001, n=17 \text{ stimuli averaged})\). This effect began within 15 seconds of superfusion with ethanol and was rapidly reversed, with recovery of the potassium component beginning within 15 seconds after the cessation of ethanol treatment (Figure 6c, \(1.88 \pm 0.02, n=17 \text{ stimuli}\)). In contrast to the effects of ethanol on potassium currents, the sodium component of the perineural waveform (Figure 6a, \( \text{Na}^+ \)) was unchanged by ethanol in this experiment \((-1.28 \pm 0.02 \text{ mV in control}; -1.22 \pm 0.05 \text{ mV in ethanol}; P=0.12\)). In all five experiments, ethanol (400 mM) produced reversible, highly significant decreases in the \( K^+ \) component of the perineural
waveform without a change in the Na\(^+\) component (for further details see Fig 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 Ca\(^{2+}\) current that mediates evoked ACh release (see Silinsky, 2004, Figure 1).

**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. As both cAMP regulated systems (Moore et al., 1998; Lai et al., 2007) and those regulated by phorbol esters (Fehr et al., 2005) have been implicated as potential targets for ethanol, it was decided to investigate the potential involvement of ethanol in these pathways. However, as found here, neither pre-treatment with the adenylate 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 employed to determine if changes in pre-junctional ionic currents or the immediately available store of ACh were directly affected by ethanol. Previously it
was found at the frog neuromuscular junction, that the application of binomial statistics in the presence of Sr\(^{2+}\) ions can provide insights into the pre-junctional mechanisms underlying the modulation of neurotransmitter release. Applying this technique to the mouse neuromuscular junction in this paper (Figure 5) it was found that phorbol esters (which acts 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. In contrast, increasing \(m\) by raising Sr\(^{2+}\) 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, it is possible to distinguish between differing mechanisms of action. With this approach, it was 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 employed, namely measuring pre-junctional ionic currents. The results showed that the component of the perineural wave form associated with the pre-junctional K\(^+\) current was inhibited to an extent that would cause an increase in Ca\(^{2+}\) 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 has been described as occurring at much lower
concentrations (Gage, 1965; Inoue and Frank, 1967) than found by us in this study. One partial explanation for this might be the post-junctional interactions between ethanol and neuromuscular blockers that we recently described (Searl and Silinsky, 2010). Specifically, the spurious appearance of a pre-junctional affect of ethanol is likely to occur if the effects ethanol on EPPs in the presence of a reversible post-junctional blocking agent is compared with the effects of ethanol on MEPPs in the absence of blockade. It should be noted in this regard that the effects found in this study occurred in conditions both of low neurotransmitter output, and also 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 usual, reversible non-depolarizing 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 post-junctional and prejunctional mechanisms, ethanol, at high concentrations, is known as 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 it should be noted 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. Figure 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 employed 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 pre-synaptic store of releasable neurotransmitter.
References


Footnotes:

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Legends for Figures

Figure 1 The effects of ethanol (400 mM) on the electrophysiological correlates of evoked neuromuscular transmission. Traces in (a) and (b) show control situation, (c) and (d) after 400 mM ethanol (for further discussion-see text). In these experiments, EPPs were recorded in low Ca2+ - high Mg2+ solution. * is the stimulus artifact which has been retouched.

Figure 2 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 produced dose dependent increases in nerve-evoked EPP amplitudes and MEPP amplitudes. An increase in the evoked release of ACh was observed at 400 mM ethanol (c). Error bars represent the SEM.

Figure 3 The effect of pretreatment with forskolin (50 mM) on the actions of 400 mM ethanol on neurotransmitter release. As shown here, the adenylate cyclase activator forskolin had no effect on the potentiation of neurotransmitter release by ethanol. Error bars represent the SEM. For further details, see text.

Figure 4 The effect of pretreatment with phorbol dibutyrate (100 nM) on the actions of 400 mM ethanol on neurotransmitter release. As shown here, the presence of the phorbol ester phorbol dibutyrate (100 nM) had no effect on the increase in neurotransmitter release produced by 400 mM ethanol. Error bars represent the SEM. For further details, see text.

Figure 5. Comparison of the effects of activating the phorbol ester signaling system (a) increasing Sr2+ concentrations (b) and ethanol (c) on the binomial
parameters of release in Sr2+ solutions. The parameters are the mean number of ACh quanta released by a nerve impulse (m), the immediately available store of ACh quanta (n) and the average probability release (p). Note the increase in m(=np) produced by phorbol ester is due to an increase in n. Specifically, m was 1.8 ± 0.2 quanta in control versus 3.4 ± 0.5 quanta in 100nM PDBu, P=0.0014; n was 4.9 ± 0.7 quanta in control versus 8.5 ± 1.3 quanta in PDBu (P = 0.035); the probability of release (p) was 0.39 ± 0.04 in control versus 0.40 ± 0.01 in PDBu, P = 0.813). In contrast, the increase in m produced either by ethanol by increasing [Sr2+] is due to an increase in p. Specifically m was 1.7 ± 0.4 quanta in 1 mM Sr2+ and 4.0 ± 0.9 quanta in 1.5 mM Sr2+ (P=0.014); n was 6.2 ± 0.7 quanta in 1 mM Sr2+ and 7.9 ± 0.7 quanta in 1.5 mM Sr2+ (P = 0.124 and p was 0.28 ± 0.05 in 1 mM Sr2+ and 0.56 ± 0.09 in 1.5 mM Sr2+ (P=0.026; n=5 preparations). The increases in m produced either by ethanol is also do exclusively to an increase in p. Specifically, m was 3.2 ± 0.4 quanta in control versus 5.3 ± 0.4 quanta in ethanol, (P<0.001); n was 8.3 ± 0.4 quanta in control versus 8.7 ± 0.4 quanta in ethanol (P=0.67) and p was 0.39 ± 0.03 in control versus 0.62 ± 0.05 in ethanol (P=0.007). Error bars represent the SEM.

Figure 6 The effects of ethanol on pre-junctional ionic currents.

Note the reversible reduction in the K+ current component of the perineural recording by ethanol without an effect on the Na+ sodium component. Trace a shows control, b shows the effects of 400 mM ethanol, c shows the wash. The recordings were made in normal physiological salt solutions using (+)-
tubocurarine to reduce the EPPs below threshold. The data are the average responses to 17 stimuli (0.5 Hz). For further details see text and Silinsky 2004. As mentioned in the text, in 5 of 7 experiments, no change in the Na+ current was associated with changes in the K+ currents. In the remaining 2 experiments a reversible decrease in the Na+ current was also observed.