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Alcohol Effects on Synaptic Membrane Calcium Ion Fluxes

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MICHAELIS, M. L., E. K. MICHAELIS AND T. TEHAN. *Alcohol effects on synaptic membrane calcium ion fluxes.* PHARMACOL BIOCHEM BEHAV **18**: Suppl. 1, 19–23, 1983.—The effects of ethanol on Na^+-dependent CA^{2+} fluxes have been examined in resealed synaptic membrane vesicles assayed at 3 different temperatures. Sodium chloride-loaded vesicles were preincubated with various concentrations of ethanol for 120 sec prior to being diluted into a $^{45}CaCl_2$-containing medium in the presence or absence of an outward-directed Na^+ gradient. The effect of ethanol on Na^+-dependent Ca^{2+} transport measured at 23°C was biphasic. However, when the assay was conducted either at 16°C or at 35°C, all ethanol concentrations tested (10–300 mM) produced only inhibition of Ca^{2+} influx. The role of membrane fluidization in the ethanol-induced inhibition was explored by determining the effects of incorporating various fatty acids into the membranes. Membrane fluidizing agents such as *cis*-vaccenic acid stimulated Ca^{2+} influx whereas *trans*-vaccenic and saturated fatty acids had little effect. The fluidizing effect of incorporating *cis*-vaccenic acid into the membranes was confirmed with electron paramagnetic resonance (EPR) spectroscopy. The data obtained from these studies suggest that the inhibition of Ca^{2+} fluxes produced by alcohol and local anesthetics is not the result of a general increase in bulk phase synaptic membrane fluidity.

Ethanol Calcium ion fluxes Na^+Ca^{2+} exchange Synaptic membrane vesicles Fatty acids EPR spectroscopy

ETHANOL has been reported to influence both Ca^{2+} binding to plasma membranes and Ca^{2+} transport across plasma membranes in several types of cells [7, 8, 17, 23, 32]. The effects of ethanol on neuronal Ca^{2+} activity are of particular interest in view of the pronounced influence of ethanol on CNS functioning and the behavioral consequences of excessive and/or chronic alcohol consumption. Neurons appear to have a number of different mechanisms which participate in the maintenance of intracellular Ca^{2+} homeostasis and in the removal of the Ca^{2+} that enters nerve terminals at the time of depolarization. The proposed mechanisms include an ATP-dependent sequestration of free Ca^{2+} either by mitochondria [27] or by intraterminal vesicles [10,15], the pumping of Ca^{2+} out of the cells by a plasma membrane Ca^{2+}-Mg^{2+} ATPase [6], and the transport of Ca^{2+} by the coupled exchange of Ca^{2+} for Na^+ across the plasma membrane [1,2]. This membrane antiport system which utilizes a Na^+ gradient to transport Ca^{2+} has been studied in this laboratory through the use of isolated synaptic plasma membrane vesicles. The resealed synaptic membrane vesicle preparation permits one to manipulate the ionic gradients on either side of the plasma membrane and thus to examine the influence of various ionic species on membrane transport processes [20,21].

We have recently described some effects of *in vitro* ethanol exposure on the Na^+-dependent Ca^{2+} fluxes in resealed synaptic membrane vesicles [22]. It was observed that low (< 50 mM) concentrations of ethanol increased, while higher concentrations (100–600 mM) decreased, the activity of this ion counter transport system. Charged local anesthetics such as tetracaine and dibucaine also were found to inhibit the Na^+-dependent Ca^{2+} fluxes [22]. The Na^+-Ca^{2+} antiport activity may involve a carrier molecule which moves within the membrane to transport ions [2], and such a carrier system is likely to be quite sensitive to any alterations in the physical state of the synaptic membranes. Since ethanol, other aliphatic alcohols, and local anesthetics have been reported to disrupt membrane organization as determined by various physical probes of membrane structure [4, 5, 9, 11, 12, 13, 14, 16, 29], such a membrane disordering effect may explain the alterations in Ca^{2+} transport observed in these vesicles. One of the best described effects of ethanol is to increase fatty acid chain motion within the lipid bilayer, an effect often referred to as "fluidization" of the membrane [4, 5, 9, 14, 29]. It is also possible to enhance membrane fluidity by raising the temperature of the surrounding medium or by introducing into the membranes unsaturated fatty acids in the *cis*-configuration [28, 30, 31]. The following report describes the effect of using these two manipulations of the physical state of the synaptic membranes on the activity of the Na^+-Ca^{2+} exchange carrier. These studies were undertaken to determine whether such general increases in bulk phase synaptic membrane fluidity would affect this ion transport system in a manner analogous to that produced by the alcohols and local anesthetics.

METHOD

Preparation of Synaptic Membrane Vesicles and Measurement of Ca^{2+} Fluxes

Synaptic membranes were prepared from pooled whole

brains from male Sprague-Dawley rats (200–350 g) essentially as described elsewhere [20,21]. The final membrane pellet was resuspended in 0.32 M sucrose-50 μM $MgCl_2$ at 9–12 mg protein/ml, and small aliquots were frozen in liquid N_2 and stored at −80°C for periods no longer than 4 weeks. The Na^+-dependent Ca^{2+} fluxes were measured as described in detail elsewhere [20,22]. Briefly, aliquots of frozen membranes were loaded internally with Na^+ by rapidly thawing them at 37°C for 10 min in the presence of 7–8 volumes of 160 mM NaCl-50 μM $MgCl_2$-2 mM Tris/HCl, pH 7.4. The vesicles were then allowed to cool to 23°C, and 50 μl aliquots (40–70 μg protein) were transferred to tubes containing 10 μM or, where indicated, varied concentrations of $^{45}CaCl_2$, 0.07 μCi/sample (New England Nuclear Corporation), in 250 μl of either 160 mM NaCl or KCl in 25 mM Tris/HCl, pH 7.4. Calcium influx was allowed to proceed for the indicated time periods, depending on the temperature of the incubation medium. The incubations were terminated by addition of 2 ml ice-cold 160 mM KCl and rapid filtration through Whatman GF/B filters under moderate vaccum. The filters were washed with 2 ml of the KCl "stop" solution, dried, and the radioactivity assayed in 6 ml of toluene/Triton X-100 scintillation fluid. Background absorption of ^{45}Ca to filters and membranes was determined in samples to which 2 ml of the "stop" solution were added prior to addition of the membranes. These blank values were subtracted from the values obtained for incubated samples in the calculation of the Ca^{2+} fluxes.

When the effects of ethanol were examined, the membranes were preincubated for 120 sec with the indicated concentrations of alcohol prior to initiation of the Ca^{2+} influx. In the studies in which the effects of added fatty acids were examined, the Na^+-loaded membrane vesicles were incubated with 1 mM *cis*- or *trans*-vaccenic acid (Sigma Chemical Co.) for 10 min at 23°C. The vesicles were then precipitated at 20,000 × g for 15 min at 4°C to remove unincorporated fatty acids, and the pellets were resuspended in the NaCl loading solution and used in the assay. The protein content of membranes was determined by the method of Lowry [18].

Electron Paramagnetic Resonance (EPR) Spectroscopy of Synaptic Membranes

The EPR studies were performed with synaptic membrane vesicles that were mixed with the EPR probe 5-doxyl stearic acid (Syva) in ethanol in a mole ratio of approximately 130:1, lipid to probe. The membranes were centrifuged at 4°C for 15 min at 20,000×g and then resuspended in 160 mM NaCl-25 mM Tris/HCl for incubation with *cis*-vaccenic acid or with the methanol solvent used to dilute the fatty acid (0.1% or less final methanol concentration). Incubation was carried out at 23°C for 10 min and the membranes were again centrifuged at 20,000 × g for 15 min. Samples were then flame-sealed in 100 μl glass capillary tubes and their EPR spectra determined as described elsewhere [25]. Methods for calculation of the S parameter are described in an accompanying paper [24].

RESULTS

Ethanol Effects on Na^+-Dependent Ca^{2+} Fluxes

The Na^+-dependent Ca^{2+} fluxes were determined under all conditions by subtracting the amount of ^{45}Ca bound to or passively taken up by the membranes in the absence of a Na^+ gradient (i.e., vesicles loaded internally with NaCl and incubated in a NaCl medium) from the Ca^{2+} taken up in the presence of an outward-directed Na^+ gradient (i.e., Na^+-loaded vesicles incubated in a KCl medium). The Na^+-loaded synaptic membrane vesicles exhibited fairly low ^{45}Ca uptake in the absence of a Na^+ gradient. This background flux generally constituted about 20% of the ^{45}Ca signal in the presence of the lower Ca^{2+} concentrations and tended to increase to about 35% of the total signal as the external Ca^{2+} concentration approached 100 μM. The background Ca^{2+} flux was measured under all experimental conditions and was used to determine the Na^+ gradient-dependent component of the Ca^{2+} transport activity. Previous studies have revealed that this ion transport system was bidirectional [20]. The activity of the Ca^{2+} uptake system was linear over the range of protein concentrations tested (0.08–0.4 mg/ml final concentration in the assay) and was dependent on the concentration of Ca^{2+} in the external medium over the range from 5 μM to 100 μM $CaCl_2$ [22]. Studies on the time kinetics revealed that the Na^+-dependent CA^{2+} influx was linear for varying periods of time depending on the incubation temperature. Thus, incubation times within the linear phase for each of the temperatures were selected when temperature conditions were varied as in Fig. 1.

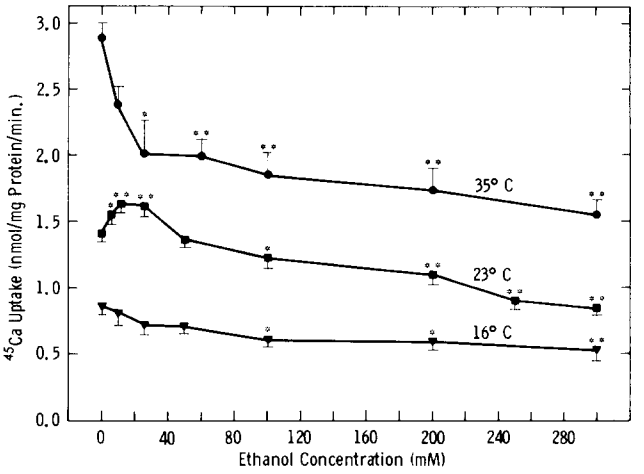

FIG. 1. Effects of ethanol on Na^+-dependent Ca^{2+} uptake into synaptic membrane vesicles measured at 3 different incubation temperatures. NaCl-loaded vesicles were incubated in the presence or absence of an outward-directed Na^+-gradient with 10 μM $^{45}CaCl_2$ and indicated ethanol concentration in the external medium. Calcium uptake in the absence of a Na^+ gradient was subtracted to obtain the Na^+-dependent uptake values. Membranes were preincubated for 120 sec with ethanol prior to being transferred to appropriate salt solutions where incubations were carried out for 15 sec at 16° and 23°C, and for 8 sec at 35°C in order to measure transport during the initial linear phase. All values were extrapolated to nmol Ca^{2+} taken up/min. Each point is the mean±S.E.M. for 10–20 determinations from 3–4 different membrane preparations. Calcium uptake values which differ significantly from those obtained for a given temperature in the absence of ethanol are indicated with asterisks, $*p<0.05$ and $**p<0.01$. Statistical significance of differences between control and ethanol samples was determined by means of Student's *t*-test for unpaired samples with a minimum of 18 *df* for any set of values tested.

The effects of several ethanol concentrations on the Na^+-dependent Ca^{2+} uptake measured at 16°, 23°, and 35°C are shown in Fig. 1. It can be seen in this figure that the predominant effect of ethanol is to decrease the Ca^{2+} trans-

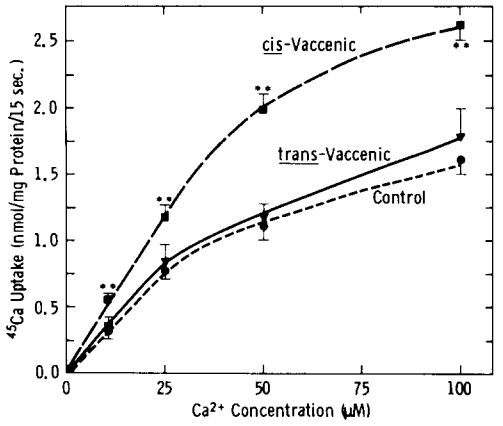

FIG. 2. Effects of pretreatment of membranes with *cis*- or *trans*-vaccenic acid on the Na^+-dependent Ca^{2+} uptake measured at 4 concentrations of $CaCl_2$. Membrane vesicles were preincubated with 1 mm *cis*- or *trans*-vaccenic acid or appropriate amount of methanol for the controls as described in the Method section. Sodium dependent Ca^{2+} uptake was measured for 15 sec at 23° in the presence of the indicated concentrations of Ca^{2+} in the external medium. Each point is the mean±S.E.M. for 8–12 determinations from 3 different membrane preparations. Calcium uptake values which differ significantly from controls are indicated (**$p<0.01$) with a minimum of 14 *df* for any set of values tested.

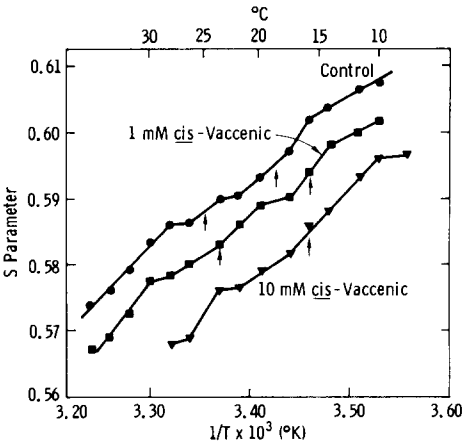

FIG. 3. Arrhenius-type plot of the effects of *cis*-vaccenic acid on membrane organization of synaptic membrane vesicles labelled with the 5-doxyl stearic acid spin probe. Electron paramagnetic resonance spectra were recorded at the indicated temperatures following pre-treatment of the membranes with 1 mM or 10 mM *cis*-vaccenic acid as described in the Method section. Data are from a single experiment which was repeated 2 times with similar results.

port activity regardless of the incubation temperature. The one notable exception, however, is the small but statistically significant enhancement in Ca^{2+} flux observed at the lower ethanol concentrations (<50 mM) when the assay was conducted at 23°C. More recent experiments have shown that ethanol concentrations as low as 2.5 mM decrease the Ca^{2+} transport acitivity measured at 35°C but increase activity at 23°C (data not shown). It should be noted that the data are graphed as the amount of Ca^{2+} taken up per mg protein per min though the actual incubation times differed for each temperature in order for the measurements to be obtained during the initial linear phase of the uptake process at a given temperature in the presence of 10 μM $CaCl_2$.

Incorporation of Fatty Acids into Synaptic Membranes

The effects of preincubating the synaptic membranes with the monounsaturated fatty acids vaccenic acid (Δ^{11-12}-C_{18}) in either the *cis* or the *trans* configuration are shown in Fig. 2. The Na^+-dependent Ca^{2+} uptake is plotted across increasing Ca^{2+} concentrations in the medium with all incubations carried out for 15 sec at 23°C. It is readily apparent that pretreatment with the *cis* isomer increased the transport activity while the *trans* isomer had very little effect. Thus it appears that the two isomers have very different effects on this Ca^{2+} transport system with the membrane disordering or fluidizing isomer causing a substantial enhancement in Na^+-dependent Ca^{2+} uptake. In a related series of experiments pretreatment of the synaptic membranes with the fully saturated C_{18} fatty acid stearic acid at 0.5 mM concentration also had no effect on the Na^+-dependent Ca^{2+} uptake (data not shown).

The membrane disordering actions of *cis*-vaccenic acid under the conditions used in these studies were confirmed by EPR analysis with a 5-doxyl stearic acid spin label probe. Preincubation of the synaptic membranes with *cis*-vaccenic acid led to concentration-dependent decreases in the order parameter measured across temperatures between 8° and 36°C. Similar experiments with *trans*-vaccenic acid revealed no such effects (data not shown). The conclusion that decreases in the S parameter after preincubation with *cis*-vaccenic acid were indicative of increased lipid motion within the membrane bilayer was confirmed by the observed decreases in the S parameter caused by raising the temperature of the chamber (Fig. 3). Heating of the synaptic membrane samples revealed the presence of two apparent transition phases. The first transition phase (16–22°) had a midpoint transition temperature (T_c) of 19°C while the second transition phase (24–26°C) had a T_c of 25°C (Fig. 3). Incubation of the synaptic membranes with 1 mM *cis*-vaccenic acid lowered the T_c of the first transition phase (14–18°C) to 16°C, markedly broadened the second transition phase (20–28°), and lowered the T_c to 24°C. The introduction of 10 mM *cis*-vaccenic acid produced even more dramatic changes in the thermotropic behavior of the synaptic membranes. There was only one apparent transition phase which was rather broad (10–24°C) and had a T_c of 16°C (Fig. 3).

DISCUSSION

The synaptic membrane Na^+ gradient-dependent Ca^{2+} transport activity appears to be quite sensitive to the presence of ethanol as well as other aliphatic alcohols and local anesthetics [22]. At nearly physiologic temperatures, low ethanol concentrations (<25 mM) inhibited the activity of this transport system by approximately 30%. Inhibition by pharmacologically relevant ethanol concentrations of a neuronal membrane system that may be involved in the extrusion of intracellular free Ca^{2+} and in the maintenance of low intraneuronal Ca^{2+} concentrations could have important implications for neuronal excitability and transmitter release. For example, the enhancement by ethanol of the activity of Ca^{2+}-dependent K^+ channels in hippocampal neurons has been attributed to a rise in the intraneuronal free Ca^{2+} concentration [3]. Activation of these K^+ channels

leads to prolonged hyperpolarization of the affected neurons and subsequent decreases in excitability.

It is difficult to explain the process by which ethanol produces the biphasic effects on Na^+-Ca^{2+} antiport activity that was measured at 23°C, since at both 16°C and 35°C ethanol caused only inhibition of Ca^{2+} transport. One plausible explanation might be related to a presumed physicochemical interaction of ethanol with the lipid environment surrounding this transport carrier, leading to differential effects on its activity at different temperatures. It may be significant with respect to such ethanol-lipid interactions that both the 16°C and 35°C incubation temperatures are outside the transition phases of the synaptic membrane lipid organization as determined by means of EPR spectroscopy (Fig. 3). Thus, the activation of Na^+-Ca^{2+} exchange transport by low ethanol concentrations at 23°C could be the result of a cooperative interaction between ethanol and clusters of lipids around the carrier that are undergoing a transition from the gel to the liquid-crystalline phase. It has been suggested that anesthetic agents may act primarily by changing the size and cooperativity of the lipid clusters of either the gel or liquid phase [26]. It has yet to be demonstrated that ethanol at low concentrations can produce such changes in synaptic membrane lipid cooperativity during temperature-induced phase transitions. This is in part due to the complexity of the lipid composition of these membranes as compared to the model membrane systems used in the previously cited studies of anesthetic-induced changes in lipid organization [26].

One of the best documented physical effects of ethanol on synaptic membrane lipid organization is the enhancement in lipid motion produced by concentrations of this alcohol as low as 8–20 mM [4,9]. If bulk phase lipid "fluidization" were an important determinant of ethanol's effects on the Na^+-Ca^{2+} antiport system, then one would predict that agents which produced similar or greater degrees of lipid fluidization should have the same effect as ethanol on this membrane Ca^{2+} carrier. The unsaturated fatty acids *cis*-vaccenic and oleic acid have been shown to increase membrane lipid motion whereas the *trans* isomer of vaccenic acid had no effect on membrane lipid fluidity [28,33]. These findings were confirmed with synaptic membranes in our studies showing that *cis*-vaccenic acid, but not the *trans* isomer caused decreases in the S parameter of the 5-doxyl stearic acid-labelled membranes. Yet the primary effect of *cis*-vaccenic acid on the Na^+-Ca^{2+} exchange transport was a substantial stimulation of transport rather than inhibition as produced by ethanol. The *trans*-vaccenic isomer had no effect on the transport activity. Similar differential effects of the *cis* and *trans* isomers of vaccenic acid were reported for D-glucose transport activity in adipocyte plasma membranes [30]. It would appear that a generalized fluidization of the bulk lipid phase may be an inadequate concept to explain the actions of ethanol on this neuronal membrane ion transport system. Alternative explanations of the actions of ethanol may include a more specific interaction with a sub-class of membrane lipids that surround this membrane carrier or possibly a more direct interaction of ethanol with the cation binding sites of the carrier complex.

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