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CALCIUM BINDING TO BRAIN SYNAPTOSOMES

EFFECTS OF CHRONIC ETHANOL INTAKE*

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Abstract—The effects of chronic ethanol intake (16 days) on the Ca^{2+} binding activity of rat brain synaptosomes were explored. Two different procedures were used for the determination of Ca^{2+} interactions with the synaptosomal membranes. The chelator fluorescent probe chlorotetracycline was employed for fluorescence measurements of Ca^{2+}-membrane interaction, and the direct assay of ^{45}Ca binding to the same preparations was used as an index of Ca^{2+} binding capacity at micromolar Ca^{2+} concentrations. Both methods gave qualitatively very similar results. Chronic ethanol treatment resulted in a significant decrease in the ^{45}Ca binding capacity of the synaptosomal membranes which was found to be related to a decrease in the maximum number of binding sites: 11.24 nmoles·mg^{-1} protein for the controls as opposed to 6.10 nmoles for the ethanol-treated animals. No significant changes in the dissociation constant of this ^{45}Ca binding to the membranes was observed. In addition, the membrane preparations from the chronically treated subjects were found to be relatively resistant to the *in vitro* effects of ethanol on Ca^{2+} interaction with the synaptosomes. Ethanol at concentrations from 5 to 100 mM caused biphasic increases of both ^{45}Ca binding to, and CTC fluorescence of, synaptosomes from controls, though it caused minimal changes in either measure in synaptosomes from chronically ethanol-treated animals. These results are suggestive of the appearance of tolerance to the activity of ethanol at the membrane level.

The interaction of ethanol with biological membranes has been shown to affect the activity of membrane attached enzymes, ionophores, uptake carriers, and neurotransmitter receptors [1–6]. Some of these effects of ethanol on plasma membranes are probably related to the changes in nerve cell excitability produced by acute and chronic ethanol intake [7, 8]. Since many of these membrane-related systems are controlled by the activity of the divalent cation, calcium (Ca^{2+}), its interaction with biological membranes in the presence of ethanol has attracted considerable attention recently. The *in vitro* exposure of erythrocyte ghosts to ethanol has been shown to bring about increases in the Ca^{2+} binding activity of these preparations [9]. On the other hand, *in vivo* administration of ethanol to animals causes a decrease in the Ca^{2+} content of several brain regions [10]. It was shown subsequently that the subcellular fraction which exhibits the greatest reduction in Ca^{2+} content following the acute administration of ethanol is the synaptic membrane subfraction [11]. The effects of ethanol on the Ca^{2+} content of brain tissue are suggestive of an inhibition of Ca^{2+} binding to the synaptic membranes as a result of their acute exposure to this alcohol. In fact, such an *in vitro* effect of ethanol on the Ca^{2+} binding activity of synaptic membranes has already been reported [12].

In the studies by Ross *et al.* [12], the *in vitro* effects of ethanol on synaptic membrane Ca^{2+} binding activity were tested only over a very low concentration range (0.1 to 100 μM) of ethanol which is probably not totally representative of the ethanol concentration achieved in brain tissue *in vivo*. We were led to believe that ethanol may cause complex shifts in Ca^{2+} interaction with these membranes because of our observations that the *in vitro* exposure of synaptosomal membrane preparations to various concentrations of ethanol (5–200 mM) caused a biphasic effect on glutamate binding to the receptor sites on these membranes [6]. The pattern of this response was very similar to the biphasic changes in glutamate binding produced by progressive increases in the Ca^{2+} concentration of the medium [13]. These observations, coupled with the previous demonstration that ethanol at concentrations higher than 20 mM enhances Ca^{2+} binding to some biological membranes such as the erythrocyte ghosts [9], made it apparent that the *in vitro* effects of this alcohol on synaptosomal Ca^{2+} binding should be reevaluated. In particular, we have examined the possibility that ethanol over a wide range of concentrations may have a biphasic action on the Ca^{2+} interaction with synaptosomal preparations just as it does for glutamate binding to these membranes [13]. We relied primarily on ^{45}Ca radioligand binding assays to detect such changes in Ca^{2+} binding capacity, but we have also employed the fluorescent Ca^{2+}-chelating probe chlorotetracycline (CTC) [14, 15] as a measure of altered interactions.

In addition to an examination of Ca^{2+} binding interactions, the present study was designed to explore a possible Ca^{2+}-related index of the development of physical tolerance to ethanol. The continued administration of ethanol for 14 days has been shown to result in a gradual reversal of the acute effects of ethanol, thereby bringing about an increase in Ca^{2+} content and a decrease in Ca^{2+} binding capacity of the synaptic mem-

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branes [11]. In the present study, we have followed the changes in the binding activity of synaptosomal preparations as a means of determining the effects of chronic ethanolism. The main focus has been placed, however, on an exploration of the development of physical tolerance to the effects of *in vitro* exposure to ethanol in synaptosomal preparations from chronically intoxicated animals.

MATERIAL AND METHODS

Animals and diets. Adult male Sprague–Dawley rats were maintained on laboratory chow and water *ad lib.* until the start of the experiment when they reached a body wt of 300 g. They were then fed either a liquid diet containing 6% (w/v) ethanol (experimental animals) or an isocaloric amount of sucrose (control animals) as has been described in a previous study [6]. Average daily consumption of this diet by each animal was 70–90 ml, which corresponds to 4.2 to 5.4 g of daily ethanol intake for the experimental animals (14–18 g/kg body wt). All animals were maintained on these diets for 16 days. The choice of this period of exposure was based on our previous studies in which it was determined that the largest changes in synaptosomal glutamate binding occur by 16 days and that the animals are tolerant to the effects of ethanol since they exhibit no loss of the righting reflex at that time [6].

Tissue preparation and protein determination. All the animals were killed by cervical dislocation and the brain tissue was rapidly removed, weighed, and placed in ice-cold 0.32 M sucrose where it was finely minced in 10 vol. of the sucrose solution/g brain wt. The homogenization and centrifugation procedures employed to obtain the crude mitochondrial subfraction and to separate the membranous subcellular fractions from the mitochondria were the same as described previously [6]. These membranous structures have been shown to consist of synaptosomes (approximately 40 per cent of particulate in the crude mitochondrial subfraction), plasma membranes of neuronal and glial origin, and myelin fragments [16, 17].

These crude "synaptosomal" membranes were resuspended by hand homogenization in 15 ml of 50 mM glycylglycine–NaOH buffer, pH 7.5, allowed to stand on ice for 5 min and precipitated by centrifugation at 27,000 *g* for 10 min. The final pellet was resuspended in 50 mM glycylglycine–NaOH buffer to a volume sufficient to bring the final protein concentration to 1.5 mg/ml. Protein concentration was determined according to the method of Lowry *et al.* [18] using bovine serum albumin as the standard. All steps in the preparation of the synaptosomal membranes were performed at 0–4° and all assays were conducted on the same day of tissue preparation.

Fluorescent Ca^{2+} chelator probe studies. The formation of the Ca^{2+}–CTC complexes on the crude synaptosomal membrane preparations was measured by following the increases in fluorescence produced by additions to the assay medium of either increasing amounts of Ca^{2+} or of ethanol. An Aminco–Bowman spectrophotofluorometer was used in all of these studies. The excitation wavelength was set at 390 nm and the emission wavelength was set at 530 nm unless excitation and emission spectra were being monitored. All measurements were conducted with excitation and emission slit widths of 1 mm. Each cuvette contained 0.75 mg protein of the synaptosomal preparation suspended in a final volume of 2 ml of 50 mM glycylglycine–NaOH buffer. This buffer system was found to give lower blanks for Ca^{2+}–CTC chelates than either Tris–HCl or Tris–Tricene buffers. Additions of increments of Ca^{2+} in the absence or in the presence of ethanol in cuvettes that contained only glycylglycine–NaOH buffer medium and CTC (0.07 mM final concentration) produced less than 10 per cent of the increase in fluorescence observed when tissue was included in the cuvette. These increases in fluorescence of CTC in the buffer medium were taken as the blank values and subtracted from the values obtained from the samples that contained tissue. Corrections were also made for any dilutional effects produced by the addition of very small volumes (0.5 to 20 μl) of the solutions that contained Ca^{2+} or ethanol. The contents of the cuvette were stirred rapidly and fluorescence measurements were read directly off the ammeter scale at 30 sec after the addition of each agent.

CTC binding to synaptosomal preparations. The amount of CTC bound to the synaptosomal membranes under various conditions was determined as follows. Suspensions of membranes (0.75 mg protein/2 ml final volume) were allowed to equilibrate with various concentrations of CTC alone or of CTC and Ca^{2+} or CTC and ethanol for 60 sec at room temperature and were subsequently precipitated by centrifugation at 27,000 *g* for 10 min (at 4°). The supernatant fraction was decanted, the tube walls were dried, and the pellets were resuspended and solubilized in 2 ml of a 1% (w/v) sodium dodecyl sulfate (SDS) in water solution. The amount of CTC associated with the membranes was determined by measuring the absorbance of the solubilized membrane solution at 388 nm in a Varian Associates scanning UV spectrophotometer (model 634S). A spectrophotometric scan conducted on a standard solution of CTC in 2 ml of a 1% (w/v) solution of SDS revealed a maximum absorbance of CTC at 380 nm.

^{45}Ca binding assays. Many of the synaptosomal membrane preparations used in the fluorescence assays of Ca^{2+}-membrane interactions were also employed on the same day for ^{45}Ca binding assays. These measurements were conducted by incubating at room temperature (23°) for 1 min 0.75 mg protein with variable concentrations of ^{45}Ca (1.33 to 14.6 μM) or with a constant concentration of ^{45}Ca (2.5 μM) and variable concentrations of ethanol (2.5 to 100 mM). The incubation medium had a total volume of 0.5 ml of glycylglycine–NaOH buffer in which the concentration of ^{45}Ca was maintained at micromolar levels by the use of a Ca–EGTA * buffer system as described by Katz *et al.* [19]. Each assay tube contained 0.02 to 0.04 μCi ^{45}Ca. The incubation was initiated by the addition of tissue and terminated by the rapid filtration (5–10 sec) of each sample through Millipore filters (HAWP 0.45 μm). The filters were washed with 5 ml of ice-cold 0.32 M sucrose. Duplicate samples that contained tissue were processed consecutively with their respective blank sample that contained only buffer. The amount of

* Abbreviations used: EGTA, ethylene glycol-bis-(β-aminoethylether) *N,N'*-tetra acetic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]-benzene; phenyl-oxazolylphenyl-oxazolylphenyl.

radioactivity retained by the filters was determined by liquid scintillation spectrometry in ethylene glycol monoethyl ether–toluene (1:4, v/v) scintillation fluid that also contained 5 g PPO and 0.1 g POPOP per liter of solution. All filters were dried at 90° for 15 min and dissolved in 1 ml of ethylene glycol monoethyl ether prior to the addition of the scintillation fluid.

Mathematical and statistical analysis. The effects of Ca^{2+} or ethanol on the CTC fluorescence are expressed as the enhancement of fluorescence intensity per mg of tissue protein (I_e) which is defined as

$$I_e = \frac{I_2 - (I_1 + I_0)}{\text{mg protein}}$$

where I_2 = the intensity of fluorescence of the membrane–CTC–ligand complex, I_1 = the intensity of fluorescence of the membrane–CTC complex, and I_0 = the intensity of fluorescence of the ligand–CTC complex.

The differences on ^{45}Ca binding between the control and the ethanol-treated animals were analyzed for significance by Student's *t*-test and ANOVA. Statistically significant differences are indicated in the respective figures.

Materials. Chlorotetracycline and glycylglycine were obtained from the Sigma Chemical Co., St. Louis, MO. The sources of ^{45}Ca were the New England Nuclear Corp., Boston, MA (4 Ci/m-mole) and Amersham/Searle Corp., Arlington Heights, IL (0.91 Ci/m-mole).

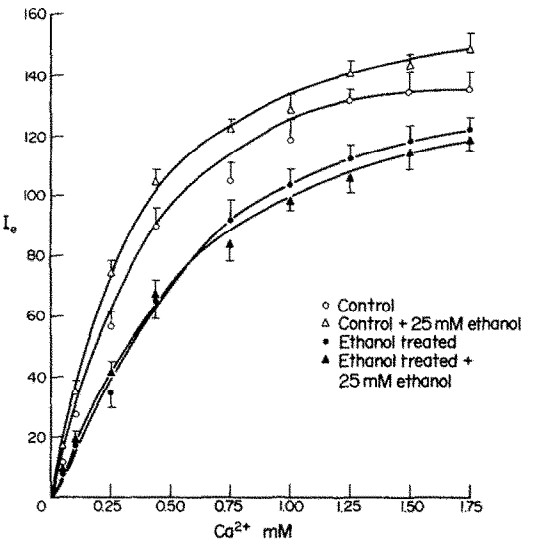

Fig. 1. Increases in fluorescence intensity (I_e) of the CTC–synaptosome mixture from control and ethanol-treated animals in response to increments in Ca^{2+} concentration. Each point is the mean (± S.E.) of seven to eight determinations from seven animals in each group. Changes in fluorescence intensity were measured separately either in the absence or in the presence of 25 mM ethanol. All measurements were obtained as described in Materials and Methods.

RESULTS

Ca^{2+}-synaptosomal interaction and CTC fluorescence. The chelation by CTC of Ca^{2+} or Mg^{2+} is known to cause an increase in CTC fluorescence [14]. The Ca^{2+}–CTC complex, however, exhibits, different excitation and emission maxima (390 nm and 520 nm, respectively, in 90% methanol–1 mM Tris–HCl solution) from those of the Mg^{2+}–CTC complex (375 and 510 nm) [15]. We have found that in an 80% methanol–25 mM glycylglycine–NaOH solution the fluorescence of 70 μM CTC in the presence of 0.5 mM Ca^{2+} exhibited a small excitation maximum at 310 nm, a larger excitation maximum at 398 nm, and an emission maximum at 522 nm. Similarly, synaptosomal membranes in the presence of 70 μM CTC alone exhibited a fluorescence excitation maximum at 400 nm and an emission maximum at 520 nm (data not shown). These spectral characteristics could be interpreted as being indicative of the primary sensitivity of CTC fluorescence to the Ca^{2+} that is present in the synaptosomal membranes. The fluorescence intensity of the CTC–membrane complex (I_1) was dependent on the protein concentration of the membrane suspensions employed in the assay but it was somewhat variable from one membrane preparation to the next. In order to minimize such variability, all data were obtained employing the same protein concentration in each assay (0.75 mg/2 ml) and changes in the intensity of fluorescence as a result of the addition of various ligands were expressed as I_e (See Methods), a measure which was found to be more stable from preparation to preparation.

A comparison of the effects of increasing amounts of Ca^{2+} on the fluorescence intensity of the CTC–membrane complexes from control and ethanol-treated animals is shown in Fig. 1. The inclusion of ethanol in the diet of the experimental animals has produced an apparent decrease in Ca^{2+}–membrane interaction as manifested by the decreased change in CTC fluorescence intensity brought about by the addition of Ca^{2+} to membranes from chronically ethanol-treated animals. The time kinetics for the increases in fluorescence intensity as a result of Ca^{2+} additions to the incubation medium were very rapid for membranes from both the control and the alcohol-intoxicated animals, with the maximum reached within 30 sec. After 60 sec and up to 5 min there was only a small decrease in the fluorescence intensity.

The differences between the responses of membranes from control and chronically ethanol-treated animals were even more remarkable when CTC fluorescence changes in response to Ca^{2+} increases were measured in the presence of 25 mM ethanol (Fig. 1). Whereas the synaptosomes from the controls showed an enhanced response to Ca^{2+} in the presence of 25 mM ethanol, the membranes from the ethanol-treated animals exhibited essentially no change from their baseline pattern of response to Ca^{2+}. This lack of response to the *in vitro* addition of ethanol in the membranes of the chronically ethanol-exposed animals may represent a component of the phenomenon of physical tolerance.

The possibility that the synaptosomal membranes of the alcohol-treated animals are somewhat resistant to the *in vitro* actions of ethanol was examined further through a determination of the effects of increasing ethanol concentrations on the fluorescence of the CTC–membrane complex. These experiments were conducted without the addition of Ca^{2+} to the medium. Only the endogenous Ca^{2+} that was already bound to the membranes from the two tissue sources was available for participation in the formation of CTC–CA^{2+}–membrane complexes. As is shown in Fig. 2, ethanol

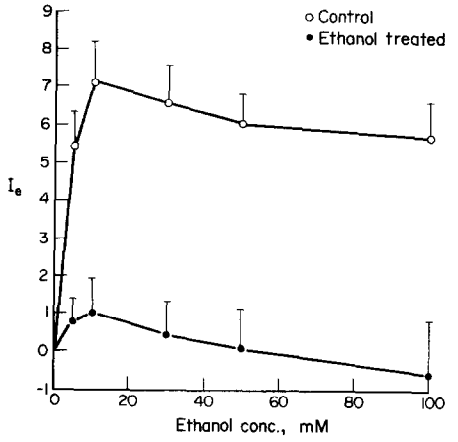

Fig. 2. Changes in baseline fluorescence intensity (I_e) of the CTC-synaptosomal membrane preparations from control and chronically ethanol-exposed animals brought about by *in vitro* ethanol additions. Each point represents the mean (± S.E.) of five determinations from five animals in each group.

concentrations from 5 to 100 mM produce a biphasic effect on the CTC–membrane fluorescence in both the control tissue and the synaptosomes from alcoholic animals. However, the increases in I_e of the control membranes that were produced by ethanol in the range of concentrations from 5 to 10 mM were six to seven times as large as the increases in I_e produced in the synaptosomes from the ethanolic animals. In addition, the synaptosomes from this latter group of animals responded with rather small and variable increases in I_e only in the ethanol concentration range of 5–25 mM

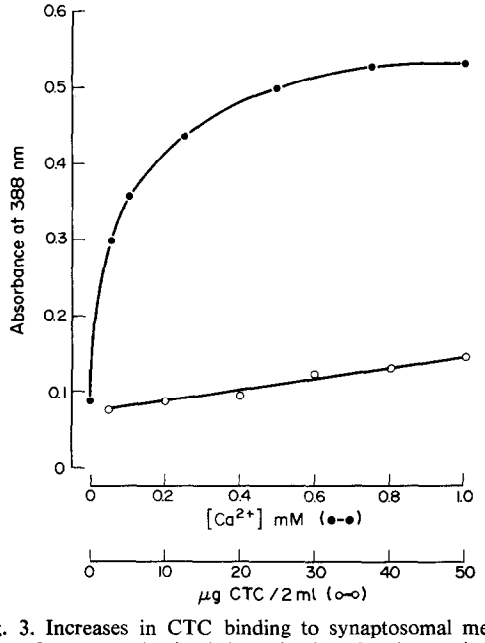

Fig. 3. Increases in CTC binding to synaptosomal membranes from control animals brought about by changes in the Ca^{2+} concentrations of the medium or by increases in the amount of CTC added to the incubation solution. The effects of Ca^{2+} on CTC binding to the membranes were measured in the presence of a constant concentration of CTC (0.07 mM). All values are the mean of two determinations from two animals.

and exhibited a rapid return to baseline fluorescence values with higher ethanol concentrations. Thus, it would appear that the *in vitro* ethanol exposure of the control membranes, even in the absence of exogenously added Ca^{2+}, can increase the formation of CTC–membrane complexes. This enhanced formation of CTC-membrane complexes may represent either an intrinsic difference in the CTC binding activity of membranes from control and ethanolic animals in response to exposure to ethanol, or it may indicate that ethanol can affect differently the established equilibrium between $(Ca^{2+})_{bound}$ and $(Ca^{2+})_{free}$ for these two groups of synaptosomal preparations. The first possibility examined was whether CTC binding to synaptosomal preparations is influenced by Ca^{2+} and whether it is affected by *in vitro* additions of ethanol.

CTC binding to synaptosomal membranes It can be seen in Fig. 3 that increasing the CTC concentration from 5 to 50 μg in the absence of Ca^{2+} in the medium produced only a slight increase in the amount of CTC bound to the synaptosomes, whereas addition of increasing amounts of Ca^{2+} in the presence of CTC resulted in a much larger increase in CTC binding. This observation illustrates the relative importance of the Ca^{2+}–CTC complex in causing CTC attachment to the membranes as compared to the simple dissolution of CTC from the surrounding medium into the membrane matrix. Exposure of the synaptosomal membranes from control and ethanol-treated animals to increasing Ca^{2+} concentrations led to a considerable enhancement in membrane bound CTC, with the control membranes showing a slightly greater overall response to the presence of Ca^{2+} (Fig. 4A). The same is true for the response of the two types of synaptosomal membranes with respect to the effects of increasing concentrations of ethanol in the incubation medium. The synaptosomes from the control animals exhibited a greater increase in membrane-bound CTC than those from the ethanol-treated animals (Fig. 4B). In addition, this enhancement of CTC binding to the synaptosomal membranes occurred over the same ethanol concentrations as the increase in CTC fluorescence. The response of synaptosomal CTC binding to the presence of ethanol was once again biphasic in nature, just as the CTC fluorescence was, and the synaptosomes from the ethanolic animals exhibited only minimal changes in CTC binding. These observations, then, provide a reasonable correspondence between the changes in fluorescence seen as a result of Ca^{2+} or ethanol exposure and the amount of CTC that is bound by the synaptosomal membranes. However, despite the strong effect of Ca^{2+} on both CTC binding and CTC fluorescence, these findings do not necessarily demonstrate that most of these changes are the direct result of shifts in membrane-bound Ca^{2+}. It is for this reason that the Ca^{2+} binding activity of these preparations was determined directly through the use of the ^{45}Ca radioligand binding assays.

Ca^{2+} binding to brain synaptosomes. The Ca^{2+} binding capacity of the synaptosomal membranes from several of the same animals that were used in the fluorescence assays was determined by measuring the ability of these membranes to bind ^{45}Ca. Of particular interest in these studies was the exploration of the high affinity Ca^{2+} binding sites of these membrane preparations. Consequently, all of these studies were conducted

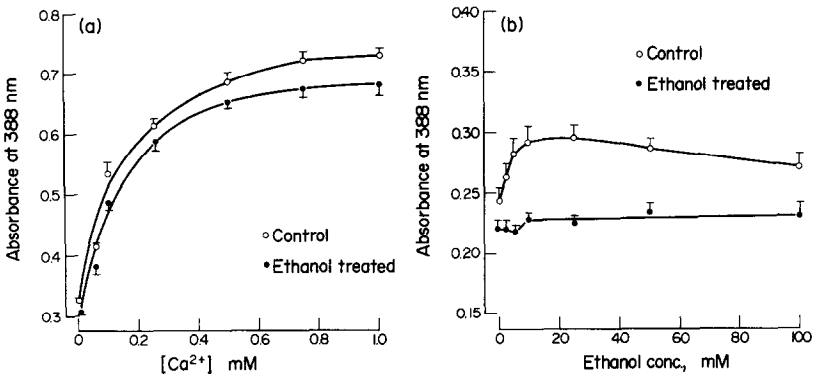

Fig. 4. Effect of increasing Ca^{2+} (A) and increasing ethanol (B) concentrations on the CTC binding to synaptosomal membranes from control and ethanol-treated animals. The membrane preparations from three animals in each group were incubated with the various agents and CTC binding was measured as described in Materials and Methods. Each point is the mean of three determinations (±S.E.).

at micromolar Ca^{2+} concentrations which were obtained through the use of Ca^{2+}–EGTA buffer systems [19]. The effect of a 16-day ethanol treatment was a significant decrease in ^{45}Ca binding activity of the synaptosomal membranes from these chronically intoxicated animals (Fig. 5). Analysis of the data by the method of Klotz [20] was done in order to determine the dissociation constant (K_D) and the maximum number of binding sites (N) of the membranes from the control and ethanol-treated animals. The values for the apparent K_D were 2.89×10^{-5} M for the controls and 2.73×10^{-5} M for the ethanolic animals. The estimated values for N were 11.24 nmoles ^{45}Ca bound·mg^{-1} protein for the controls and 6.10 nmoles ^{45}Ca bound·mg^{-1} protein for the membranes from the ethanol-treated subjects.

The differences between the synaptosomal membranes from control animals and ethanol-treated rats were not confined solely to the ^{45}Ca binding activity of these membranes but were once again detectable in their response to *in vitro* additions of ethanol. Figure 6 reveals that increasing concentrations of ethanol in the assay medium cause a significant increase in ^{45}Ca binding to the synaptosomal preparations from controls without causing a comparable effect on the ^{45}Ca binding activity of the synaptosomes from the ethanolic animals. This pattern of response to *in vitro* ethanol additions is quite similar to that observed in measurements of CTC fluorescence changes and of CTC binding to membrane preparations. Also, the ^{45}Ca binding activity of the membranes from the ethanol-exposed animals is at a lower initial state and changes very little with increases in the concentration of ethanol in the medium (Fig. 6).

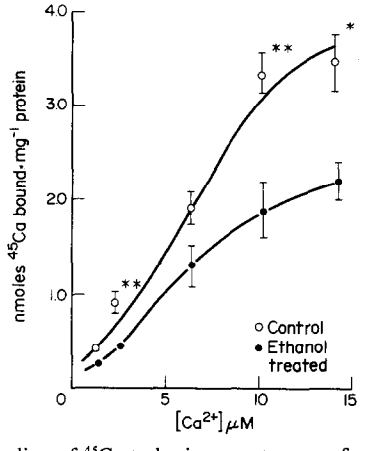

Fig. 5. Binding of ^{45}Ca to brain synaptosomes from control and ethanol exposed animals. All assays were conducted using the Millipore filtration procedure as described in Materials and Methods. Each point is the mean (± S.E.) of five to six determinations from four animals in each group. Differences which are significant at the 0.05 (*) and at the 0.01 (**) level are indicated. Analysis of variance of the group data for the controls and ethanol-treated animals revealed a highly significant difference ($P < 0.01$) between the trends of the two groups.

DISCUSSION

The present study was designed to explore the effects of ethanol on the Ca^{2+} binding capacity of synaptosomal preparations obtained from normal animals and from animals which were chronically exposed to

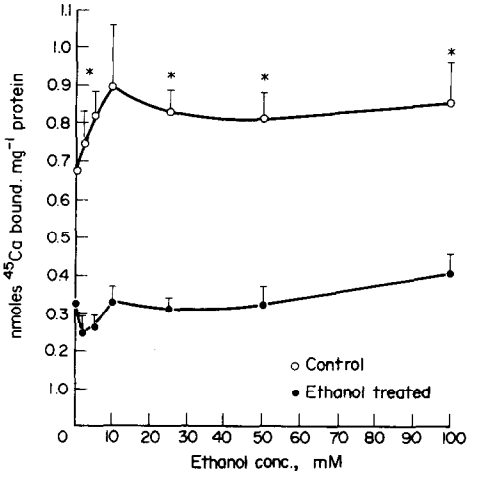

Fig. 6. Effect of increasing ethanol concentrations in the incubation medium on ^{45}Ca binding to synaptosomal membranes from control and ethanol treated animals. Each point is the mean (± S.E.) of six to ten determinations from five animals in each group. ^{45}Ca binding which is significantly different ($P < 0.05$) from baseline binding at zero ethanol concentration is indicated (*).

ethanol. Two issues were of particular interest: (1) Could *in vitro* exposure of these membrane preparations lead to an enhancement of Ca^{2+} binding activity? and (2) Would the sensitivity of these membranes to alcohol be altered as a result of prolonged exposure of the organism to ethanol? The two methods that were employed in the study of Ca^{2+} binding interactions yielded qualitatively very similar results with regard to these two questions.

The ^{45}Ca radioligand binding assays revealed that Ca^{2+} binding to the synaptosomal membranes from chronically ethanol-treated animals was considerably lower than that observed with membranes from control animals. An analysis of these binding interactions showed that, as a result of the chronic ethanol treatment, the maximum number of high affinity Ca^{2+} binding sites was decreased to about one-half the number for the controls although the affinity of these membrane sites for Ca^{2+} in the ethanolic animals was not different from that of the controls. These observations are in very good agreement with the decreases in ^{45}Ca binding to synaptic membranes from chronically (14 days) ethanol-treated animals reported by Ross [11], although the magnitude of the decrease observed by Ross was somewhat smaller (approximately 40 per cent.) No mention is made in that particular report of any changes in the affinity of the binding sites for Ca^{2+}.

The *in vitro* exposure of the synaptosomal membranes from control animals to various concentrations of ethanol in the range of 2.5 to 100 mM led to a significant increase in Ca^{2+} binding for these membrane preparations. This response of the synaptosomal membranes is similar to the previously reported ethanol-induced increases in Ca^{2+} binding activity of erythrocyte ghosts [9]. However, such ethanol-induced enhancement of Ca^{2+} binding to the synaptosomes is difficult to reconcile with the earlier observations by Ross *et al.* [10, 11] that acute administration of ethanol to animals causes Ca^{2+} depletion from the synaptic membranes and that the *in vitro* exposure of synaptic membranes to micromolar concentrations of ethanol leads to inhibition of high affinity Ca^{2+} binding [12]. Since synaptosomal Ca^{2+} levels were not measured directly in our studies, it would be difficult to judge whether changes in Ca^{2+} levels similar to those reported previously [11] were achieved following chronic treatment with ethanol. The discrepancy between the results obtained in this study with respect to ethanol effects on Ca^{2+} binding, and those of Ross *et al.* [12] may be due primarily to differences in methodology, especially in the concentrations of ethanol employed in these assays. In general, the observations from this study and from previous studies point to a rather complex pattern of interactions between ethanol and the Ca^{2+} binding systems of neuronal membranes.

The results obtained by measuring the increases in the fluorescence of CTC in response to changes in Ca^{2+} or ethanol concentration were qualitatively very similar to the patterns obtained through direct determinations of ^{45}Ca bound by the synaptosomal membranes. Based on the observation that addition of Ca^{2+} to the membrane suspension caused a marked enhancement of CTC binding to the synaptosomes, it seems reasonable to interpret the changes in CTC fluorescence as indicative of shifts in Ca^{2+}–membrane interactions. This assumption fits well with the demonstration that the Ca^{2+}–induced enhancement of CTC fluorescence was considerably smaller in membrane preparations from ethanolic animals just as their ^{45}Ca binding capacity was. Similarly, since ethanol additions *in vitro* were shown to shift the equilibrium between $(Ca^{2+})_{bound}$ and $(Ca^{2+})_{free}$ in favor of $(Ca^{2+})_{bound}$, it would be expected then that ethanol additions would enhance CTC binding to the membranes from control animals as well as CTC fluorescence, and both of these effects were observed. On the other hand, since *in vitro* additions of ethanol did not significantly affect the amount of ^{45}Ca bound to membranes from chronically ethanol-treated animals, then it would be expected that ethanol would not have enhanced either CTC binding or CTC fluorescence. Even though the results obtained qualitatively are consistent with the idea that all of these changes are tracing shifts in Ca^{2+} binding, it is difficult to exclude totally the possibility that these changes are the result of ethanol effects on the membrane binding and fluorescence quantum yield of CTC itself.

The additional demonstration in this study that the Ca^{2+} binding activity of membranes from ethanol-treated animals is resistant to the effects of increasing amounts of ethanol added to the medium is of considerable interest. This type of resistance is what one might expect to find in association with a process that has the capacity to undergo adaptive changes that lead to the development of physiological tolerance to the effects of ethanol. The observations of an apparent resistance of the synaptosomal membranes from chronically ethanol-treated animals to the effects of *in vitro* ethanol exposure are in agreement with the findings of Chin and Goldstein [21] who observed a definite resistance of synatosomes from chronically ethanol-treated mice to the membrane-fluidizing effects of ethanol. These membrane-fluidizing effects were shown to be related to the greater mobility of the fatty acid chains of membrane lipids [21]. Thus, both the findings reported in that study as well as our findings suggest that not only are neuronal membranes an important site for the activity of ethanol in the nervous system, but that the phenomenon of physical tolerance to the effects of this agent can actually be observed at the molecular level in the response patterns of these membranes to ethanol additions.

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