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# ACUTE AND CHRONIC ACTIONS OF ETHANOL ON ENDOGENOUS CALMODULIN CONTENT IN SYNAPTIC PLASMA MEMBRANES FROM RAT BRAIN

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**Abstract**—The present study was designed to demonstrate that endogenous calmodulin (CaM) content in synaptic plasma membranes (SPM) is altered by acute and chronic administration of ethanol and is a sequel to the kinetic characterization of ethanol inhibition of [$^{125}$I]CaM binding to SPM reported in our previous study. In rats, an acute ethanol injection (2 g/kg, i.p.) rapidly reduced CaM content in SPM from cerebral cortex, whereas chronic ethanol treatment [6% (w/v) in a liquid diet for 3 weeks] led to an up-regulation of the CaM content. In both cases, the alteration of CaM content in SPM occurred in the EGTA-dissociable pool of CaM (77% of total membrane CaM); the EGTA-nondissociable pool (23% of total CaM) was not affected. In animals receiving chronic ethanol treatment, CaM content in SPM was not altered significantly by the acute ethanol dose that produced rapid reduction of CaM content in control animals, indicating that resistance to ethanol develops. This resistance to ethanol can be attributed to alterations of membrane properties. In control SPM, ethanol at 50 mM markedly accelerated the temperature-dependent dissociation of endogenous CaM, whereas in SPM from animals chronically treated with ethanol, significant acceleration of CaM dissociation required ethanol concentrations as high as 150–200 mM. These findings on SPM *in vitro* were consistent with the data on CaM content obtained *in vivo*. Since CaM mediates a variety of biochemical processes in synaptic membranes, we hypothesize that the effects of ethanol in altering the content of membrane-bound CaM may lead to a cascade of consequences in synaptic membrane function.

*Key words:* ethanol; alcohol tolerance; calmodulin; calcium; synaptic plasma membranes

Ethanol produces extensive alterations in neuronal plasma membrane function, and the list includes adenylate cyclase [1], phospholipase $A_2$ [2], $Na^+$, $K^+$-ATPase [3], $Ca^{2+}$-ATPase [4], voltage-dependent $Na^+$ and $Ca^{2+}$ channels [5, 6], opiate receptors [7], $\alpha$-adrenergic receptors [8], $\beta$-adrenergic receptors [9], 5-$HT_3$‖ receptors [10], nicotinic acetylcholine receptors [11], $GABA_A$ receptors [12], and two types of glutamate receptors: *N*-methyl-D-aspartate (NMDA) and kainate receptors [13, 14]. Clearly, the neuronal plasma membranes are a major cellular target site for the actions of ethanol in the brain. In a previous study [15], we have shown that ethanol inhibits the binding of $^{125}$I-labeled CaM to SPM from rat brain, and that in animals chronically treated with ethanol, the SPM are more resistant to the inhibitory effect of ethanol on [$^{125}$I]CaM binding. These data, obtained *in vitro*, imply that ethanol may produce inhibition of CaM binding to synaptic membranes *in vivo* and that this membrane action may be involved in the development of neuronal tolerance to ethanol.

The present study was designed to demonstrate that endogenous CaM content in SPM is indeed altered by ethanol administration and is a sequel to the kinetic characterization of ethanol action on [$^{125}$I]CaM binding reported in the previous study [15]. By determining endogenous CaM content, it provides an opportunity to detail the effects of ethanol on the synaptic membranes not only *in vitro* but also *in vivo*. CaM mediates a variety of cellular activities, including biochemical processes in the plasma membranes [16], and its role in the regulation of synaptic function in the brain has long been recognized [17]. The brain, of all mammalian tissues examined, has the highest content of CaM, and approximately 50% of the total CaM is bound to membrane fractions [18]. Obviously, binding with the membranes is the initial event in the regulation of specific membrane processes by CaM. Alteration of CaM binding to SPM by ethanol thus may lead to a cascade of consequences in synaptic membrane function.

## MATERIALS AND METHODS

*Animals and materials.* Male Sprague–Dawley

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‖ Abbreviations: 5-$HT_3$, 5-hydroxytryptamine$_3$; GABA, $\gamma$-aminobutyric acid; CaM, calmodulin; SPM, synaptic plasma membranes; IgG, immunoglobulin G; RIA, radioimmunoassay; ED-CaM, EGTA-dissociable calmodulin; and END-CaM, EGTA-nondissociable calmodulin.

rats (approximately 200 g) were obtained from a commercial breeder (Sasco King Animal Laboratories, Oregon, WI). Monospecific anti-CaM antibody (sheep), purified CaM (bovine brain) and $^{125}$I-labeled CaM were supplied by NEN Research Products (Boston, MA). Anti-sheep IgG (donkey), normal rabbit serum, and other biochemicals were obtained from the Sigma Chemical Co. (St. Louis, MO).

*Preparation of SPM.* SPM were prepared by a procedure described previously [4, 15, 19]. The cerebral cortex was homogenized in 5 vol. of 0.32 M sucrose–10 mM Tris–HCl, pH 7.4. Nuclei and other cellular debris were pelleted by centrifuging at 1000 *g* for 10 min. The synaptosomes remaining in the supernatant were pelleted by a subsequent centrifugation at 10,000 *g* for 20 min. The pellet was resuspended in the homogenizing buffer and placed on a discontinuous gradient of 0.85, 1.0, and 1.2 M sucrose. After centrifuging at 90,000 *g* for 90 min, the synaptosomes were removed from the interface between the 1.0 and 1.2 M sucrose. The synaptosomal fraction was diluted with 4 vol. of 0.25 M sucrose–10 mM Tris–HCl, pH 7.4, and centrifuged for 20 min at 15,000 *g*. In a previous study, it has been shown that in the absence of $Ca^{2+}$, membrane-bound CaM dissociates substantially, even at 0° [19]. Therefore, in subsequent steps for the isolation of SPM from whole synaptosomes, $Ca^{2+}$ was added in all solutions to prevent endogenous CaM from dissociation. The synaptosomes were lysed by resuspending the pellet in 10 mM Tris–HCl, pH 7.4, containing 0.1 mM $CaCl_2$. The osmotically lysed synaptosomes were layered on a discontinuous sucrose gradient (0.85, 1.0, and 1.2 M, all containing 0.1 mM $CaCl_2$) and centrifuged at 90,000 *g* for 90 min. The band containing the SPM fraction at the interface between 1.0 and 1.2 M sucrose was removed and diluted with 5 vol. of 10 mM Tris–HCl, pH 7.4, and 0.1 mM $CaCl_2$. The SPM were then pelleted at 15,000 *g* for 20 min. The purity of the SPM was monitored by the activity of 5′-nucleotidase, a plasma membrane marker, as described previously [20, 21].

*Acute and chronic ethanol administration.* For acute ethanol administration, the rats were given ethanol (2 g/kg, i.p.) in a volume of 2 mL in saline. The control animals were given isocaloric sucrose instead of ethanol. For chronic ethanol administration, the rats were given a liquid diet (Liquidiet RAT L/D '82, BioServ, Inc., Frenchtown, NJ) containing 6.0% (w/v) ethanol *ad lib.* for 3 weeks, as described in our previous studies [4, 15]. This diet is based on the Lieber–DeCarli formulation [22]. One group of control animals was given a control liquid diet, without ethanol but containing instead an isocaloric amount of maltose-dextrin, pair fed to the volume consumed by the ethanol-treated animals. Normal control animals were given standard laboratory food (Purina rat feed, PMI Feeds, St. Louis, MO) and water *ad lib.* All animals were killed at 9:00–10:00 a.m. for the preparation of SPM.

*Ethanol incubation of SPM in vitro.* In experiments where CaM dissociation in the presence of ethanol *in vitro* was determined, SPM (20–25 μg protein) were equilibrated with ethanol for 20 min at 0° in 200 μL of 50 mM Tris–HCl, pH 7.4, 0.5 mM $CaCl_2$, 2 mM dithiothreitol, 50 μM phenylmethylsulfonyl fluoride, and 0.2% bovine serum albumin (buffer A). Temperature-dependent dissociation of membrane-bound CaM was initiated by placing the incubation mixture in a shaker-bath at 37° and terminated by the addition of 5 vol. of ice-cold buffer A. The membranes were pelleted immediately at 0° (15,000 *g* for 20 min) for the determination of CaM content.

*Radioimmunoassay of CaM.* The RIA procedure for CaM was essentially that described by Chafouleas *et al.* [23]. For total CaM, the SPM pellet was resuspended in 10 mM Tris–HCl, pH 7.4, and 2 mM dithiothreitol (buffer B). For ED-CaM, the SPM pellet was resuspended and washed twice in buffer B containing 2 mM EGTA, and the washings were pooled. The CaM remaining in the membranes was the END-CaM. The dilution of all preparations was such that the CaM content fell in the range of 2–20 ng/100 μL. All preparations were heated at 95° for 10 min to extract the CaM [24]. After centrifugation at 10,000 *g* for 10 min, the supernatant was used for the determination of CaM. The sample preparation, 100 μL, was added to 200 μL of an assay buffer (125 mM sodium-borate, pH 8.4, 75 mM NaCl, 1 mM EGTA, 0.2% bovine serum albumin and 0.1% sodium azide) containing the anti-CaM antibody (sheep) and [$^{125}$I]CaM tracer (0.02 μCi). The mixture was incubated at 0° for 16 hr. An anti-sheep IgG (donkey), diluted in 500 μL of the assay buffer containing 5% normal rabbit serum, 5.6% polyethylene glycol 8000 and 0.5% rice starch, was then added, and the incubation continued at 0° for 30 min. After centrifugation at 5000 *g* for 15 min, the precipitate was determined for $^{125}$I-radioactivity. For the standard curve, CaM standards were heated at 95° under the same conditions as the sample preparation, and a series of concentrations (0.30 to 100 ng/100 μL) were included in each assay. All determinations on samples and standards were made in triplicate.

## RESULTS

*Endogenous CaM content in SPM.* For baseline information, endogenous CaM bound to SPM was determined by RIA, and the data are shown in Fig. 1. In control animals, the total amount of CaM in SPM was determined as 3.27 μg/mg membrane protein. Following repeated washing of the membranes with EGTA, the amount of CaM dissociated was 2.52 μg/mg (77% of total), which represents $Ca^{2+}$-dependent binding of CaM to the membranes (this pool of CaM is termed "EGTA-dissociable CaM" and abbreviated 'ED-CaM'). The CaM remaining on the membranes after EGTA washing was 0.75 μg/mg (23% of total). This tightly bound CaM (termed "EGTA-nondissociable CaM" and abbreviated 'END-CaM'), which is resistant to dissociation by $Ca^{2+}$-chelators and requires a detergent for its extraction, represents $Ca^{2+}$-independent CaM binding to membranes [25, 26] and is present in the plasma membranes of a variety of cell types including neuronal cells [27].

The effects of acute and chronic administration of ethanol on endogenous CaM content in SPM are summarized in Fig. 1. Following an ethanol injection

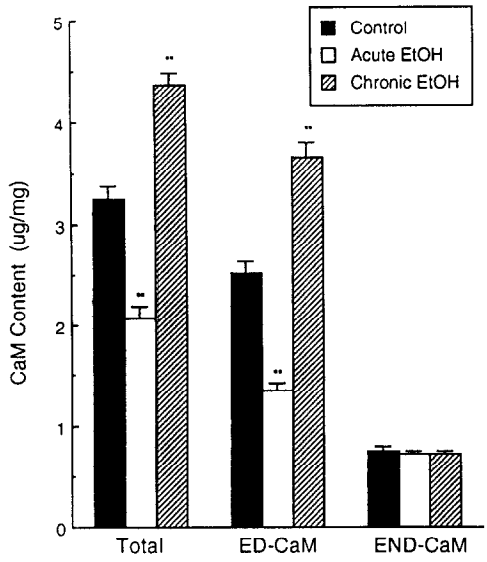


Fig. 1. Endogenous CaM content in SPM. Total: total CaM; ED-CaM: EGTA-dissociable CaM; END-CaM: EGTA-nondissociable CaM. Control: untreated rats; acute EtOH: rats receiving an acute dose of ethanol (2 g/kg, i.p.; 20 min); chronic EtOH: rats chronically treated with ethanol [6% (w/v) in a liquid diet; 3 weeks]. See text for details on ethanol administration. Each value is the mean ± SEM from six SPM preparations. Significant difference from the corresponding control value: (**) $P < 0.001$.

(20 min after 2 g/kg, i.p.), the content of total CaM in SPM was lowered (by 36%), and this reduction of membrane-bound CaM was localized exclusively in the ED-CaM fraction (a reduction of 47%). Following chronic ethanol treatment [3 weeks with a liquid diet containing 6% (w/v) ethanol], the content of total CaM in SPM was elevated (by 34%), and the increase of CaM in the membranes was localized again in the ED-CaM pool (by 46%). Thus, acute ethanol exposure resulted in a reduction of CaM bound to the membranes by the $Ca^{2+}$-dependent process, whereas chronic ethanol exposure led to the up-regulation of the membrane content of this CaM. In both cases, the content of END-CaM, the fraction tightly bound to the membranes by the $Ca^{2+}$-independent process, was not affected. It should be emphasized that for controls in the chronic ethanol experiment, the SPM from animals pair-fed with a control liquid diet yielded identical values of CaM content (not shown) as the SPM from control animals receiving standard laboratory food (shown in Fig. 1). The dietary difference did not pose as a variable to CaM content in SPM.

*Effects of ethanol* in vivo *on CaM content.* To detail the ethanol effects *in vivo*, the alteration of CaM content in SPM was determined in a time–course after the acute dose of ethanol (2 g/kg, i.p.). In control animals (i.e. rats without prior exposure to ethanol), a significant decrease in the ED-CaM content in SPM occurred within 5 min after the ethanol injection, and the reduction of ED-CaM reached nearly 50% below the control value at 20 min (Fig. 2A). In the saline-injected group, there was a small and transient rise of ED-CaM content in SPM (20% above control) after the saline injection. This transient increase, detected only at the 5-min point after the saline injection, could be due to the release of adrenal corticosterone induced by the injection stress, since glucocorticoid administration has been shown to elevate the endogenous content of ED-CaM in SPM [28]. In any event, it is evident from the data on ethanol-injected animals that the acute dose of ethanol resulted in a rapid reduction of ED-CaM content in the synaptic membranes.

To compare with the response in control animals, the acute dose of ethanol was administered to animals chronically exposed to ethanol, and the ED-CaM content in SPM was determined. As shown in Fig. 2B, the initial ED-CaM content in these animals was higher than that in control animals because of the chronic ethanol exposure. Moreover, following the acute dose of ethanol, there was only a small decline in ED-CaM content in SPM, and the differences from the 0-time value (7–13%) were not statistically significant. Thus, in contrast to the response in control animals, chronic treatment with ethanol resulted in the resistance of the animals to the acute effect of ethanol in altering ED-CaM content. It is also noted that saline injections did not produce a transient rise of ED-CaM content in these animals, as in the case of saline-injected control animals shown in Fig. 2A. It appears that chronic ethanol treatment resulted in the resistance not only to the acute effect of ethanol, but also to the effect of injection stress on ED-CaM content.

The content of END-CaM in SPM was monitored in both control and chronically ethanol-treated animals. As shown in Fig. 2 (A and B), this small pool of tightly bound CaM was not altered by ethanol at any time point in either group of animals.

*Effects of ethanol* in vitro *on CaM dissociation.* To compare the SPM from control and chronically ethanol-treated animals *in vitro*, the effects of ethanol on temperature-dependent dissociation of endogenous CaM in SPM were examined. The approach was based on our previous kinetic observations that [$^{125}$I]CaM bound to EGTA-washed SPM (i.e. membranes with CaM binding sites fully exposed) is stable at 0° if $Ca^{2+}$ is present, but the bound [$^{125}$I]CaM dissociates from the membranes rapidly at 37° even in the presence of $Ca^{2+}$ [19]. In this experiment, SPM were first equilibrated with ethanol (100 mM) at 0°, and the temperature-dependent dissociation of endogenous CaM was initiated at 37°. In control SPM, ED-CaM underwent rapid dissociation at 37°, with a 30% dissociation occurring at 5 min and a 44% dissociation at 20 min (Fig. 3). This time–course of the dissociation of endogenous ED-CaM is similar to that of the dissociation of preloaded [$^{125}$I]CaM in EGTA-washed SPM reported previously [15]. In the presence of 100 mM ethanol, the dissociation of ED-CaM was increased markedly, with a 56% dissociation occurring at 5 min and a 73% dissociation at 20 min. Clearly, ethanol *in vitro* accelerated the temperature-dependent dissociation of endogenous ED-CaM. At

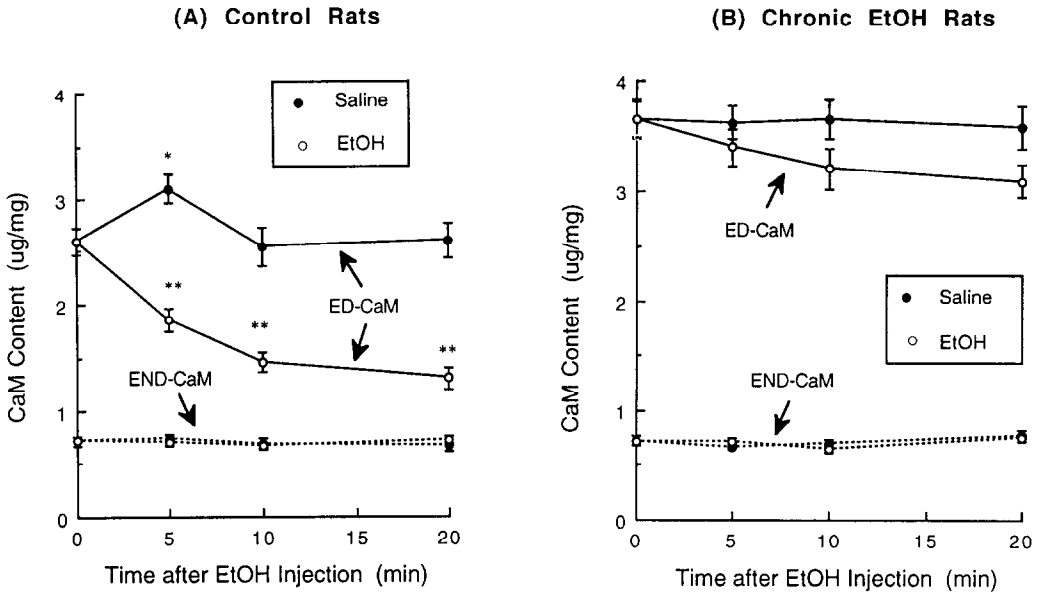


Fig. 2. Effects of ethanol administration on CaM content in SPM. (A) Control rats without prior exposure to ethanols. (B) Rats chronically treated with ethanol. In both animals, ethanol was injected at a dose of 2 g/kg (i.p.) in saline; the control group received saline containing isocaloric sucrose. Each value is the mean ± SEM from six animals. Significant difference from 0-time value: (*) $P < 0.01$; and (**) $P < 0.001$.

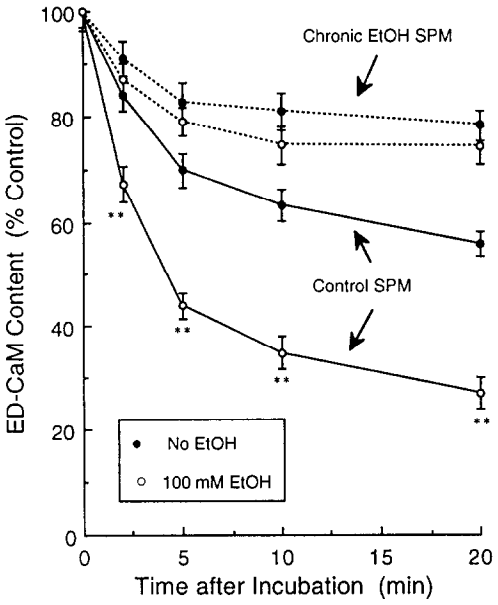


Fig. 3. Effects of ethanol *in vitro* on ED-CaM dissociation. Control SPM: SPM from control rats without prior exposure to ethanol. Chronic EtOH SPM: SPM from rats chronically treated with ethanol. SPM were first equilibrated with ethanol (100 mM) at 0° for 20 min, and temperature-dependent ED-CaM dissociation was initiated at 37° (see text for details). The 0-time value for control SPM was 2.52 ± 0.08 μg/mg; for chronic EtOH, SPM was 3.65 ± 0.14 μg/mg. Both values were normalized to 100% to allow comparison. Each value is the mean ± SEM from six experiments. Significant difference from 0-time value: (**) $P < 0.001$.

0°, no dissociation of ED-CaM occurred and neither did ethanol provoke the dissociation (data not shown). It should also be mentioned that the small pool of endogenous END-CaM in SPM did not undergo dissociation even at 37° [28].

In SPM from animals chronically exposed to ethanol (Fig. 3), it is evident that the dissociation of endogenous ED-CaM occurred at a significantly slower rate than that in SPM from control animals. After incubation at 37°, the dissociation of ED-CaM was only 17% at 5 min and 22% at 20 min. Thus, chronic treatment with ethanol resulted in SPM that were resistant to the temperature effect on ED-CaM dissociation *in vitro*. For control of the liquid diet effect, there was no difference in the dissociation behavior in SPM from animals pair-fed with a control liquid diet (not shown), as compared with animals given standard laboratory food. Therefore, the dietary difference alone did not affect membrane dissociation of CaM. Of particular note is the lack of a significant effect of ethanol *in vitro* on ED-CaM dissociation in SPM after chronic ethanol exposure. At 100 mM, ethanol accelerated ED-CaM dissociation only marginally, and the small increases at all time points were not statistically different from the control (in the absence of ethanol). These data on the resistance of the SPM to the CaM-dissociating effect of ethanol *in vitro* are in agreement with the finding *in vivo* that these animals were resistant to the alteration of CaM content by an acute dose of ethanol.

To assess the difference between control and chronically ethanol-treated animals in a quantitative manner, the effect of ethanol in accelerating the dissociation of ED-CaM in SPM was determined over an ethanol concentration range of 25–200 mM.

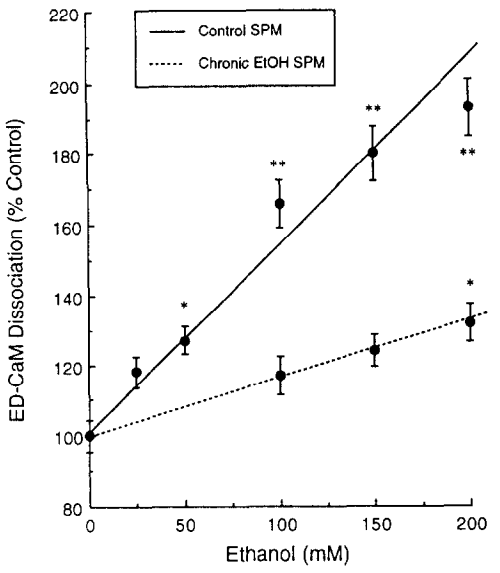


Fig. 4. Concentration effects of ethanol *in vitro* on ED-CaM dissociation. SPM were first equilibrated with ethanol at the indicated concentration for 20 min at 0°. All SPM were incubated at 37° for 20 min, and the amount of ED-CaM dissociated from the membranes was determined as μg/mg/20 min (see text for details). The amount dissociated in the presence of ethanol was compared with that dissociated in the absence of ethanol (control) as 100%. (See legend of Fig. 3 for control values.) Each value is the mean ± SEM from six experiments. Significant difference from control (value at 0 mM ethanol): (*) $P < 0.01$; and (**) $P < 0.001$.

The SPM were incubated at 37° for 20 min, and the amounts of ED-CaM dissociated in the presence and absence of ethanol were calculated as percent of control (Fig. 4). In control SPM, ethanol at 25 mM produced only a threshold and insignificant increase (18%) of ED-CaM dissociation; as the ethanol concentration reached 50 mM, the increase of dissociation (27%) was significant. At 200 mM ethanol, the increase of ED-CaM dissociation was 93% over the control. In SPM from animals with chronic ethanol exposure, ethanol as high as 100 mM was without a significant effect on ED-CaM dissociation. Only when the ethanol concentration reached 150 mM did an increase in dissociation become substantial (24% over control); at 200 mM, ethanol increased the dissociation by 32%. Thus, under the incubation condition *in vitro*, the acceleration of ED-CaM dissociation at 150 mM ethanol was comparable to that at 50 mM ethanol found in SPM from control animals.

## DISCUSSION

Methodologically, a critical step in our study was the addition of $Ca^{2+}$ (0.1 mM) in sucrose and other media to preserve endogenously bound CaM during fractionation of plasma membranes following hypotonic disruption of whole synaptosomes. In kinetic analysis of [$^{125}$I]CaM–SPM complexes, it has been shown that in the absence of $Ca^{2+}$, CaM dissociates from the membranes at a slow but steady rate, even at 0° [19]. Thus, at 0°, a substantial amount of endogenously bound CaM can dissociate over a prolonged period of time, such as that required in subcellular fractionation of plasma membranes, if $Ca^{2+}$ is not present in the media. In earlier studies that determined the endogenous content of membrane-bound CaM in the brain [18, 23, 25, 26], this precaution was apparently not undertaken. Moreover, in those studies, CaM was measured in crude particulate preparations, which contained heterogeneous subcellular elements from neuronal as well as glial cells. Therefore, the data from the earlier studies, whether obtained by cyclic nucleotide phosphodiesterase activation assay or by RIA, cannot be quantitatively compared with those in the present study.

There are three relevant results of our study. First, we demonstrated *in vivo* that acute ethanol administration produced a rapid reduction of CaM content in SPM, occurring within 5 min after the dose of ethanol. Moreover, chronic ethanol administration led to an up-regulation of the CaM content in SPM, as shown by a significant increase of the CaM content following the ethanol treatment for 3 weeks. In highly purified SPM, as examined in our study, about 77% of total endogenous CaM could be dissociated by EGTA. Our data indicate that this main pool of CaM involving $Ca^{2+}$-dependent binding is altered by acute and chronic ethanol treatment. The other 23% was resistant to repeated washing by EGTA, and the tight binding has been attributed to the presence of high amounts of a neurospecific protein, P-57, which has high affinity for CaM even in the absence of $Ca^{2+}$ [29]. This small pool of tightly bound CaM involving $Ca^{2+}$-independent binding is not affected by either acute or chronic administration of ethanol.

Second, we demonstrated, also *in vivo*, that adaptive responses occurred in animals chronically exposed to ethanol. In addition to the up-regulation of CaM content in SPM, these animals were resistant to a challenge dose of ethanol that could produce a substantial reduction of the CaM content in control animals. Several cases are known where similar adaptive responses occur in neuronal membrane-bound processes after chronic ethanol treatment. For example, ethanol acutely inhibits brain $Na^+$, $K^+$-ATPase activity, whereas the ATPase activity is up-regulated in animals chronically exposed to ethanol and the membrane-bound enzyme becomes resistant to the inhibitory effect of ethanol [3]. $GABA_A$ receptor-mediated chloride uptake in brain synaptosomes is stimulated by ethanol acutely, whereas chronic ethanol treatment leads to the reduction of the chloride uptake [30, 31]. Such adaptive responses result in desensitization of the neurons to ethanol and are believed to be the mechanisms in the development of ethanol tolerance and dependence [32].

Third, we demonstrated, by examining temperature-dependent dissociation of endogenous CaM *in vitro*, that the resistance to ethanol found *in vivo* can be attributed to adaptive changes in the synaptic membranes. In control SPM, endogenous CaM

dissociated rapidly at 37° and ethanol *in vitro* accelerated the dissociation. In SPM from animals chronically treated with ethanol, CaM dissociation occurred at a significantly slower rate and, further, higher concentrations of ethanol were required to produce the acceleration of dissociation, indicating that the membranes are resistant to the ethanol effect. These findings on CaM dissociation *in vitro* are consistent with the data on ethanol alteration of CaM content *in vivo*. The resistance of the SPM to ethanol found here may be another consequence of the biophysical changes in membrane properties known to occur after chronic ethanol exposure (see below).

Two earlier studies have dealt with total CaM levels in brain regions after ethanol administration [33, 34]. In one study, total CaM levels in cerebral cortex and striatum remained unchanged after acute and chronic ethanol treatment [33]. In another study, chronic ethanol treatment resulted in an increase of total CaM levels in cerebral cortex as well as several other brain regions reported [34]. The reason for the discrepancy is not clear. It is generally believed that total CaM levels in the tissue do not reflect particular regulatory events, since CaM is present in large excess in the cell relative to the proteins it modulates [35]. Moreover, except during the growth cycle, changes in total cell or tissue levels of CaM do not occur under a variety of physiological and hormonal conditions examined [35]. Translocation of CaM within the cell, on the other hand, appears to be an important regulatory phenomenon. For example, during the release of luteinizing hormone induced by gonadotropin releasing hormone, there is an increase of CaM bound to the pituitary plasma membranes [36]. In some cases, the redistribution of CaM, including binding to the membranes, is secondary to the alteration of $Ca^{2+}$ distribution in the cell. In the case of ethanol action described in this study, the acceleration of temperature-dependent dissociation of CaM in SPM was demonstrated *in vitro* under saturating $Ca^{2+}$ conditions (0.5 mM). Therefore, the action of ethanol is independent of $Ca^{2+}$ influence. The alteration of membrane affinity for the $Ca^{2+}$–[$^{125}$I]CaM complex by ethanol has been kinetically characterized in detail in our previous study [15].

In Scatchard analysis of [$^{125}$I]CaM binding in EGTA-washed SPM, maximal binding was estimated as 283 pmol or approximately 5 $\mu$g of CaM per mg of membrane protein [19]. In the present study, endogenous CaM bound to SPM by the $Ca^{2+}$-dependent process was estimated to be 2.5 $\mu$g per mg of membrane protein. Therefore, SPM are endogenously not saturated with CaM and membrane binding of CaM can be regulated upwards, such as that following chronic ethanol administration shown in this study. It is relevant to mention that glucocorticoid administration to rats also increases CaM content in SPM *in vivo* [28] and that [$^{125}$I]CaM binding to the synaptic membranes is enhanced by the steroids *in vitro* [37]. Thus, glucocorticoids and acute ethanol have opposite effects on CaM binding in SPM, one stimulatory and the other inhibitory. In a behavioral study, co-administration of glucocorticoids in mice antagonizes the sedative action of ethanol, as shown by the reduction of sleep time following a hypnotic dose of ethanol [38]. This antagonistic effect of the steroids on the central depressant action of ethanol is consistent with their opposite effects on CaM binding in SPM.

The mechanism of ethanol action on membrane binding of CaM has been a subject of our recent investigation. In Arrhenius analysis of CaM binding to SPM, the binding exhibits a biphasic function in temperature dependency, indicating that binding of $Ca^{2+}$-CaM (the form of CaM that binds through the $Ca^{2+}$-dependent process) is influenced by conformational changes of the membranes [19]. Ethanol lowers the transition temperature of the biphasic function in the Arrhenius analysis, suggesting that ethanol inhibits CaM binding by increasing membrane fluidity [15]. Moreover, the increase of lipid fluidity in SPM by ethanol and other short-chain alcohols, as determined by diphenylhexatriene fluorescence polarization, is directly correlated with the inhibition of CaM binding by these alcohols [39]. These findings, taken together, provide evidence that ethanol inhibits CaM binding (or promotes CaM dissociation) by increasing lipid fluidity in SPM. It has long been known that chronic ethanol administration causes biophysical changes in membranes that result in the resistance of the membranes to the fluidizing effect of ethanol [40]. These adaptive biophysical changes are related, at least in part, to the alteration of lipid composition of the membranes [41].

As noted at the beginning of this article, ethanol produces extensive alterations in neuronal plasma membrane function, ranging from the activities of membrane-bound enzymes to the activities of ion channels and neurotransmitter receptors. The molecular mechanism(s) underlying these diverse alterations appears intriguing. For nearly two decades, the notion has been pursued that ethanol acts on plasma membranes primarily by increasing membrane fluidity [32, 40–42]. More recent findings indicate that certain membrane-bound proteins, such as $GABA_A$ receptors and NMDA receptors, are affected by low concentrations of ethanol (less than 50 mM), whereas other membrane-bound proteins, such as voltage-dependent $Ca^{2+}$ channels, are affected only by higher concentrations of ethanol [43]. This differential sensitivity suggests that ethanol may act directly and selectively on certain proteins, probably by interacting with hydrophobic regions of the proteins. Thus, membrane lipid fluidization and direct interaction with proteins may both be involved in the diverse ethanol actions on membrane functions. Furthermore, it is also possible that lipid fluidity may not directly affect the activity of certain membrane-bound proteins, particularly those that are subjected to complex biochemical regulation, such as receptor-gated ion channels. Rather, the alteration of membrane fluidity may perturb regulatory events that, in turn, affect the activity of these membrane-bound proteins. In this regard, the inhibitory action of ethanol on membrane binding of CaM is of particular interest.

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