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Calcium Enhancement of Alcohol and Drug-Induced Sleeping Time in Mice and Rats[1]

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ERICKSON, C. K., T. D. TYLER, L. K. BECK AND K. L. DUENSING. *Calcium enhancement of alcohol and drug-induced sleeping time in mice and rats.* PHARMAC. BIOCHEM. BEHAV. 12(5) 651–656, 1980.—Calcium injected intracerebroventricularly (IVT) at various times before a hypnotic dose of ethanol significantly enhanced the duration of sleeping time (loss of righting reflex, LRR) in mice and rats. This cation also enhanced the duration of LRR induced by t-butanol and chloral hydrate, but not that induced by sodium pentobarbital. Other cations injected IVT (manganese, cadmium, and zinc) also enhanced ethanol-induced LRR. The synergistic effects of the ionophores X537A and A23187 on calcium-enhanced LRR, and the antagonism of ethanol-induced LRR by EDTA and EGTA suggest the involvement of a membrane-associated calcium pool in the hypnotic effect of ethanol. These studies show the generality of cation enhancement of alcohol-induced sleeping time in mice and rats, and confirm earlier reports which suggested that calcium is involved with the central nervous system depressant effects of alcohols.

Calcium Ethanol t-Butanol Chloral hydrate Pentobarbital Ionophores EDTA EGTA Sleeping time

RECENT neurochemical studies have shown that ethanol produces changes in membrane fluidity [3,4], membrane lipid composition [17,21], and receptor binding activity [22]. Ethanol has also been shown to affect neurotransmitter release both *in vitro* [2] and *in vivo* [16]; to alter cyclic nucleotide levels [29]; and to affect electrical activity of the brain [19]. Such reports confirm that ethanol has multiple effects on various components of neuronal systems. Conceivably an alteration in calcium function at the nerve membrane may underly many of these effects, or at least be associated in some way with the multiple membrane actions of ethanol.

Calcium is thought to be involved in the membrane effects of anesthetics [33], opiates [32,41], barbiturates [23], tranquilizers [20], ethanol [10], amphetamines [38], and other therapeutic agents [28]. There may also be a common effect of calcium in the action of many of these drugs [34]. Morphine and ethanol have been reported to decrease brain calcium concentrations [31]; while these may not be direct effects of the drugs [14,15], the observation does provide a rationale for looking at similarities between the two drugs. Furthermore, it was observed that morphine analgesia can be antagonized by intracerebroventricularly-injected cations [11,18]. Earlier studies in our laboratories found that calcium and certain other cations, when injected intracerebroventricularly, would enhance ethanol-induced loss of righting reflex (LRR, sleeping time, unconsciousness) in mice, but not ethanol-induced hypothermia [7].

The present experiments are extensions of the earlier work presented by Erickson *et al.* [7] and Harris [10]. They provide further evidence for the generality of the cation-enhancing effect on ethanol intoxication in another species and with certain other central nervous system (CNS) depressants.

METHOD

Materials

The drug and chemical sources were as follows: ethanol, 95 and 99.5% v/v; t-butanol, Matheson, Coleman and Bell Manuf. Chem. (Norwood, OH); chloral hydrate, Merck and Co., Inc. (West Point, PA); sodium pentobarbital, Abbott Labs, (North Chicago, IL); ethylenediamine tetraacetic acid (EDTA), J.T. Baker Chem. Co. (Phillipsburg, NJ); [ethylene-bis (oxyethylenenitrilo)] tetraacetic acid (EGTA), Sigma Chemical Co. (St. Louis, MO); calcium chloride and zinc chloride, Mallinckrodt, Inc. (St. Louis, MO); manganese chloride and cadmium chloride, Fisher Scientific Co. (Fair Lawn, NJ); A23187, a gift from Eli Lilly and Co. (Indianapolis, IN); X537A, a gift from Hoffman-LaRoche, Inc. (Nutley, NJ).

Animals

Female albino Swiss-Webster mice of the HA/ICR strain

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and female albino rats of the MCR(SD) strain were obtained from Mid-Continent Research Laboratories (Shawnee, KS). They were housed in groups of 6–8 mice or 4–5 rats in wire bottomed stainless steel cages on a 12/12 hour light/dark cycle at 25°. Food (Lab Blox, Purina) and water were available ad lib. The mice weighed 20–25 g, the rats 200–220 g, at the start of the experiment.

Intracerebroventricular Injections

Intracerebroventricular (IVT) injections were performed in conscious mice as described by Clark *et al.* [5]. The procedure in conscious rats was the same as that described by Popick [27]. An injection volume of 5 μl/animal was used. All drugs and chemicals were dissolved in 0.9% saline. Pilot studies showed no consistent or significant effects on ethanol-induced LRR of injection versus sham puncture, osmolarity, or pH, when a fixed injection volume of 5 μl was used. Calcium was injected IVT in 4 doses, calculated on body weight, but adjusted for brain weight: ave. brain weight (mouse)=approximately 400 mg; ave. brain weight (rat)=approximately 1500 mg. Thus a dose of 15 μmol calcium/kg body weight in a mouse was equivalent to 6 μmol calcium/kg body weight in a rat, when adjusted for the size of the brain and injected in 5 μl volumes. The actual concentrations of injected calcium were therefore approximately 20–80 mM. These doses produced no obvious behavioral effect when injected into the ventricle, in preliminary studies.

Administration of Cations During Loss of Righting Reflex Induced by Various CNS Depressants

Chloride salts of several cations (calcium, manganese, cadmium, and zinc) or saline were injected IVT in mice or rats. After various time periods, either ethanol (4.5 g/kg, 20% w/v, mice; 3.5 g/kg, 20% w/v, rats), t-butanol (1.0 g/kg, mice; 1.1 g/kg, rats), chloral hydrate (400 mg/kg, mice; 300 mg/kg, rats), or sodium pentobarbital (50 mg/kg, mice; 30 mg/kg, rats) was injected intraperitoneally (IP). A time-response study was performed, after which 30 min was chosen as a standard time between cation administration and CNS depressant administration.

The effects of several other ions on ethanol-induced LRR have previously been reported [7,10]. Selection of ions and their dosages were based on studies by Harris *et al.* [11]. Shortly after IP drug administration, each animal was placed on its back and the LRR was measured to the nearest minute from beginning of sleeping time to the time that the animal righted itself twice within one minute. Sleep variables such as room temperature and extraneous sounds were controlled. No attempt was made to measure the onset of LRR.

In separate experiments, EDTA and EGTA (4 μmole/kg, mice; 1.6 μmole/kg, rats) were administered IVT 5 minutes before ethanol IP.

Still other experiments utilized the IVT administration of the ionophores X537A (mice only, 2 μmole/kg) and A23187 (1 μmole/kg, mice and rats) simultaneously with calcium chloride (5 μmole/kg, as the salt) in saline, in a total volume of 5 μl. This injection preceded a hypnotic dose of IP ethanol by 30 min.

Statistical Methods

Differences among means in each experiment were analyzed by a one-way analysis of variance. The Student-Newman-Keuls procedure was used to determine significant differences between specific means.

TABLE 1

TIME-RELATED DIFFERENCES IN THE ENHANCEMENT OF ETHANOL-INDUCED SLEEPING TIME BY INTRACEREBROVENTRICULAR CALCIUM IN MICE AND RATS

	Sleeping time (min ± SE)
Mice	
Ethanol alone	68.6 ± 9.0 (21)
Ethanol before calcium	
5 min	112.5 ± 10.9 (12)*
Calcium before ethanol	
15 sec	130.6 ± 11.8 (14)*
15 min	164.1 ± 13.3 (14)†
30 min	180.6 ± 14.8 (14)†
60 min	137.6 ± 11.8 (14)*
120 min	110.3 ± 11.9 (12)*
Rats	
Ethanol alone	157.0 ± 6.7 (3)
Calcium before ethanol	
5 min	204.3 ± 11.2 (7)*
15 min	200.7 ± 9.9 (7)*
30 min	204.9 ± 4.8 (9)*
60 min	167.8 ± 7.3 (9)

IVT $CaCl_2$ doses: mice, 15 μmole/kg; rats, 8 μmole/kg
IP Ethanol doses: mice, 4.5 g/kg; rats, 3.5 g/kg.
( )=number of animals.
*=$p<0.05$, compared to ethanol alone.
†=$p<0.01$, compared to ethanol alone.

RESULTS

Time-Response Study of Calcium Effects on Ethanol-Induced Loss of Righting Reflex

Table 1 shows the results of a study in which IVT calcium chloride was given at various times before or after IP ethanol in mice, and at various times after IP ethanol in rats. In mice the administration of ethanol before calcium caused an enhancement of ethanol-induced LRR. Varying the times that calcium was injected before ethanol produced a time-related effect with the greatest enhancement of LRR occurring when calcium was given 30 min before ethanol. In rats, the time-response curve was flatter, with 5–30 min pretreatment giving essentially the same degree of enhancement. The time period of 30 minutes was therefore chosen as a standard time period between cation administration and CNS depressant administration.

Cation Enhancement of Ethanol-Induced Loss of Righting Reflex in Mice

Table 2 indicates that the chloride salts of calcium, manganese, cadmium and zinc in equivalent doses (as determined by Harris *et al.* [11]) significantly enhance ethanol-induced LRR when injected IVT in mice. As reported earlier [7] IVT calcium gluconate gave results similar to those with calcium chloride, and IP calcium gluconate or chloride also enhanced ethanol-induced LRR, but there was no clear dose-response curve when this route of administration was

TABLE 2

THE EFFECT OF VARIOUS CATIONS ON ETHANOL-INDUCED SLEEPING TIME IN MICE

Cation	IVT dose (μmole/kg)	N	Sleeping time (min ± SE)
Saline	—	82	69.0 ± 4.1
$CaCl_2$	15	12	141.9 ± 19.3*
$MnCl_2$	15	23	184.5 ± 14.9*
$CdCl_2$	1	19	121.8 ± 12.2*
$ZnCl_2$	5	14	222.1 ± 20.5†

Cations were injected IVT 30 min before ethanol, 4.5 g/kg IP.
*$p<0.01$, compared to saline.
†$p<0.005$, compared to saline.

used. Finally, we have reported that not all cations enhance ethanol-induced LRR; for example, magnesium chloride (10–20 μmole/kg), nickel chloride (10 μmole/kg), and barium chloride (0.4 μmole/kg) do not [7].

Cation Enhancement of Loss of Righting Reflex Produced by Other CNS Depressants

Figure 1 compares the calcium enhancement of ethanol-induced LRR with the effects of calcium on LRR produced by t-butanol, chloral hydrate, and sodium pentobarbital. As seen in Fig. 1 and as reported earlier [7], there was a significant enhancement of ethanol-induced LRR in mice with 10, 15, and 20 μmole/kg $CaCl_2$. The dose relationship is even clearer in rats (Fig. 1). There is a similar enhancement of t-butanol LRR, but the effect is significant only in mice. A clear dose relationship with calcium enhancement of chloral hydrate LRR is seen in mice, but significant enhancement is seen only with the two highest doses of calcium. In rats, only the 8 μmole/kg dose of calcium significantly enhanced chloral hydrate LRR. Finally, there was no enhancement of pentobarbital-induced LRR by calcium, except with the modest dose of 4 μmole/kg in rats. The studies with pentobarbital in Fig. 1 are representative of a larger number of experiments using mice and rats, in which we have been unable to consistently alter barbiturate-induced LRR with IVT calcium.

Ionophore Enhancement of Low-Dose Calcium Effects on Ethanol-Induced Loss of Righting Reflex

Ionophores are substances which are capable of solubilizing cations in a lipid phase and transporting them across membranes [13]. They can thus be used as tools to aid in identifying the site of action of a cation at the nerve membrane. Figure 2 illustrates a significant enhancement of calcium's effect on ethanol-induced LRR with ionophores in both mice and rats. The ionophores alone had no effect on the duration of LRR, but combined with the minimally-effective dose of calcium, there was a significant enhancement of calcium-induced prolongation of LRR. In mice, A23187 was more effective than X537A in enhancing the effect of calcium. The enhancement of calcium's effect by A23187 was not as dramatic in rats as in mice.

Reduction of Ethanol-Induced Loss of Righting Reflex by Cation-Chelating Drugs

EDTA, a non-specific chelator, and EGTA, a specific calcium chelator, significantly reduced the duration of ethanol-induced LRR, in both mice and rats (Fig. 3). These chelators do not readily cross lipid membranes and are useful for studying extracellular calcium binding [36].

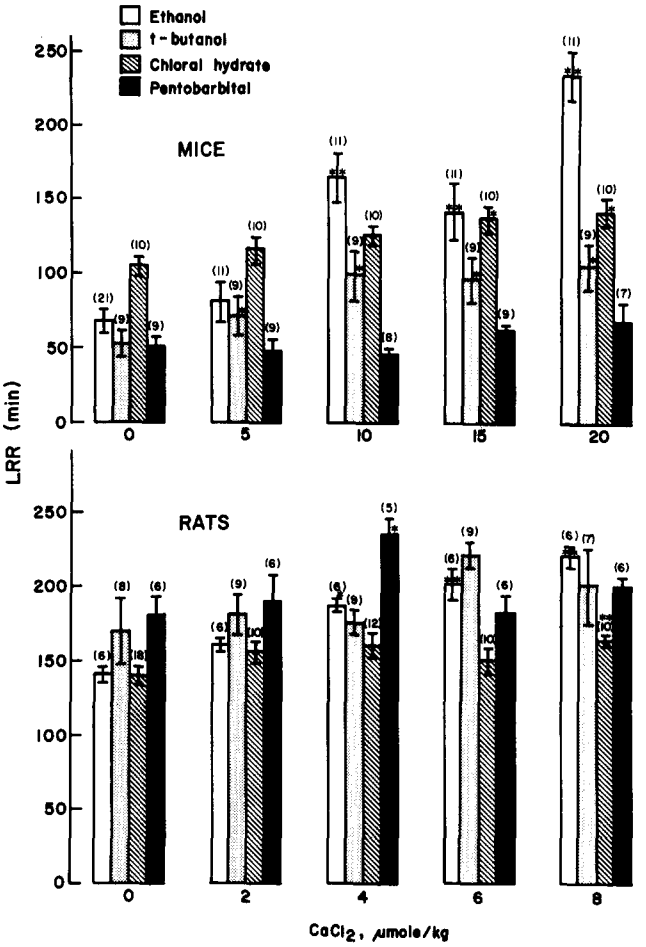

FIG. 1. Dose-relationship of calcium enhancement of drug-induced loss of righting reflex (LRR) in mice and rats. Calcium chloride ($CaCl_2$) was injected IVT in a volume of 5 μl, 30 min before each drug. 0=saline control in a volume of 5 μl. See text for drug doses. Bars represent mean LRR for ( ) subjects, ± SE; *=$p<0.05$, **=$p<0.01$, compared to "0" controls.

DISCUSSION

The results of this study confirm the earlier observations by Harris [10], who showed that ethanol-induced LRR was significantly enhanced by various doses of IVT calcium chloride. In that study, doses of 0.2–0.4 μmole/mouse were used, which are roughly equivalent to the doses of 5 and 15 μ mole/kg used in the present study. Since the enhancement of ethanol effects by calcium was remarkably similar in Harris' study and the present study, there do not appear to be significant sex, strain, species, ethanol hypnotic dose or IVT injection methodological variables involved (see details in [7,10]). The dose-related enhancement of ethanol-induced LRR in both mice and rats points directly to a pharmacologic interaction between the cation and ethanol at similar sites of action, perhaps at the postsynaptic or axonal nerve mem-

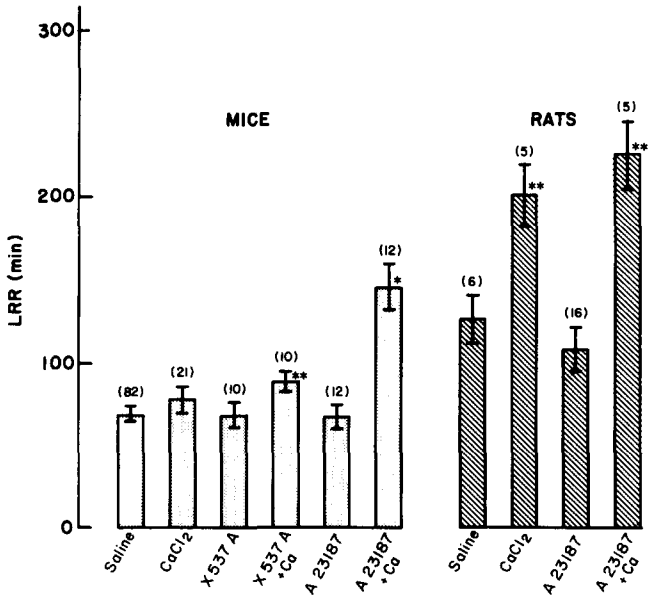

FIG. 2. Effects of ionophores on calcium (Ca)-enhancement of ethanol-induced loss of righting reflex (LRR) in mice and rats. Calcium chloride (Ca Cl_2, 5 μmole/kg) or calcium chloride plus ionophore (X537A, 2 μmole/kg; A23187, 1 μmole/kg) in saline (total volume=5 μl) was injected 30 min before a hypnotic dose of ethanol. Bars represent mean LRR for ( ) subjects, ± SE; *=$p<0.05$, **=$p<0.01$, compared to saline.

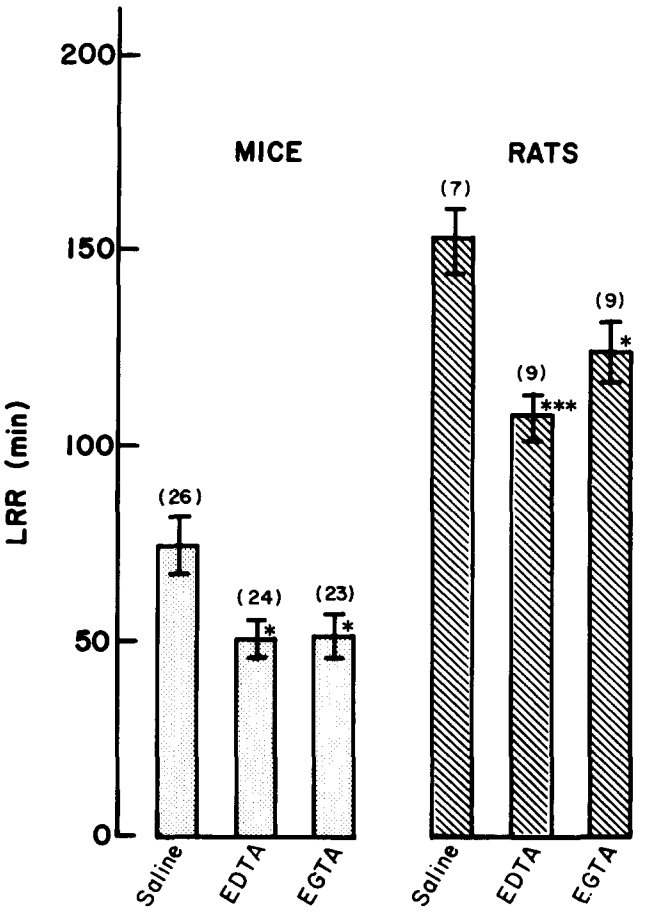

FIG. 3. Chelating drug effects on ethanol-induced loss of righting reflex (LRR) in mice and rats. Chelators (EDTA and EGTA) were injected IVT 5 minutes before a hypnotic dose of ethanol. Chelator doses were: mice, 4 μmole/kg; rats, 1.6 μmole/kg in saline in a volume of 5 μl. Bars represent mean LRR for ( ) subjects, ± SE; *=$p<0.05$, ***=$p<0.001$, compared to saline.

brane [33] or at the presynaptic neurotransmitter release site [8]. This suggestion is further supported by the present results which show that (a) ionophores enhance the effects of calcium on ethanol-induced LRR, and (b) cation chelating agents significantly shorten the effects of ethanol. Ionophores are felt to be valuable tools for altering calcium transmembrane movement, since they stimulate neurotransmitter release through enhanced stimulus-secretion coupling [13], and they also enhance the morphine analgesia antagonist effect of a low dose of calcium, while not altering the effects of morphine given alone [11]. Colburn *et al.* [6] have shown that A23187 promotes amine neurotransmitter release by a calcium-mediated mechanism, whereas X537A does not require calcium to mediate amine transport. Our results reflect a similar difference between the ionophores: A23187 was more effective in enhancing the effects of calcium than X537A (Fig. 2). In the other experiments, the ability of EDTA and EGTA to shorten ethanol-induced LRR indicates that extracellular calcium, readily-available for membrane entry during depolarization, may be important for the hypnotic effects of this drug. The calcium is apparently loosely bound to the membrane or is in the extracellular fluid, since these chelators do not readily cross lipid membranes [36].

Since ethanol is capable of producing marked hypothermia [30], and since hypothermia can enhance the effects of anesthetic agents [24], it is important that Harris [10] found insignificant effects of calcium on ethanol-induced hypothermia. The differential effect of calcium on the LRR and the hypothermia produced by ethanol indicates that different mechanisms may be responsible for these two effects. In addition, it is clear that the enhancement of LRR by calcium cannot be explained by an alteration of the hypothermic effect of ethanol.

The LRR-enhancement by calcium is not limited to a single dose of ethanol, nor to ethanol alone. In the present study we used a 4.5 g/kg IP dose of ethanol; in the study by Harris [10] a 4.0 g/kg IP dose was used, and in a previous report we showed that IVT calcium could significantly enhance the effects of low doses of ethanol (0.7–1.6 g/kg IP in rats) on impairment of treadmill coordination [7]. Furthermore, the present results show that calcium enhances t-butanol-induced LRR (at least in mice), and chloral hydrate-induced LRR (at least with high doses of calcium). Chloral hydrate is known to be metabolized to trichloroethanol [12]; thus it appears that calcium can enhance the action of alcohols but not non-alcohols, such as pentobarbital. Differences have been seen in neurotransmitter release [37] and presynaptic mechanisms [8,20], but not membrane mechanisms [34], of alcohols and barbiturates. The differences in calcium effects with these two depressant classes might therefore suggest differences in the presynaptic involvement of calcium in their hypnotic effects.

Like any pharmacological agent, IVT-administered calcium demonstrates a time-dependent response in affecting ethanol's action (Table 1). The optimum time of 30 minutes may reflect the time required to diffuse or penetrate to the site of interaction with ethanol. Based upon ethanol's known actions, this site could be the reticular formation or the cerebral cortex [25]. It is also clear that several other divalent cations mimic the effects of calcium in enhancing ethanol-induced LRR (Table 2). Manganese and cadmium are classified as calcium antagonists [26,40]; and manganese stimulates while zinc depresses adenylate cyclase activity in rat brain homogenates, synaptosomes, isolated synaptic membranes and slices [39]. Such effects are difficult to relate to a calcium-like action in the present study; however, the interaction may be as straightforward as a simple additive effect of the cations on a known central neurotransmitter-altering effect of ethanol. For example, ethanol is known to decrease regional brain acetylcholine levels with chronic administration [16]; cadmium has the same chronic effect [35]. Unfortunately, the acute central effects of these cations on neurotransmitter function have not been studied. Studies on membrane and central neurotransmitter effects of various divalent cations would perhaps explain why certain cations can enhance ethanol-induced LRR while others (e.g., other calcium antagonists such as magnesium, nickel and barium [7]) cannot.

Since both ethanol and morphine have been reported to decrease brain levels of calcium [31], and since morphine analgesia is antagonized by IVT calcium, the enhancement of ethanol's action in the present study was unexpected. Based upon recent unsuccessful attempts by Hood and Harris [14] to replicate the earlier work of Ross *et al.* [31], and the apparent lack of a direct action of morphine on calcium binding sites on the membrane [15], it appears that ethanol and morphine may actually have different mechanisms with respect to calcium function. It is possible, for example, that morphine decreases neurotransmitter release through decreased stimulus-secretion coupling due to its confirmed reduction in synaptosomal calcium content [14] and calcium transport [9], while ethanol and calcium produce an additive stabilizing effect on the nerve membrane, similar to the classical local anesthetic stabilization mechanism [1].

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