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The interaction of ethanol with reconstituted synaptosomal plasma membrane Ca^{2+}-ATPase

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Abstract

The primary effect of ethanol is on the central nervous system. However, the molecular mechanisms responsible for the physiological symptoms of ethanol intoxication are still unknown. Low concentrations of ethanol were observed to stimulate the activity of the calcium pump from reconstituted synaptosomal plasma membrane Ca^{2+}-ATPase (PMCA), and ethanol inhibited Ca^{2+}-ATPase activity at concentrations above 5%. The greatest stimulating effect was obtained with 5% (v/v) ethanol and was lipid-dependent, being 74% when the protein had been reconstituted in phosphatidylcholine (PC) and less when the reconstituted protein had previously been activated by calmodulin or after removal of a 9-kDa autoinhibitory site by controlled trypsinization. Stimulation of the pump by ethanol was lower for the native or trypsin-digested protein in the presence of phosphatidylserine than in PC. These results suggest a direct ethanol–protein interaction, because the activating effect depended on the state of Ca^{2+}-ATPase (native or truncated, or in presence of calmodulin). The activating mechanism of ethanol may involve opening an autoinhibitory domain located close to the calmodulin binding domain.

Keywords: Synaptosomal calcium pump; Ethyl alcohol; Activity

1. Introduction

Calcium regulates various cell functions and neuronal processes. The resting intracellular concentration of free calcium is in the nanomolar range while the extracellular calcium concentration is in the millimolar range. In response to specific signals, the concentration of calcium in the cytosol increases to trigger a variety of effects, such as action potential generation or release of neurotransmitters at synapses, inter alia, thereby playing a key role in synaptic transmission [1]. It is therefore very important that excess calcium is removed from the cytosol [2] in order to avoid possible neurological disorders due to high levels of intracellular calcium. Plasma membrane Ca^{2+}-ATPase (PMCA) has a high affinity for calcium ($K_{m(Ca)}$<300 nM) and is directly involved in pumping Ca^{2+} out of the cell. The ATPase is encoded by alternatively spliced transcripts from four genes [3–6]. In addition, the primary gene transcripts can be spliced. The synaptosomal protein has been purified and characterized [7–10]. A common feature of the PMCA pump from different tissues is that it contains an autoinhibitory domain in the C-terminal region that reduces the activity of the protein by hindering Ca^{2+} access and ATP binding [11,12]. Calmodulin (CaM) binds with high affinity to the protein, opening the autoinhibitory domain and stimulating its activity. Phospholipids and controlled proteolysis are also stimulators of PMCA activity [7,11–13].

The effects of ethanol have long been associated with its action on the lipid membrane [14,15]. Several studies have shown, however, that alcohols affect membrane

Abbreviations: CaM, calmodulin; PMCA, plasma membrane Ca^{2+}-ATPase; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PC, phosphatidylcholine; PS, phosphatidylserine

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proteins from erythrocytes [16–18]. Changes in neuronal calcium levels may be an important aspect of ethanol neurotoxicity [15]. It is therefore of interest to investigate the interaction of ethanol with structures involved in calcium homeostasis in the nervous system, such as synaptosomal PMCA. In the present work, we investigated the effect of ethanol on PMCA purified from pig brain. The results suggest a direct interaction of ethanol with the protein, this interaction being strongly dependent on the conformational state of the reconstituted protein in the presence of different lipids.

2. Materials and methods

2.1. Isolation of purified PMCA

The fractions were prepared from adult pig brain, as described by Salvador and Mata [7]. Briefly, synaptic plasma membrane (SPM) vesicles were prepared by osmotic lysis of synaptosomes. The Ca^{2+}-ATPase was obtained by solubilization of SPM with 0.6% (w/v) Triton X-100 and loading the solubilized proteins onto a calmodulin affinity column. The purified fraction was eluted in a delipidated form with a buffer containing 15% glycerol, 0.06% Triton X-100, and 2 mM EDTA. The protein content was measured by the Bradford method [19].

2.2. Ca^{2+}-ATPase activity

The ATPase activity was determined using a coupled enzyme assay, measuring the rate of decrease in NADH concentration at 25 °C in a medium containing 10 IU pyruvate kinase and 28 IU lactate dehydrogenase in 1 ml of a buffer containing (in mM): 50 HEPES/KOH pH 7.4, 100 KCl, 5 Na_3N, 2 $MgCl_2$, 0.00316 free Ca^{2+}, 0.11 NADH, and 0.42 phosphoenolpyruvate. The purified ATPase (5 μg) containing 0.06% Triton X-100 was mixed with 5.3 μg of total phospholipids (phosphatidylcholine (PC) type XI-E from egg yolk and/or phosphatidylserine (PS) from bovine brain; Sigma) previously dried as specified in Palacios et al. [13], incubated for 3 min at 25 °C and then diluted in the assay medium. Bovine brain calmodulin (0.32 μg/ml) was also added when indicated directly into the reaction medium. The SPM vesicles (40 μg) were added directly to the assay medium containing 100 nM of thapsigargin, in order to inhibit the ATPase activity due to SERCA protein. Samples were incubated for 4 min at 25 °C, and the reaction was started by adding 1 mM ATP. Ethanol at the indicated percentage was added immediately after ATP addition. The Ca^{2+}-ATPase activity of membrane vesicles was determined from the total activity by subtraction of the Mg^{2+}-ATPase activity obtained on adding 3 mM EGTA. The significance of the increases in activities in ethanol with respect to control activities was determined by the one-tailed Wilcoxon signed-rank test (paired samples), and differences between groups were evaluated by the one-tailed Wilcoxon rank-sum test (unpaired samples).

2.3. Trypsin digestion

The delipidated Ca^{2+}-ATPase (0.18 mg/ml) was incubated for 20 min with TPCK-trypsin (Sigma) at a ratio of 10 mg ATPase/mg trypsin. The Ca^{2+}-ATPase activity was determined as described above after a preliminary incubation for 4 min at 25 °C of 5-μg purified protein with the phospholipid indicated.

2.4. Electrophoresis and immunoblotting

Electrophoresis was performed by the method of Laemmli [20], using 6.5% polyacrylamide gels. Protein transfer to PVDF membrane was carried out in a semi-dry system from Bio-Rad. Immunodetection was performed using the following primary antibodies: a pbPMCA polyclonal antibody raised against the purified synaptosomal PMCA from pig brain [7]; the monoclonal antibody 5F10 against purified human PMCA from erythrocytes (Affinity Bioreagents) that binds to all PMCA isoforms [21,22]; and four PMCA isoform specific antibodies kindly provided by Prof. J. Penniston (Mayo Clinic Foundation, Rochester, MN).

3. Results

Although the purified synaptosomal PMCA is inactive because it is delipidated during the purification procedure [7], it can be fully reactivated by reconstitution in phospholipids, the reactivation being dependent on phospholipid structure and on the native or truncated form of the protein [7,13]. The Ca^{2+}-ATPase activity of the reconstituted PMCA is regulated by ethanol in a dose-dependent manner. Thus, Fig. 1 shows that low concentrations of ethanol stimulated the activity of the PMCA reconstituted in PC, a maximum activation of 74±5% (Wilcoxon signed-rank test, $P<0.002$) being reached at 5% (v/v) ethanol. However, at concentrations above 5%, ethanol inhibited the ATPase activity. The activating effect of ethanol on the native protein was always greater (Wilcoxon rank-sum test, $P<6.10^{-5}$) in the absence of the activator calmodulin than in its presence, and activation was also less (Wilcoxon rank-sum test, $P<0.001$) for the truncated protein. The dual effect of ethanol on the activity was also seen when the native or trypsin-digested protein was reconstituted in the acidic phospholipid PS (Fig. 2).

To test for possible competition between calmodulin and ethanol for binding to the protein in the presence of PC, activities were measured following the addition of calmodulin and ethanol at the concentrations that produced maximum activation. As shown in Fig. 3, direct addition of

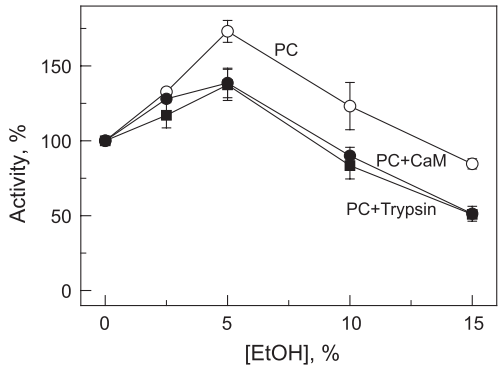


Fig. 1. Effect of ethanol on the Ca^{2+}-ATPase activity of the purified PMCA reconstituted in PC. The ATP hydrolysis of the native protein (5 μg) was measured as indicated in Section 2, in the absence (○) or presence (●) of 0.32 μg/ml calmodulin, and for the trypsin digested protein (■). Data are means±S.D. (bars) of six experiments performed in duplicate. Values in the presence of 5% ethanol are significantly different ($P<0.002$ ○, $P<0.003$ ●, and $P<0.02$ ■) from those without ethanol. The 100% activity corresponds to 0.91±0.7, 1.73±0.25 and 1.73±0.11 μmol phosphate min^{-1} mg^{-1} of protein for the native PMCA in the absence or presence of calmodulin and for the trypsinized protein, respectively.

5% ethanol to PC-reconstituted native PMCA caused a 73±2.7% stimulation of ATPase activity. The further addition of calmodulin increased this activity to a maximum value of 2.70±0.1 μmol Pi min^{-1} mg^{-1}. The addition of calmodulin to the PC-reconstituted native PMCA in the absence of ethanol resulted in an 87% increase in activity, and the subsequent addition of 5% ethanol produced a further 51% stimulation, the final activity being the same as when ethanol was added first, followed by calmodulin. Fig. 3 also shows the activating effect of 5% ethanol on trypsin-treated PMCA. The activity of the PC-reconstituted protein was greatly increased by ~86% after its treatment with trypsin, and the addition of ethanol resulted in a further ~63% increase, the final activity being the same as for the native PMCA in the

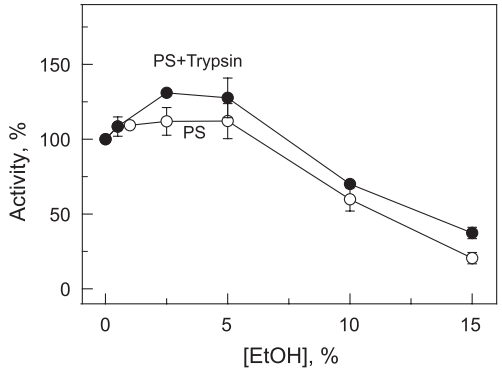


Fig. 2. Effect of ethanol on the Ca^{2+}-ATPase activity of the native (○) and the trypsinized (●) PMCA reconstituted in PS. The activity of the protein (5 μg) was measured as indicated in Section 2. Data are means±S.D. (bars) of four experiments performed in duplicate. The 100% activity corresponds to 2.33±0.3 and 1.81±0.16 μmol phosphate min^{-1} mg^{-1} of protein for the native and the trypsinized PMCA, respectively.

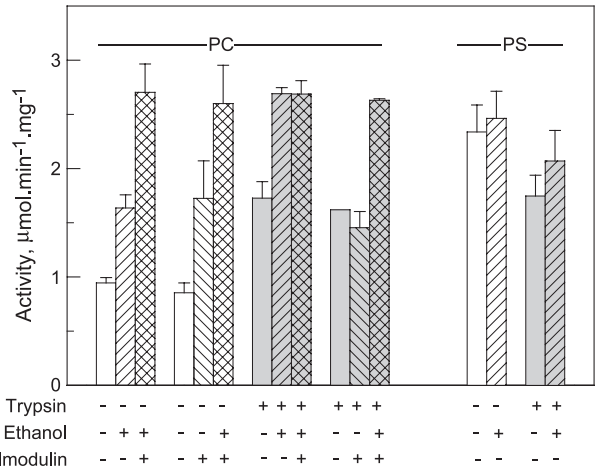


Fig. 3. Effect of 5% ethanol, 0.32 μg/ml calmodulin, and trypsin on the activity of the Ca^{2+}-ATPase reconstituted in PC or PS. The activity determination and treatment with trypsin were as described in Section 2. Data are means±S.D. (bars) of five experiments performed in duplicate and from different standard preparations.

presence of ethanol and calmodulin. The activity of the truncated form was not enhanced by calmodulin, but did increase ~68% after the subsequent addition of ethanol. When PS was used to reconstitute the protein, a standard preparation of the native PMCA showed an initial activity value of 2.33±0.30 μmol Pi min^{-1} mg^{-1}, which was only about 5% stimulated by ethanol, the final activity again being the same as observed in the other systems. The

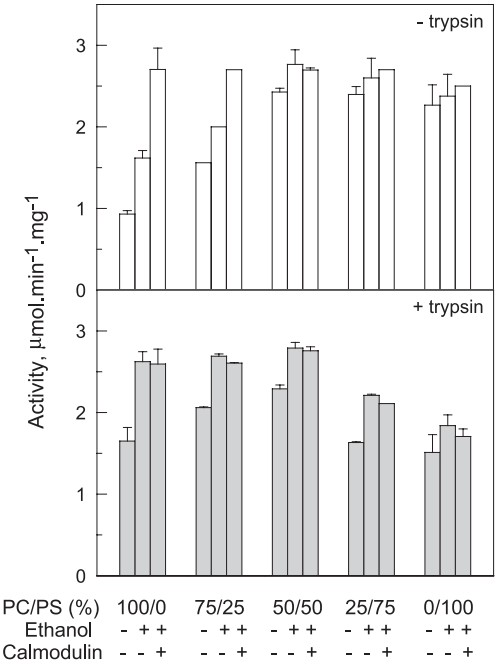


Fig. 4. The effect of ethanol, calmodulin, and trypsin on the ATPase activity of reconstituted PMCA as a function of lipid composition. All conditions were as described for Fig. 3 except that the protein (5 μg) was reconstituted with 5.3 μg of total phospholipids, varying the percentages of PS and PC as indicated. Data are means±S.D. (bars) of six experiments performed in duplicate.

initial activity of the truncated protein in PS was, however, only 1.81±0.16 μmol Pi min^{-1} mg^{-1}, and was stimulated by 29±8% on addition of 5% ethanol. Since the low activity of the fragment could have been related to the nature of the lipid used for reconstitution, the PMCA (5 μg) was reconstituted in different percentage mixtures of PC and PS (26.5 μg of total lipids), and its activities were measured as above, in its native state or after its digestion by trypsin. As can be observed in Fig. 4, the activity of the native protein increased by factors of up to 2.5 with the additional PS content, the maximum increase being for a PC/PS ratio of 1:1. With the addition of PS, the activating effects of ethanol and calmodulin diminished, with no effect at PC/PS ratios less than or equal to 1:1. It can also be observed that the stimulation by calmodulin appeared to be additive to that of ethanol since it always reached the maximum activity. This value did not change significantly with an increasing amount of PS in the reconstitution medium. With respect to the trypsinized fragment, the activity also increased by addition of PS, again reaching the maximum activity at a PC/PS ratio of 1:1. However, PC/PS ratios less than or equal to 1:1 led to about 25% reduction of the basal activity. Ethanol, but not calmodulin, stimulated the activity by about 65% in pure PC, this stimulatory effect being less in the presence of PS down to 20–25% for a PC/PS ratio less than or equal to 1:1.

The dual effect of ethanol was also observed under more physiological conditions, using synaptosomal plasma membrane vesicles (Fig. 5).

In order to assign the effect of ethanol in the brain to specific PMCA isoforms, an immunoblot of the purified fraction was performed (Fig. 6), using a pbPMCA antiserum raised against the purified fraction [7], the antibody 5F10 that cross-reacts with all isoforms in different tissues [21,22], and antibodies specific for the four PMCA isoforms [22]. The pbPMCA antiserum (Lane 1) labeled a narrow band around 130 kDa, while the stain with the 5F10 antibody (Lane 2) appeared as a broad band corresponding to different isoforms of the PMCA pump [22]. The

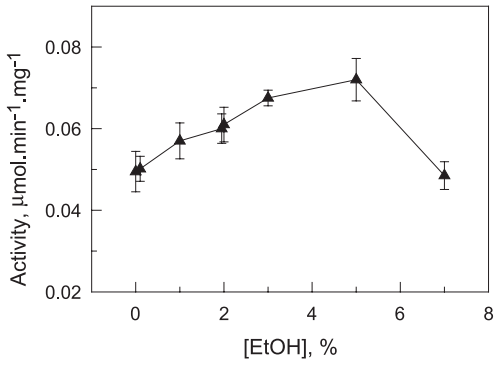


Fig. 5. Effect of ethanol on the Ca^{2+}-ATPase activity of synaptosomal plasma membrane vesicles. The enzymatic activity of the protein (40 μg) was measured as indicated in Section 2. Data represent means±S.D. (bars) of five experiments performed in duplicate.

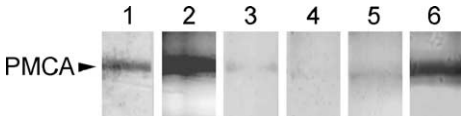


Fig. 6. Identification of isoforms in the pig brain purified PMCA. Electrophoresis was performed in a 6.5% Laemmli gel. The gel was cut into strips, and each strip (containing 5 μg of protein) was incubated with one of the following primary antibodies: lane 1, antiserum (1:100 dilution) raised against the pig brain purified PMCA; lane 2, monoclonal antibody 5F10 (1:2000 dilution) that recognized all PMCA isoforms; lanes 3, 4, and 5, polyclonal antibodies NR1, NR2, and NR3 (1:100 dilution) raised against rat brain PMCA isoforms 1, 2, and 3, respectively; lane 6, monoclonal antibody JA9 (1:800 dilution) that selectively binds to rat brain isoform PMCA4. The blot presented is representative of three assays performed with different preparations of purified PMCA.

immunoreaction of the purified fraction was very weak with all the isoform-specific antibodies except with that specific for PMCA4 (Lane 6).

4. Discussion

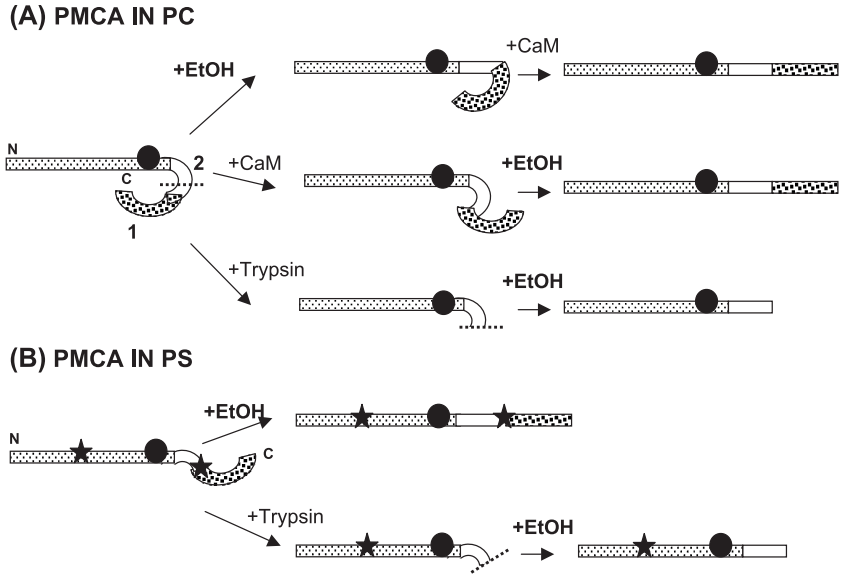
Ethanol at low concentrations stimulated the activity of purified synaptosomal PMCA after its reconstitution in PC or PS. The activation was dependent on the lipid, on the presence of the activator calmodulin, and on the protein state. Thus, the native delipidated ATPase reconstituted in PC (Figs. 1 and 3) had lower activity in the absence of calmodulin than in its presence, and a lower activity than the trypsin-digested protein. In PC, the C-terminal region of ATPase acts as an autoinhibitory domain in the absence of calmodulin [23], leading to a low activity (Fig. 1). We found that the activity in this state was very sensitive to 5% ethanol. Calmodulin binds with high affinity to ATPase, removing the effect of the autoinhibitory domain and stimulating PMCA activity. A similar level of stimulation is seen on digestion with trypsin because of removal of the inhibitory domain. In both cases, the activity of the protein is further stimulated by ethanol (Fig. 3). The somewhat lower increase of the ATPase activity produced by ethanol on the native protein in the presence of calmodulin or after trypsin treatment strongly suggests that the effect of ethanol is mediated by direct binding to the protein, because in all cases the protein was reconstituted in PC. The activation that ethanol produces in synaptosomal PMCA is similar to that previously reported in human erythrocyte PMCA eluted in PC, and has also been related to a protein–drug interaction [16–18]. Indeed, Suju et al. [17] suggested the presence of a putative autoinhibitory "site" on the PMCA as the ethanol binding site, besides the autoinhibitory calmodulin binding domain. Cervino et al. [18] observed an isoform-dependent activation of PMCA by ethanol, suggesting that the observed differences were related to interaction of ethanol with the membrane protein rather than with the lipid bilayer, since the isoforms only differ in a C-tail which is not membrane intrinsic.

The observation that calmodulin still activates the synaptosomal PMCA in the presence of ethanol (Fig. 3) can be understood in terms of the model depicted in Scheme 1. The scheme indicates that ethanol could interact with brain PMCA at an autoinhibitory domain (site 2) adjacent to the inhibitory calmodulin binding domain (site 1) in the C-terminal region. The binding of ethanol to site 2 could open this domain and partially remove site 1, thus stimulating ATPase. Subsequent binding of calmodulin to site 1 would completely remove the domain from the active site, increasing the activity of the protein to its maximum value. The additive effect of ethanol and calmodulin suggests that binding of ethanol to the protein does not interfere with calmodulin binding. If ethanol is added to the protein already activated by calmodulin, a further activation is produced because ethanol could completely displace the C-terminal autoinhibitory domain. In the presence of trypsin, ATPase activity increases because of the removal of the calmodulin binding domain, but the ethanol binding site 2 must still be present because ethanol increases the activity of the fragment.

The stimulation of PMCA activity by ethanol was different when the synaptosomal protein had been reconstituted in the acidic phospholipid PS (Figs. 2 and 3). Although the mechanism of PMCA activation by acidic phospholipids is unknown, it has been reported that these lipids bind to the protein at two separate phospholipid binding sites, activating the protein [24,25], and that the proteolyzed Ca^{2+}-ATPase lacks one of these sites [25] (see Scheme 1). Ethanol increased the activity of the native PMCA slightly, and the activity of the trypsin fragment more extensively. In both conformations, ethanol interacts with site 2, stimulating the maximum activity. The lower activity of the trypsinized fragment with respect to that of the native PMCA reconstituted in PS could be due to a loss of stability of the fragment in the acidic phospholipid, because the trypsinized fragment presents increasing activity with increasing PC content in the reconstitution medium relative to PS (Fig. 4). Suju et al. [17] report greater activation of erythrocyte PMCA by ethanol than by PS or other acidic phospholipids, the greatest stimulation being when ethanol was added in the presence of PS. They used a non-delipidated protein eluted and stored in PC, and found a maximum twofold stimulation by ethanol when lipids were present at a PC/PS ratio of 1:9. However, Fig. 4 shows no significant stimulation of ATPase activity by ethanol when the protein was reconstituted in a mixture of lipids at any PC/PS ratio less than or equal to 1:1, since the protein was already at its maximal activity.

As mentioned above, the effect of ethanol seems to be isoform specific. Thus, Cervino et al. [18] find that in erythrocytes the activities of PMCA2 and PMCA4 isoforms were highly stimulated by 0.5% and 5% ethanol, respectively. In the present work, the brain Ca^{2+}-ATPase activity was maximally stimulated by the presence of 5% ethanol (Fig. 1). Immunoblotting with isoform specific antibodies (Fig. 6) showed that the PMCA fraction purified from pig brain is a mixture of isoforms, the principal isoform present being PMCA4. The finding that 5% ethanol is required to maximally stimulate the protein and that the membrane is enriched in PMCA4 isoform is fully coherent with the specific activation of this isoform by 5% ethanol in erythrocytes [18].

The following conclusions can be drawn from this work: (1) ethanol directly interacts with brain PMCA, with maximal stimulation at 5% ethanol; (2) this interaction depends on the



Scheme 1. A model of the interaction of ethanol with the purified PMCA reconstituted with PC (A) or PS (B). Calmodulin (CaM) and ethanol additions and trypsin treatment were as indicated. (●) Active site; (1) calmodulin binding site; (2) putative binding site for ethanol; (★) binding sites for PS; (- - -) trypsin cleavage site.

state of the PMCA, whether it is reconstituted with neutral or acidic phospholipids, or whether it is stimulated by calmodulin or trypsin; (3) in all cases, the effect of ethanol on brain PMCA is additive to the stimulation produced by other modulators of ATPase activity; (4) ethanol probably acts by opening another autoinhibitory domain next to the calmodulin binding domain.

A high concentration of ethanol could inhibit ATPase activity by affecting the structure of the lipid bilayer, and hence the optimal reconstitution of the protein. In fact, it has been reported that a high concentration of ethanol in PS membranes affects the fluidity and thickness of the lipid bilayer [15,26]. The concentrations of ethanol needed for maximal activation of the PMCA protein are higher than those associated with ethanol intoxication in vivo. However, in the present work we used a purified enzyme, reconstituted in an artificial bilayer not comparable to the environment of the synaptosomal PMCA in vivo. Taking these differences into account, one suspects that ethanol ingestion may well overstimulate the PMCA, pumping calcium out of the synaptic terminals and thus altering intracellular Ca^{2+} homeostasis. Neurotransmitter release and nerve signal transmission could consequently be drastically affected.

Acknowledgments

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