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ALCOHOL AND LOCAL ANESTHETIC EFFECTS ON Na^+-DEPENDENT Ca^{2+} FLUXES IN BRAIN SYNAPTIC MEMBRANE VESICLES

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Abstract—Resealed synaptic plasma membrane vesicles exhibit Na^+-dependent Ca^{2+} transport activity which may participate in regulation of free Ca^{2+} concentrations in nerve endings. Sodium chloride-loaded vesicles took up Ca^{2+} from the external medium (150 mM KCl–25 mM Tris/HCl) in the presence of an outward-directed Na^+ gradient, a Ca^{2+} concentration of 38.6 μM producing half-maximal uptake at 23°. Methanol (5–200 mM) and low concentrations of ethanol (5–25 mM) enhanced the Na^+-dependent Ca^{2+} influx measured at 23°. Higher ethanol concentrations (100–600 mM), as well as 1-propanol and 1-butanol (10–200 mM), produced only inhibition of Ca^{2+} fluxes. Dixon plot analysis of the inhibitory phase revealed that ethanol inhibited Ca^{2+} uptake in an apparently competitive manner with respect to Ca^{2+} concentration, and the K_i obtained from these experiments was 1.01 M ethanol. The inhibition of Ca^{2+} fluxes by butanol was non-competitive, and the K_i was 68.6 mM. The local anesthetics dibucaine and tetracaine also inhibited Ca^{2+} fluxes with IC_{50} values of 1.8 mM for tetracaine and 0.46 mM for dibucaine. The possible physiologic consequences of this inhibition of Na^+–Ca^{2+} countertransport is synaptic membranes by the alcohols and local anesthetics are discussed with regard to neuronal transmission and membrane conductance.

The *in vitro* exposure of cells or isolated membranous organelles to various concentrations of ethanol and other alcohols leads to changes in the activities of membrane-related entities such as ion channels, enzymes, uptake carriers, ion binding sites, and neurotransmitter receptors [1–14]. In addition, the observation has been made frequently that resistance to the *in vitro* effects of ethanol on these systems develops after chronic treatment of organisms with ethanol [8–10, 13, 15, 16]. This type of resistance to the "acute" effects of ethanol on membrane-related functions has been considered to be a cellular or molecular analog of the tolerance that appears in organisms that have been chronically exposed to this alcohol. The functional changes produced following ethanol interaction with biological membranes are also related to physical–chemical changes, brought about by exposure of biological membranes to ethanol, that may involve increases in fatty acid chain motion within the membrane lipid bilayer, expansion of membrane volume, and changes in membrane lipid and protein composition and conformation [17–26]. It is worth noting that chronic exposure of organisms to ethanol alters the chemical composition of some biological membranes and brings about a certain level of resistance to the effects of ethanol on membrane organization [17, 18, 21, 23]. Thus, it has been assumed that many of the acute pharmacological actions of ethanol and other short-chain aliphatic alcohols, as well as some of the physiological changes brought about by chronic exposure to ethanol, are produced through alterations in the structure and function of biological membranes (e.g. Refs. 27–29).

Some of the effects of ethanol on plasma membranes are probably related to changes in nerve cell excitability and in neutrotransmitter release [1–4, 13, 14, 30]. Since calcium ions (Ca^{2+}) are known to be involved in the regulation of voltage-dependent and neurotransmitter-induced ion fluxes and in the initiation of stimulus-induced transmitter release from nerve endings (e.g. Refs. 31 and 32), a number of studies have been conducted to explore the effects of ethanol on Ca^{2+} binding to neuronal [9, 10, 33] and erythrocyte plasma membranes [34] and on Ca^{2+} flux or transport processes in neuronal preparations [16, 35]. For example, the introduction of ethanol into the incubation media of either intact neurons or of isolated nerve endings (synaptosomes) has been shown to increase the release of neurotransmitter in unstimulated and stimulated preparations [30, 36, 37]. This enhanced neurotransmitter release may be related either to increases in Ca^{2+} binding to neuronal membranes produced by ethanol [10, 34] or to increases in the activity of free Ca^{2+} within neurons [30, 35]. It has been suggested that these effects of ethanol on Ca^{2+} binding or fluxes may be caused by the membrane "expansion" or "fluidization" that is brought about by exposure of biological membranes to increasing concentrations of ethanol [36].

An additional possible mechanism for enhanced intraneuronal accumulation of free Ca^{2+} in the presence of ethanol might be the inhibition by ethanol of Ca^{2+} transport processes involved in Ca^{2+} extrusion or Ca^{2+} sequestration. One such transport process which is thought to be operating at the level of the neuronal plasma membrane and to be important

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in maintaining low intraneuronal Ca^{2+} levels is the Na^+–Ca^{2+} antiport system described by Baker, Blaustein, and their colleagues [38–40]. In the present study, we have explored the *in vitro* effects of several short-chain aliphatic alcohols and some local anesthetic agents on the Na^+–Ca^{2+} antiport process of isolated-resealed synaptic plasma membrane vesicles that we have described previously [41].

MATERIALS AND METHODS

Preparation of synaptic membrane vesicles. Male Sprague–Dawley rats (250–350 g), obtained from the Charles River Breeding Laboratories, were used in all studies reported. The resealed synaptic membrane vesicles were prepared essentially as described elsewhere [41] with only minor modifications. The synaptosome-enriched subfraction recovered from the discontinuous 7.5–13% Ficoll gradients was washed with 0.32 M sucrose, and the pellets were resuspended in a large volume of cold deionized H_2O and stirred at 4° for 30 min. After the osmotic lysis step, the suspension was centrifuged at 20,000 *g* for 20 min, and the membrane pellets were resuspended in 0.32 M sucrose–50 μM $MgCl_2$ at a final protein concentration of 9–12 mg/ml. The membranes were divided into small aliquots (3–4 mg protein), quickly frozen in liquid N_2, and stored at −80° for periods no longer than 4 weeks.

Measurement of Na^+*-dependent* Ca^{2+} *fluxes.* Aliquots of frozen membranes were internally loaded with Na^+ by rapidly thawing them at 37° in the presence of 7–8 vol. of 160 mM NaCl–50 μM $MgCl_2$–25 mM Tris/HCl, pH 7.4. After 10 min at 37°, the membranes were allowed to cool to 23° before the assay was begun. Aliquots (40–60 μg protein) of the Na^+-loaded membrane vesicles were transferred to tubes containing either 10 μM or, where indicated, varied concentrations of $^{45}CaCl_2$ (0.07 μCi) in 250 μl of either 160 mM KCl–25 mM Tris/HCl or 160 mM NaCl–25 Tris/HCl, pH 7.4. All incubations were carried out at 23° for 15 sec or for variable periods of time as indicated. Incubations were terminated by the addition of 2 ml of ice-cold (0–4°) 160 mM KCl–25 mM Tris/HCl, pH 7.4, and rapid filtration through Whatman GF/B filters under moderate vacuum. The filters were then washed with 2 ml of the ice-cold KCl solution, dried, and assayed for radioactivity after dissolution in 6 ml of toluene/Triton X-100 scintillation fluid. Background adsorption of $^{45}Ca^{2+}$ to the filters and the membranes was determined in samples to which 2 ml of the "stop" solution was added prior to addition of the membranes. These samples were immediately filtered and washed identically to the others, and the values obtained in this way were subtracted from the values for the incubated samples in the calculation of Ca^{2+} influx.

When the effects of alcohols or local anesthetics on Ca^{2+} influx were determined, the membranes were preincubated for 120 sec with the indicated concentrations of the drugs. Membranes were then transferred to the tubes containing either NaCl or KCl with $^{45}CaCl_2$ and the same concentration of the drug with which the membranes had been preincubated. The $^{45}Ca^{2+}$ uptake into the membrane vesicles was allowed to proceed for 15 sec, and the reaction was terminated as described above.

Other procedures. The protein concentration of the membrane preparations was determined by the method of Lowry *et al.* [42] with bovine serum albumin as a standard. Statistical analysis of the significance of differences between control and drug-treated samples was performed using Student's *t*-test for unpaired samples.

RESULTS

Na^+-dependent Ca^{2+} fluxes in synaptic membrane vesicles. Synaptic plasma membrane vesicles internally loaded with 160 mM NaCl showed a very low $^{45}Ca^{2+}$ uptake or binding activity when incubated in the presence of 160 mM NaCl–10 μM $^{45}CaCl_2$ (0.0945 ± 0.016 nmole $Ca^{2+} \cdot (\text{mg protein})^{-1} \cdot (15\text{ sec})^{-1}$, mean ± S.E., N = 22), as compared to the same membrane vesicles incubated in a medium that contained 160 mM KCl–10 μM $^{45}CaCl_2$ (0.425 ± 0.011 nmole $Ca^{2+} \cdot (\text{mg protein})^{-1} \cdot (15\text{ sec})^{-1}$, N = 29). The $^{45}Ca^{2+}$ influx into the membrane vesicles determined in the presence of an outward-directed Na^+ gradient across the vesicle membranes was considered to be the Na^+-dependent Ca^{2+} uptake in these preparations. The dependence of this Ca^{2+} transport activity on the presence of a Na^+ gradient (in → out in these experiments) has been demonstrated previously in synaptic membrane vesicles [41] as well as with other types of neuronal and non-neuronal tissues (e.g. Refs. 38–40 and 43). This Na^+-dependent Ca^{2+} uptake into resealed synaptic membrane vesicles was linear over the range of protein concentrations tested (0.08 to 0.4 mg/ml, final concentration in the assay). Kinetic studies revealed that the Na^+-dependent Ca^{2+} uptake was linear for at least 20 sec at 23°. Therefore, the incubation period selected as being representative of the initial velocity of the transport activity in all subsequent studies conducted at 23° was 15 sec. This Na^+-dependent Ca^{2+} flux was not inhibited by the (Na^+–K^+) ATPase blocker ouabain, and no (Na^+–K^+) ATPase activity could be detected in these membranes unless the substrate ATP was added to the membranes. This indicated that there were no endogenous levels of ATP associated with the synaptic membrane preparations, whereas low activity of (Na^+–K^+) ATPase could be detected in intact synaptosomes without addition of exogenous ATP.

Calcium influx in the presence of an outward-directed Na^+ gradient was dependent on the concentration of Ca^{2+} in the external medium over the range from 5 to 100 μM $CaCl_2$ (Fig. 1A). The Ca^{2+} concentration which led to half-maximal uptake activity (K_{act}) under these conditions was estimated from double-reciprocal plots to be 38.6 μM (Fig. 1B), and the maximal transport activity in this Ca^{2+} concentration range was found to be 1.52 nmoles $Ca^{2+} \cdot (\text{mg protein})^{-1} \cdot (15\text{ sec})^{-1}$. The Ca^{2+} uptake activity shown in Fig. 1 and subsequent figures represents the Na^+-dependent Ca^{2+} uptake after the passive Ca^{2+} flux in the absence of the Na^+ gradient has been subtracted.

Ethanol and Na^+-dependent Ca^{2+} influx. The

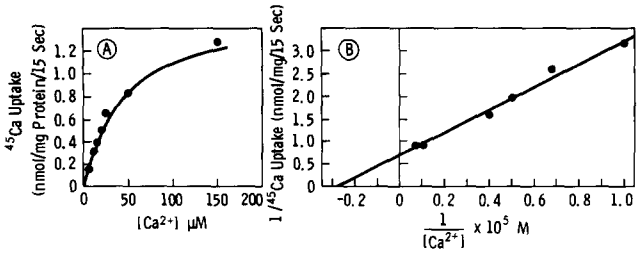

Fig. 1. Na^+-dependent Ca^{2+} uptake into synaptic membrane vesicles in the presence of increasing concentrations of $CaCl_2$ in the incubation medium. (A) NaCl-loaded vesicles were incubated in the presence or absence of an outward-directed gradient with increasing Ca^{2+} concentrations for 15 sec at 23° as described in Materials and Methods. Calcium uptake in the absence of a Na^+ gradient was substracted to obtain the Na^+-dependent uptake values. Each point is the mean of six determinations from two different preparations. (B) Inverse plot of data shown in A, with the line fitted by the least squares method. The constants obtained from this inverse plot were used to calculate the curve shown in A.

effects of several ethanol concentrations on the Na^+-dependent Ca^{2+} uptake are shown in Fig. 2. It can be seen in this figure that the *in vitro* exposure of the synaptic membranes to ethanol produces a biphasic effect on the Ca^{2+} transport activity. Exposure of the membranes to low ethanol concentrations (5–25 mM) led to a significant enhancement of Ca^{2+} influx, whereas ethanol concentrations above 100 mM substantially inhibited the Na^+-dependent Ca^{2+} fluxes. Possible effects of ethanol on passive Ca^{2+} binding to the synaptic membrane vesicles are corrected for by subtraction of the Ca^{2+} uptake signal in the absence of a Na^+ gradient from that in the presence of such a gradient. Thus, the data shown in Fig. 2 represent ethanol effects on the Na^+ gradient-stimulated Ca^{2+} transport activity. It was observed that under these assay conditions ethanol had only small effects on the Ca^{2+} binding and/or passive influx in the synaptic membrane vesicles that was measured in the absence of a Na^+ gradient.

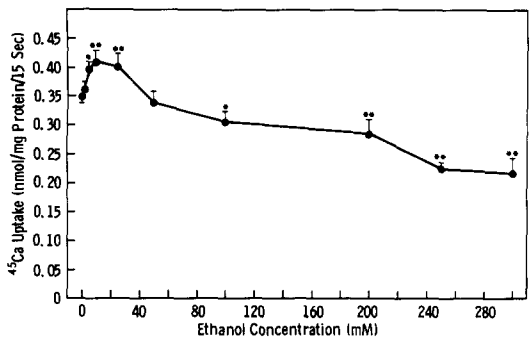

Fig. 2. Effects of ethanol on the Na^+-dependent Ca^{2+} influx in synaptic membrane vesicles. The Ca^{2+} uptake into NaCl-loaded vesicles was measured as described for Fig. 1. The various concentrations of ethanol were preincubated with the membrane vesicles for 120 sec prior to transfer of the membranes to the incubation media containing 10 μM $^{45}Ca^{2+}$ and the appropriate salt solutions. The corresponding ethanol concentrations were also present in the incubation tubes. Each point represents the mean ± S.E. of eighteen to twenty-four determinations from four different membrane preparations. Calcium uptake values which differ significantly from those obtained in the absence of ethanol are indicated with asterisks: (*) $P < 0.05$, and (**) $P < 0.01$.

The inhibitory effect of the higher ethanol concentrations on the Na^+–Ca^{2+} exchange process was explored in the presence of three different Ca^{2+} concentrations (5, 10 and 25 μM Ca^{2+}) and three of the higher ethanol concentrations (200, 400 and 600 mM). A Dixon plot analysis of the data from these experiments is shown in Fig. 3. The type of inhibition produced by these high concentrations of ethanol appeared to be competitive in nature with respect to Ca^{2+} concentrations. The inhibitory constant for ethanol obtained from these studies varied between K_i of 0.98 M and 1.04 M. The average estimated K_i for ethanol inhibition was 1.01 M.

Effects of other aliphatic alcohols and local anesthetics on Na^+*-dependent* Ca^{2+} *transport.* The effects of three other short-chain alcohols—methanol, 1-propanol, and 1-butanol—were examined in order to determine whether differences in carbon chain length of the alcohols led to different effects on this Ca^{2+} transport system. Of the four alcohols examined—methanol, ethanol, 1-propanol, and 1-butanol—only ethanol showed a biphasic type of effect on the Na^+-dependent Ca^{2+} influx within the concentration range of 2.5 to 200 mM. Methanol was found to enhance the activity of the Na^+–Ca^{2+} antiport process, whereas 1-propanol and 1-butanol produced only concentration-dependent inhibition of Ca^{2+} transport over the same concentration range (Fig. 4A). The concentration of the aliphatic alcohols ethanol to 1-butanol that produced 25% inhibition of the basal activity of the Na^+ gradient-dependent Ca^{2+} transport (IC_{25}) varied inversely with respect to the aliphatic chain length (Fig. 4B). Since propanol and butanol partition into the membrane bilayer to a greater extent than ethanol does, the effects of lower concentrations of these two alcohols on the Na^+–Ca^{2+} exchange system were also explored. At concentrations ranging from 0.25 to 2.5 mM in three different membrane preparations, neither 1-propanol nor 1-butanol produced significant enhancement of the Na^+ gradient-dependent Ca^{2+} transport. A small increase in Ca^{2+} influx was observed with 0.75 mM butanol [0.39 vs 0.43 nmole $Ca^{2+} \cdot (mg\ protein)^{-1} \cdot (15\ sec)^{-1}$ for 0 and 0.75 mM butanol samples respectively]. However, although this small enhancement was observed consistently, the magnitude of the increase was not statistically significant.

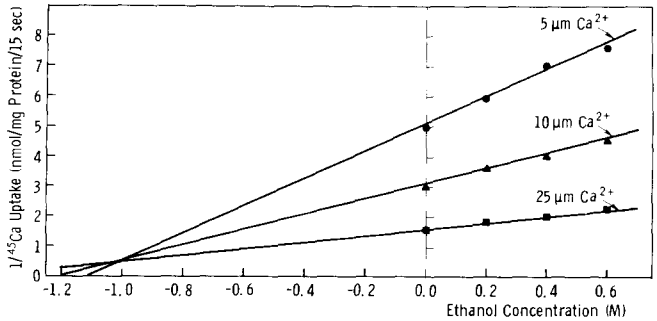

Fig. 3. Dixon plot analysis of the inhibition of Ca^{2+} transport produced by ethanol. The NaCl-loaded vesicles were preincubated for 120 sec with the indicated concentrations of ethanol before being transferred to the incubation media containing 5 μM $CaCl_2$ (●), 10 μM $CaCl_2$ (▲) or 25 μM $CaCl_2$ (■) and incubated for 15 sec at 23°. Each point was obtained from the mean of nine to twenty determinations from three to five different preparations, and the lines were fitted by the least squares method. The standard error was less than 10% for all means.

High concentrations of the aliphatic alcohols are known to cause general as well as local anesthesia [28]. Since the concentrations of ethanol, 1-propanol, and 1-butanol that inhibited the Na^+–Ca^{2+} antiport activity in these membrane vesicles were within the ranges producing local anesthetic activity [28], it was considered appropriate to examine the effects of some local anesthetic agents on the Ca^{2+} transport process and to compare them to the actions of the aliphatic alcohols. The local anesthetics dibucaine and tetracaine had a very pronounced inhibitory effect on the synaptic membrane Na^+-dependent Ca^{2+} transport (Fig. 5). The concentrations of local anesthetics that caused 50% inhibition of the basal Ca^{2+} transport activity (IC_{50}) were determined by log-probit analysis of the data shown in Fig. 5 and were calculated to be 0.46 mM for dibucaine and 1.81 mM for tetracaine. Hill plot analysis of the inhibition produced by the local anesthetics and by butanol gave linear plots with the following slopes (or Hill coefficients): dibucaine, 0.50; tetracaine, 0.55; and 1-butanol, 0.51. Thus, the local anesthetics and butanol appeared to inhibit the Na^+–Ca^{2+} antiport process in synaptic membranes in a manner that involved negatively cooperative interactions (i.e. Hill coefficients were less than 1.0). Since the inhibition produced by ethanol appeared to be competitive with respect to Ca^{2+} activation of the Na^+–Ca^{2+} exchange process (Fig. 3), we decided to explore the type of inhibition that was produced by butanol under identical conditions. The results obtained are shown in Fig. 6 in the form of a Dixon plot and clearly indicate a non-competitive type of inhibition of the Na^+ gradient-dependent Ca^{2+} uptake. These results were consistent with the apparently negative cooperativity of butanol-induced inhibition of this transport process. The K_i for the inhibition of the Ca^{2+} activation of Na^+–Ca^{2+} exchange calculated from the data shown in Fig. 6 ranged from 66 to 71.3 mM, with a mean K_i = 68.6 mM. This value was very similar to the K_i that was calculated from the IC_{50} value for the butanol inhibition of Na^+-dependent Ca^{2+} uptake (Fig. 4). The IC_{50} was obtained from the data shown in Fig. 4 by log-probit analysis where IC_{50} = 68.8 mM, and $K_i = 54.5$ mM ($K_i = IC_{50}\ K_{act}/[Ca^{2+}] + K_{act}$).

DISCUSSION

Synaptic plasma membranes were shown previously to have an active Na^+ gradient-dependent

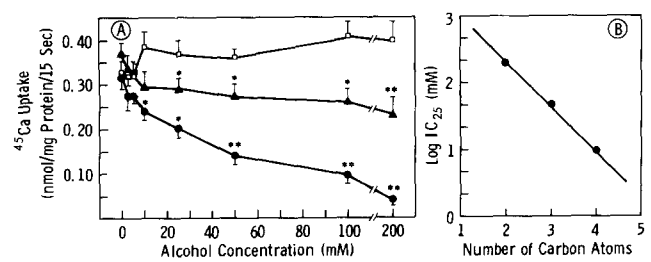

Fig. 4. Effects of short-chain aliphatic alcohols on the Na^+-dependent Ca^{2+} influx. (A) The NaCl-loaded vesicles were preincubated for 120 sec with the indicated concentrations of methanol (□), 1-propanol (▲), and 1-butanol (●) prior to transfer of the vesicles to incubation media containing 10 μM $^{45}CaCl_2$. The $^{45}Ca^{2+}$ uptake activity was allowed to proceed for 15 sec at 23°, and the indicated concentration of the alcohols was maintained throughout this incubation period. Each point is the mean ± S.E. of twelve to fifteen determinations with three different membrane preparations. Samples which differed significantly from the zero alcohol values are indicated with an asterisk: (*) $P < 0.05$ and (**) $P < 0.01$. (B) Alcohol concentrations required to inhibit Na^+-dependent Ca^{2+} uptake by 25% plotted against the number of carbon atoms of *n*-alcohols. These values were obtained from Fig. 2 and Fig. 4A.

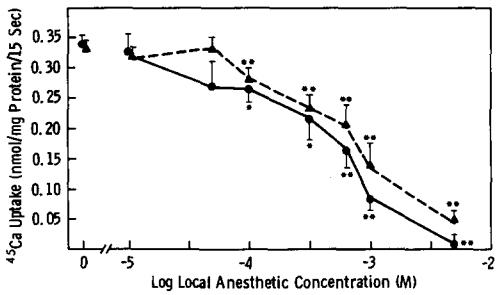

Fig. 5. Effects of increasing concentrations of local anesthetics on the Na^+-dependent Ca^{2+} influx. The assays were conducted as described for Fig. 2, except that tetracaine (▲) and dibucaine (●) were used in place of ethanol. Each point is the mean ± S.E. for nine to twelve determinations from three different membrane preparations. Values which differ significantly from those obtained in the absence of anesthetic agents are indicated with asterisks: (*) $P < 0.05$, and (**) $P < 0.01$.

Ca^{2+} uptake process which was bidirectional in nature and which could be preserved following rapid freezing and thawing of the isolated plasma membranes under specific conditions [41]. When isolated membrane vesicles were allowed to interact with concanavalin A Sepharose, greater than 60% of the vesicle protein was retained by the con A column, suggesting that greater than 60% of the membrane vesicles resealed with their glycoprotein-glycolipid matrix oriented to the outside (unpublished observations). The bidirectionality of this uptake process and its strict dependence on the presence and level of a transmembrane Na^+ gradient were very similar to the properties of the Na^+–Ca^{2+} exchange process of internally dialyzed axons [38, 39], of intact synaptosomes [40], and of cardiac sarcolemmal vesicles [43]. The function of the Na^+–Ca^{2+} antiport system in plasma membranes of excitable cells such as neurons and cardiac myocytes is thought to be related to the extrusion of free Ca^{2+} from the intracellular

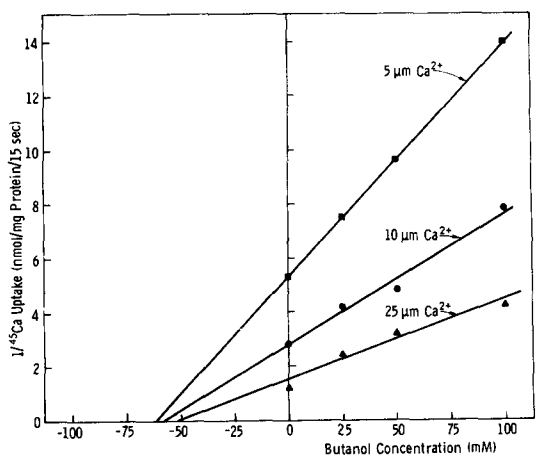

Fig. 6. Dixon plot analysis of the inhibition of Ca^{2+} transport produced by butanol. The assays were conducted as described for Fig. 3 except that butanol was used in place of ethanol. Each point was obtained from the mean of nine to twelve determinations from three different membrane preparations. The standard error was less than 10% for all means.

environment in what may be a $3\,Na^+$ for $1\,Ca^{2+}$ stoichiometry of countertransport [39]. Elimination or reversal of the normal Na^+ gradient (out → in) in physiological systems has been shown to cause gradual accumulation of Ca^{2+} within intact axons or axonal preparations [44]. Thus, stimulation of the activity of this Na^+–Ca^{2+} antiport carrier may lead to more rapid or greater Ca^{2+} extrusion from neurons or cardiac myocytes, whereas inhibition of this process would be expected to cause accumulation of Ca^{2+} intracellularly.

Ethanol produced both types of effects on this Na^+–Ca^{2+} exchange system of synaptic membranes. At subanesthetic concentrations (< 50–100 mM) ethanol caused moderate, but significant, enhancement of Na^+-dependent Ca^{2+} uptake at 23°, while at concentrations in the range of 100–600 mM it produced significant inhibition of transport. These observations might be interpreted to indicate that ethanol at anesthetic concentrations could cause accumulation of Ca^{2+} within excitable tissues such as neurons. Indeed, it has been shown that ethanol at concentrations > 100 mM enhances the spontaneous, unstimulated release of neurotransmitters from both cholinergic terminals at myoneural junctions [36, 45] and dopamine-containing synaptosomes from brain striatum [37]. Although these effects have been ascribed to ethanol-induced increases in Ca^{2+} binding to neuronal membranes [36], it is equally possible that the inhibition of Na^+–Ca^{2+} exchange described in the present study may lead to intraneuronal Ca^{2+} accumulation and increases in the spontaneous release of neurotransmitters from nerve terminals. It was shown recently that in hippocampal neurons extracellular application of ethanol produced both an increase in stimulated presynaptic release of excitatory and inhibitory neurotransmitters and an increase in postsynaptic membrane conductance that led to neuronal hyperpolarization [30]. The enhanced presynaptic release of transmitter following ethanol exposure was attributed to increased Ca^{2+} activity in neuronal terminals while the postsynaptic hyperpolarization was shown to be due to stimulation of K^+ conductance by increased Ca^{2+} activity in the intraneuronal compartment [30]. The increased intraneuronal Ca^{2+} activity was the result of a process other than the opening of the voltage-dependent Ca^{2+} channels [30]. These actions of ethanol were detected at concentrations in the range of 10–20 mM at 35°. We have found recently that, at 35°, ethanol exhibits no biphasic effect but produces only inhibition of the Na^+–Ca^{2+} antiport process, even at concentrations as low as 5 mM (unpublished observations). Thus, it is possible that the time-dependent increases in intraneuronal free Ca^{2+} activity following ethanol exposure that were demonstrated by Carlen *et al.* [30] in brain neurons may be due to inhibition of the neuronal membrane Na^+-dependent Ca^{2+} transport process at physiologic temperatures. Accumulation of intracellular Ca^{2+} may also occur as a result of inhibition of this Na^+–Ca^{2+} antiport process in axonal membranes if ethanol has a similar effect on all neuronal membranes. Such an increase in intracellular Ca^{2+} could alter the function of voltage-dependent Na^+ channels [32], a process that may be related

to the demonstrated local anesthetic actions of ethanol [1, 28].

Longer aliphatic alcohols are known to be even more effective than ethanol in terms of their general and local anesthetic activity [28]. The relative order of inhibitory activity on the Na^+–Ca^{2+} exchange system in synaptic membranes was analogous to the order of alcohol-induced general [46] and local [28] anesthesia. The K_i values for ethanol and butanol inhibition of the Na^+-dependent Ca^{2+} uptake (1.01 M and 68.6 mM respectively) were very similar to the concentrations of these alcohols that produce local anesthesia (0.5 to 0.6 M for ethanol, 68 mM for butanol) [28], but were considerably higher than the concentrations that produce general anesthesia in tadpoles (0.12 M for ethanol, 12 mM for butanol). If it is assumed that the inhibitory effects of the alcohols on the Na^+–Ca^{2+} antiport system of synaptic membranes were the result of alcohol adsorption to the membranes, then the standard free energy per methylene group of the alcohols ($\Delta G°$) can be calculated from the data of Fig. 4A and B according to the equation:

$$\Delta G° = 2.303\ RT \log \frac{a_{i+1}}{a_i} = 2.303\ RT \log \frac{C_{i+1}}{C_i}$$

The terms a_i, C_i and a_{i+1}, C_{i+1} refer to the thermodynamic activity and concentration (or mole fraction) of the ith and $(i + 1)$th homologues in the series of aliphatic alcohols. The $\Delta G°$ obtained from the plot of the log IC_{25} values versus the number of carbon atoms of the aliphatic alcohols (Fig. 4B) was $\Delta G° = -888.8$ cal/mole CH_2 at 24°. This estimate of $\Delta G°$ was very close to the free energy of transfer of each alcohol methylene group from an aqueous medium to an organic phase ($\Delta G° = -907$ cal/mol) [47] or from a buffer medium to dipalmitoylphosphatidylcholine (DPPC) vesicle membranes ($\Delta G° = -819.6$ cal/mole) [48]. Finally, if the differences in the membrane partition coefficients of ethanol and butanol are taken into consideration, then the amount of each alcohol attached to, or dissolved into, the synaptic membranes is fairly similar. The partition coefficients for alcohols in biological membranes are considered to be approximately 1/5 of those determined with phospholipid liposomes [46]. If the coefficients for ethanol and butanol partition into DPPC liposomes [48] are used in these calculations, then the membrane concentration of each alcohol at its K_i concentration in solution would be 0.51 M for ethanol and 0.84 M for butanol. These estimated intramembranous concentrations are considerably closer to each other than the K_i values for the inhibition of Na^+–Ca^{2+} exchange mentioned earlier.

In terms of pharmacological activity of the aliphatic alcohols, it would appear that the best correlation exists between the concentrations required for local anesthetic action of the alcohols and those for the inhibition of the Na^+–Ca^{2+} exchange process in synaptic membranes at 23°. On the basis of this observation one might predict that other local anesthetic agents would also inhibit the activity of the Na^+-dependent Ca^{2+} uptake in these membranes. Both local anesthetics tested in this study, tetracaine and dibucaine, inhibited the Na^+–Ca^{2+} antiport process in synaptic membranes with estimated IC_{50} values that were within the range of their local anesthetic activity [28]. However, despite the fact that inhibition of the Na^+–Ca^{2+} antiport function of synaptic membranes may appear to be correlated with local anesthetic concentrations of both neutral (alcohols) and charged (tetracaine and dibucaine) local anesthetics, the studies reported here revealed that the inhibition of Na^+–Ca^{2+} exchange produced by ethanol seemed to differ from the inhibition produced by butanol and the local anesthetics. Ethanol inhibition was competitive with respect to Ca^{2+} activation of the transport process. Butanol was shown to be non-competitive and was found to have an apparent Hill coefficient of 0.51. The Hill coefficients for dibucaine and tetracaine were almost identical to those for butanol.

The precise mechanism of inhibition of the Na^+–Ca^{2+} exchange carrier system by ethanol is obviously not known. It is possible that the interaction of ethanol with the synaptic membranes may disrupt the Ca^{2+}·carrier complex formation when the alcohol is present at high concentrations. The inhibition of Na^+-dependent Ca^{2+} uptake by 100–600 mM ethanol may appear mechanistically to be competitive with respect to Ca^{2+} activation of the membrane carriers because of possible disruption of Ca^{2+} binding to specific protein or lipid sites on the membrane. This effect of ethanol might be considered a surface-related action of this alcohol on biological membranes which secondarily affects Ca^{2+} binding. Ethanol-induced alterations in membrane surface potential have been observed in lipid monolayers [49], and changes in the membrane surface potential have been shown to affect the activity of membrane bound enzymes such as the (Na^+–K^+) ATPase [50]. The influence of the membrane surface potential on enzyme activity is due to the dependences of substrate binding on electrostatic interactions which control the availability of the substrate at the enzyme active site [50]. In view of the observations reported in this study, it is particularly relevant that brain microsomal (Na^+–K^+) ATPase activity was also inhibited by ethanol in an apparently competitive manner [3].

The longer alkane chain of the aliphatic alcohol 1-butanol may be distributed deeper within the membrane bilayer thus producing a disruption of the transport carrier that involves a step other than the formation of the Ca^{2+}·carrier complex. This may account for the negative cooperativity of butanol-induced inhibition of the Na^+–Ca^{2+} antiport activity. In this respect, the actions of butanol resembled the effects of the amphipathic molecules tetracaine and dibucaine on the same process. In addition, neither propanol, nor butanol, nor the charged local anesthetics tetracaine and dibucaine caused a significant enhancement in the Na^+–Ca^{2+} antiport activity of the synaptic membranes at 23°. Only the short-chain aliphatic alcohols ethanol and methanol within a certain range of concentrations caused such enhancement of Na^+–Ca^{2+} exchange. Biphasic actions of ethanol on other membrane-related biochemical processes have been observed previously, and these include the effects of this alcohol on brain microsomal (Na^+–K^+) ATPase [51], on L-glutamate bind-

ing to brain synaptic membrane receptor sites [13], and on Ca^{2+} binding to both brain synaptic membranes [10] and erythrocyte membranes [34]. The physiological implications of the enhancement in Na^+–Ca^{2+} exchange activity produced by ethanol in the lower concentration range at 23° are not clear at this point in time. The enhancement of Na^+-dependent Ca^{2+} extrusion from nerve endings observed at low ethanol concentrations in conjunction with the demonstrated inhibition by ethanol of depolarization-induced Ca^{2+} influx into the nerve endings [35] may be related to the inhibition of transmitter release that has been observed in a number of neuronal preparations (e.g. Refs. 52–54).

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