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DIFFERENTIAL EFFECTS OF MEMBRANE PERTURBANTS ON VOLTAGE-ACTIVATED SODIUM AND CALCIUM CHANNELS AND CALCIUM-DEPENDENT POTASSIUM CHANNELS

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One theory of the action of intoxicant-anesthetic drugs is that they disorder membrane lipids and thereby alter the function of membrane proteins. Chemically diverse compounds such as alcohols, ethers, barbiturates, and halogenated hydrocarbons all disorder membrane lipids, produce a similar spectrum of intoxication and anesthesia, and possess potencies which are correlated with their solubility in membrane lipids (1, 2). These observations provide support for the "disordered lipid" hypothesis of anesthesia, but fall short of identifying the consequences of membrane perturbation; i.e., the specific membrane proteins affected by the anesthetics. Identification of membrane proteins that are sensitive to membrane order is an important issue because some investigators suggest that it is unlikely that the changes in membrane order produced by the anesthetics are of sufficient magnitude to alter membrane function (3). Ion channels represent transmembrane proteins that may be sensitive to the physical properties of their surrounding lipids and have been suggested as a site of action for anesthetic drugs (4). There is electrophysiological evidence that *n*-alkanols decrease neuronal sodium conductance and barbiturates decrease calcium conductance (5, 6), but these studies did not compare different anesthetics and do not provide a correlation of membrane perturbation with changes in channel function. My approach to this problem was to select chemically diverse compounds and to compare their effects on sodium, calcium, and potassium transport and on membrane order.

MATERIALS AND METHODS

Synaptosomes were isolated from mouse (male DBA/2) brain by Ficoll gradient centrifugation (7). Uptake of ^{24}Na was determined by the method of Krueger and Blaustein (8) using 0.09 mM veratridine (Sigma Chemical Co., St. Louis, MO) and a 2 s uptake. The use of a short time and low concentration of sodium (5 mM) essentially eliminates sodium efflux and changes in membrane potential and allows accurate measurement of influx through voltage-sensitive channels (8). Uptake of ^{45}Ca was measured by the procedure of Nachshen and Blaustein (9) using a 3 s uptake, 68 mM KCl as the depolarizing stimulus and 0.02 mM calcium, again allowing unidirectional influx through the voltage-dependent channel. Calcium-dependent efflux of ^{86}Rb was studied in resealed human erythrocytes using an efflux period of 60 min (10). Fluorescence polarization of diphenylhexatriene (DPH) was determined using mouse brain synaptic plasma membranes (SPM) as described previously (11, 12). Experiments were performed at 30°C. Drugs were added to the membrane solutions 5 min prior to assay, except for *cis*-vaccenic acid methyl ester (*cis*-VAME, Sigma Chemical Co., St. Louis, MO), which was incorporated into the membranes for 30 min at 0°C and the unincorporated fraction was removed by washing (13, 14).

RESULTS AND DISCUSSION

The seven drugs selected for study all decreased the fluorescence polarization of DPH without altering the fluorescence lifetime, indicating that they increased the mobility of the probe in the membrane lipids (11, 12, 15). Although all of the drugs decreased fluorescence polarization, their potency in producing this effect varied over a 2,000-fold range (Table I). These results confirm and extend earlier demonstrations of the perturbation of the

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TABLE I

EFFECTS OF MEMBRANE PERTURBANTS ON ION TRANSPORT AND FLUORESCENCE POLARIZATION OF DPH

Drug	Concentration	Na Uptake (Percent Change)	Ca Uptake (Percent Change)	Ca-Stimulated K Efflux (Percent Change)	Membrane Effect‡ (Change in DPH Polarization)
	mM				
Ethanol	400	−43‖	−35§	+110§	− .018§
Pentobarbital	0.3	−16§	−41§	−31§	− .005§
Phenytoin	0.2	−63§	−27§	−62$	− .046§
Ether	30	−17§	−7	+140§	− .007§
Enflurane	1	−18§	+1	−24§	− .006§
Halothane	1	−28§	−6	−5	− .007§
cis-VAME	0.3	−55§	+5	Not Tested	−0.019§

*Uptake of ^{24}Na and ^{45}Ca were measured in synaptosomes isolated from mouse brain. Na uptake was stimulated by veratridine, Ca uptake by KCl. Ca-stimulated efflux of K was measured in human erythrocytes using ^{86}Rb. See Materials and Methods section for details.

†Drug-induced changes in fluorescence polarization of DPH were measured in synaptic plasma membranes from mouse brain.

§Significant drug effect, $p < 0.05$.

‖Values are mean from 6 to 9 experiments. The SEM was ~10% of the mean in all cases.

hydrophobic core of synaptic membranes by alcohols and barbiturates (11, 12, 16). The drugs selected for study also decreased synaptosomal uptake of ^{24}Na (Table I). In addition, their potencies in decreasing ^{24}Na uptake and disordering the membrane were linearly correlated (change in ^{24}Na uptake vs. change in DPH polarization, $r = 0.85$). In contrast, ^{45}Ca uptake was inhibited by ethanol, pentobarbital, and phenytoin, but was not altered significantly by the other membrane perturbants. The calcium-stimulated efflux of potassium was increased by ethanol and ether but was decreased by pentobarbital and phenytoin (Table I). Halothane and enflurane produced biphasic changes in potassium efflux, with low concentrations (0.01–0.1 mM for halothane and 0.1–1 mM for enflurane) increasing efflux by 20–30% and higher concentrations (10 mM) decreasing efflux by 30–40%. Examination of the concentration-response relationship for ethanol, ether, pentobarbital, and phenytoin indicated that these drugs did not produce biphasic effects on potassium efflux. It should be noted that none of the drugs altered the basal (unstimulated) fluxes of Na, Ca, or K.

These studies demonstrate that seven chemically diverse membrane perturbants inhibited the influx of sodium through voltage-sensitive neuronal channels. The correlation between decreased fluorescence polarization and decreased sodium uptake suggests that the change in sodium flux may be due to perturbation of lipids surrounding the channels. In contrast, there was no direct relationship between membrane perturbation and changes in depolarization-dependent calcium influx; calcium influx was inhibited by several membrane perturbants but was not affected by others. From the data in Table I and reference 7 it appears that a polar moiety of the drugs is important for inhibition of calcium fluxes, suggesting that the drugs may affect the channel near the membrane surface rather than in the hydrophobic regions. These results are consistent with the finding that modification of PC12 cell membrane lipid acyl group alters release of neurotransmitter produced by activation of sodium channels, but not that produced by activation of calcium channels (17). The effects of the membrane perturbants on potassium efflux appear quite complex: some agents increased efflux markedly, some inhibited strongly, and others produced modest biphasic effects. Thus, the effects of drugs on potassium efflux cannot be correlated with their action on membrane order.

In summary, inhibition of neuronal sodium channels by intoxicant-anesthetics may be due to perturbation of membrane lipids. The action of these drugs on certain calcium and potassium channels cannot, however, be due to solely to their membrane-disordering effects. The "disordered lipid" hypothesis may account for some, but not all, of the neurochemical actions of anesthetics.

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A CALMODULIN-LIKE Ca^{2+} RECEPTOR IN THE Ca^{2+} CHANNEL

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The voltage-dependent Ca^{2+} channel, or slow channel, transports Ca^{2+} across the cell membrane and is regulated by intracellular [Ca^{2+}] (1). It must, therefore, bind Ca^{2+} itself, and some domain or region of the Ca^{2+}-channel protein must be a Ca^{2+}-binding protein. Calmodulin (CDR) is perhaps the most universal Ca^{2+}-dependent regulatory protein and we have studied it as a model for the Ca^{2+} receptor in the Ca^{2+} channel. We have reported that some Ca^{2+}-antagonist drugs (Ca^{2+} channel blockers), said to be specific for the Ca^{2+} channel, bind to calmodulin (2). We have also shown that calmodulin antagonists (CDR-ANT) can act like Ca^{2+}-antagonists (Ca-ANT) and block the Ca^{2+} channel (3). Recently, allosteric interactions among Ca-ANT drug-binding sites on the Ca^{2+} channel have been reported (4, 5). The present study demonstrates allosteric interactions among the Ca-ANT–binding sites of calmodulin. This provides further, indirect, evidence of a Ca^{2+}-binding protein, similar to but probably distinct from calmodulin, serving as a regulator of the Ca^{2+} channel and a receptor for Ca-ANT drugs. With this information we propose a model of the Ca^{2+} channel and the mechanism of action of Ca-ANT.

RESULTS

Ca^{2+} binding to calmodulin is known to produce large structural changes including the exposure of 3–4 hydrophobic ligand-binding sites (7). The dihydropyridine Ca-ANT, felodipine, binds one to two of these sites on CDR with a *Kd* of 1–10 μM (8). The fluorescence spectra of felodipine (Fig. 1, inset) is not changed by the addition of CDR. The addition of Ca^{2+} (but not Mg^{2+}), only in the presence of CDR, produces a twofold fluorescence increase (Fig. 1, inset, curve 2). This suggests that Ca^{2+} binding to Ca^{2+}-specific sites on CDR exposes felodipine binding sites and that felodipine binding can be monitored by this fluorescence increase. Addition of the CDR-ANT, R24571, or the Ca-ANT, diltiazem, to Ca^{2+}-CDR-felodipine can produce a further fluorescence increase (Fig. 1, inset curve 3) only in the presence of both Ca^{2+} and CDR. This suggests that these drugs do not interact with free felodipine but bind to a Ca^{2+}-CDR complex to increase felodipine binding to CDR.

In the same concentration range where R24571 and diltiazem bind to CDR (2), we find that they produce an increase in CDR-felodipine fluorescence; at higher concentrations this fluorescence increase is reversed (6). Initially, these drugs bind to sites on CDR other than the felodipine-binding sites. Through an allosteric mechanism they increase the number and/or affinity of felodipine-binding sites, resulting in the observed fluorescence increase. At higher concentrations presumably they can bind to the felodipine-binding sites and competitively displace felodipine, resulting in the observed fluorescence decrease.

A titration of felodipine with CDR is shown in Fig. 1.

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