Ethanol: Modification of Acute Intoxication by Divalent Cations

Abstract. *Calcium, other divalent cations, and calcium antagonists were tested for their ability to alter ethanol-induced sleeping time, hypothermia, and behavioral intoxication in mice and rats. Calcium given intraventricularly significantly enhanced sleeping time and behavioral intoxication in a dose-related manner. The ionophores X537A and A23187 accentuated the effect of a low dose of calcium, whereas the calcium chelators EDTA and EGTA decreased sleeping time. Calcium also enhanced tertiary butanol– and chloral hydrate–induced sleeping time. The effects of cations on ethanol-induced hypothermia were less significant. The results suggest the existence of a central calcium pool that is involved in ethanol intoxication in rodents.*

Ethanol and other short-chain alcohols are known to affect many neuron functions, including neurotransmitter release, cyclic nucleotide concentrations, and biophysical properties of membranes (*1*). Alterations in a membrane and intraneuronal constituent such as calcium could account for most or all of the observed effects of ethanol on neurons, and thereby explain the mechanism by which ethanol produces its central depressant effect. Earlier reports have demonstrated an effect of ethanol on calcium binding to erythrocyte ghost membranes (*2*), on calcium function in muscle membrane (*3*), and on calcium concentrations in rat brain (*4*). Studies on the antagonism of morphine analgesia by centrally administered calcium and other cations (*5*) demonstrate that drug effects may be modified by divalent cations, raising the possibility of a direct involvement of membrane or intracellular cations in ethanol intoxication.

To test this hypothesis, the first step was to attempt to alter ethanol-induced sleeping time (loss of righting reflex, unconsciousness) by centrally administered divalent cations. When alterations were seen, the second step was to test the generality of the alterations by using lower doses of ethanol and measuring other behavioral and physiological parameters known to be affected by the drug. These steps were independently carried out in two different laboratories (*6*) and the results, having been independently replicated, can now be reported in more detail. In both studies, hypnotic doses (4.0 to 4.5 g/kg) of ethanol (20 percent by weight in saline) were injected intraperitoneally in male Swiss-Webster (National Laboratories and Charles River) or female HA/ICR (Mid-Continent Research Laboratories) mice and the duration of sleeping time was measured (*7*). Divalent cations were given intraventricularly in a 5-μl injection through the skull (*8*), 30 minutes before the ethanol injection. In a separate time-course study in which calcium was injected at various times before ethanol, a 30-minute interval between injections gave optimal sleeping time alterations. Cations were chloride salts dissolved in deionized water or artificial cerebrospinal fluid (*9*), and the doses were those used by Harris *et al.* (*5*), which were approximately one-fourth of the median lethal dose (LD_{50}).

Ethanol-induced sleeping time was enhanced by calcium chloride in a dose-related manner (Table 1). Even though the 15 μmole/kg dose of calcium produced about the same effect as the next lower dose, the enhancement is probably strictly dose-related because replication of this experiment in rats has produced a perfect dose relationship (*10*). Furthermore, the enhancement of ethanol effects by calcium was remarkably reproducible between the two laboratories. For example, a 10 μmole/kg dose of calcium chloride increased ethanol-induced sleeping time by 2.2 times in one laboratory and by 2.4 times in the other laboratory. In separate studies, intraventricularly administered calcium gluconate gave similar results, while intraperitoneally administered calcium gluconate or chloride also enhanced sleeping time, but the dose relationship was less clear. Intraventricular manganese chloride and cadmium chloride also significantly enhanced sleeping time, while other calcium antagonists, magnesium, nickel, and barium did not enhance sleeping time (Table 1). To study the neuronal site of the ethanol-calcium interaction, we gave the ionophores X537A and A23187 intraventricularly to enhance cation transport across the neuronal membrane (*11*). When given alone, the ionophores had no effect on sleeping time (data not shown). When given at the same time as a low dose of calcium, both ionophores significantly increased the sleep-enhancing effect of the cation. Verapamil, an antagonist of transmembrane calcium movement, and lanthanum, an antagonist of membrane calcium (*12*), had no effect on the calcium-enhancement of sleeping time. Finally, we gave the cation chelators ethylenediaminetetraacetic acid (EDTA) and [ethylene-

Table 1. The effect of various cations and cation antagonists on hypnotic- (ethanol, tertiary butanol, or chloral hydrate) induced sleeping time in mice. See text for details. All intraventricular injection volumes were 5 μl; ACSF, artificial cerebrospinal fluid.

Cation	Intraventricular dose (μmole/kg)	N	Sleeping time duration (minutes ± S.E.)
	Ethanol (4.5 g/kg)		
Saline		22	68.6 ± 9.9
$CaCl_2$	5	12	82.2 ± 13.1
$CaCl_2$	10	12	165.2 ± 16.3*
$CaCl_2$	15	12	141.9 ± 19.3*
$CaCl_2$	20	12	233.6 ± 15.9†
$MnCl_2$	15	23	184.5 ± 14.9*
$CdCl_2$	1	19	121.8 ± 12.2*
$MgCl_2$	10	12	59.4 ± 6.5
$MgCl_2$	20	11	69.0 ± 9.1
$NiCl_2$	10	11	76.9 ± 15.5
$BaCl_2$	0.4	12	83.9 ± 12.0
$CaCl_2$ plus X537A	5 and 1, respectively	33	152.4 ± 10.6‡
$CaCl_2$ plus A23187	5 and 1, respectively	12	144.5 ± 18.1‡
Verapamil	5	12	64.9 ± 8.7
Verapamil	10	12	67.0 ± 6.7
$LaCl_3$	4	12	61.0 ± 12.5
$LaCl_3$	8	11	57.6 ± 7.1
EDTA	4	24	50.3 ± 5.1‡
EGTA	4	23	51.0 ± 5.7‡
	Tertiary butanol (1 g/kg)		
ACSF		14	21.0 ± 6.2
$CaCl_2$	15	14	64.0 ± 6.8*
	Chloral hydrate (400 mg/kg)		
ACSF		14	52.2 ± 7.4
$CaCl_2$	15	14	90.8 ± 5.7*

*$P < .01$. †$P < .005$, compared to saline or ACSF. ‡$P < .05$.

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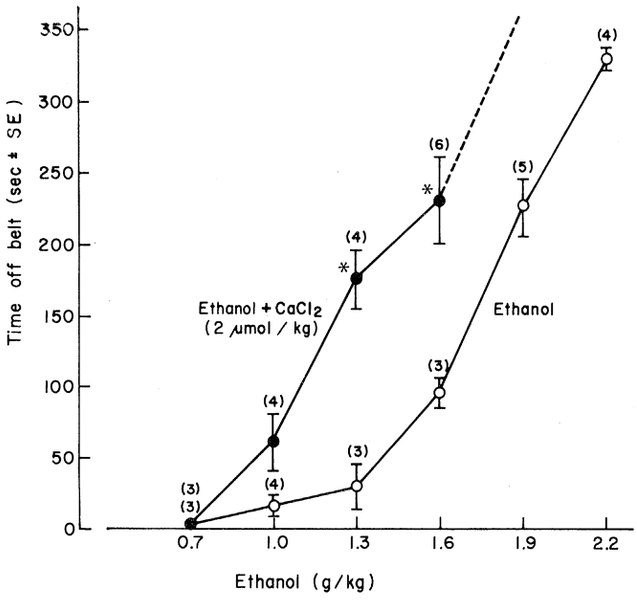

Fig. 1. The effect of ethanol and ethanol plus calcium on the performance of rats on a moving belt. Ethanol (10 percent, weight to volume) was injected intraperitoneally 20 minutes before the rats were tested. Calcium chloride in saline (5 μl was injected intraventricularly 30 minutes before the ethanol. When injected alone, this dose of calcium chloride did not significantly alter the time-off-belt. Asterisks indicate $P < .05$, one tailed t-test at each dose. The numbers of rats used are shown in parentheses.

bis(oxyethylenenitrilo)]tetraacetic acid (EGTA, which is more specific for calcium) intraventricularly 5 minutes before ethanol to study the contribution of endogenous extracellular or loosely bound membrane calcium to ethanol's action. These chemicals reduced sleeping time (Table 1). These and the preceding data suggest that ethanol produces its hypnotic effect through an interaction with a loosely bound membrane calcium pool, in much the same way that classical local anesthetic drugs exert their action (*13*).

The specificity of calcium for ethanol was tested by giving calcium intraventricularly to mice 30 minutes before injections of tertiary butanol or chloral hydrate (Table 1). Calcium also enhanced the hypnotic effects of these drugs (*14*).

The enhancement of ethanol's actions was seen in other studies in which hypothermia and ability to walk a treadmill were measured. In the hypothermia experiment, male mice were injected intraventricularly with a cation (chloride dissolved in artificial cerebrospinal fluid) 60 minutes prior to body temperature measurement with a rectal probe. In these mice, a sleep-inducing intraperitoneal dose of ethanol (4.0 g/kg) was given 30 minutes after the cation injections (*15*). Calcium, magnesium, manganese, lanthanum, and verapamil significantly lowered body temperature when injected into the ventricles. In combination with ethanol, however, only manganese (enhancement) and lanthanum (antagonism) altered ethanol-induced hypothermia. The inability of calcium to enhance ethanol-induced hypothermia represents a differential effect of this ion on ethanol action, which suggests that different mechanisms may be responsible for the soporific and hypothermic effects of ethanol.

In the treadmill experiment, female Sprague-Dawley rats were trained to walk an aluminum belt with grid shock on either side (*16*). "Time-off-belt" was measured in three 2-minute sessions per day to assess the extent of the rat's motor coordination. A dose-response effect was seen with ethanol alone (Fig. 1). The time-off-belt values increased significantly when calcium (2 μmole/kg) was injected intraventricularly (*17*), showing that even small central doses of calcium significantly potentiate the effects of low doses of ethanol in rats. This dose of calcium did not itself alter the time-off-belt.

Thus, unlike the calcium-induced antagonism of morphine analgesia reported by other workers (*5*), calcium enhances the hypnotic effects of other drugs in mice. The calcium depletion produced by ethanol and morphine (*4*) must therefore be interpreted differently for the two drugs. Since Ross *et al.* (*4*) have shown that naloxone will block the brain calcium depletion produced by morphine and ethanol, and since naloxone antagonizes many central effects of morphine (*4, 5, 18*), it is of interest that in the present studies with mice, naloxone (1 and 10 mg/kg intraperitoneally) did not significantly alter calcium-enhancement of sleeping time produced by ethanol (data not shown). In another experiment, however, naloxone significantly potentiated the rate-decreasing effects of ethanol on responding under a 2-minute fixed-interval schedule of food presentation in rats (*19*). Such disparate results suggest that the effect of naloxone on calcium is at sites other than the opiate receptor.

The dose-related effects of calcium on ethanol intoxication in the present study suggest a mechanism similar to that of ethanol. The optimal time of 30 minutes for prior treatment with calcium probably reflects the time required for the cation to reach critical sites in the brain, perhaps the reticular formation (*20*), where it can interact with ethanol. The fact that some divalent cations enhance sleeping time (calcium, cadmium, manganese) while others do not (nickel, barium, magnesium) suggests some specificity of interaction with ethanol. Manganese and cadmium are not intrinsically involved in neuronal function, and probably do not play a role in ethanol's action in vivo. On the other hand, the lack of specificity of calcium for ethanol was seen in the observation that calcium also enhanced the hypnotic effects of tertiary butanol and chloral hydrate.

Since ionophores enhance calcium effects on ethanol sleeping time, it appears that the cation must enter or cross the neuronal membrane to produce this effect. The finding that verapamil did not have the opposite effect can be rationalized by the fact that this drug has heretofore been used for studies of calcium movement in the heart, and whether or not the drug blocks transmembrane movement centrally is still unknown. Finally, the reduction in ethanol sleeping time by EDTA and EGTA indicates that endogenous calcium is important for the hypnotic effect of this drug, and that the calcium is loosely bound to the membrane or is in the extracellular fluid, since these chelators do not readily cross lipid membranes (*21*). The finding that lanthanum did not have an effect on sleeping time is surprising, since the drug has been shown to act centrally to enhance the action of morphine (*22*). This supports the suggestion that ethanol and morphine may affect different pools of brain calcium.

Whether high or low concentrations of calcium in subjects drinking ethanol may cause an altered response to ethanol remains to be tested. The results reported here, however, indicate that cations can alter the effects of various centrally depressant drugs in general, and of ethanol in particular.

CARLTON K. ERICKSON*
THOMAS D. TYLER
Department of Pharmacology and Toxicology, School of Pharmacy, University of Kansas, Lawrence 66045
R. ADRON HARRIS
Department of Pharmacology, School of Medicine, University of Missouri, Columbia 64201

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7. Duration is defined as time from loss of righting reflex to return of the reflex twice within 30 seconds, measured to the nearest minute. Sleep variables such as room temperature, extraneous sounds, food intake, and ethanol concentrations were adequately controlled.
8. Intraventricular injections in the mice were by the methods of R. A. Harris, H. H. Loh, and E. L. Way [*J. Pharmacol. Exp. Ther.* **195**, 488 (1975)] and W. G. Clark, C. A. Vivonia, and C. F. Baxter [*J. Appl. Physiol.* **25**, 319 (1968)].
9. Artificial cerebrospinal fluid was prepared by the method of D. Palaić, I. H. Page, and P. A. Khairallah [*J. Neurochem.* **14**, 63 (1967)]. Pilot studies showed no consistent or significant effects on sleeping time of injection versus sham puncture, osmolarity, or *p*H, when a fixed injection volume of 5 μl was used.
10. The dose-response relationship in rats was seen with calcium chloride (2 to 8 μmole/kg) given intraventricularly 30 minutes before a dose of ethanol (3.5 g/kg). These and related results have been reported by C. K. Erickson, L. K. Beck, K. L. Duensing, and R. M. Huff [*Abst. Soc. Neurosci.* **3**, 290 (1977)].
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14. Attempts to enhance sleeping time induced by pentobarbital by the administration of calcium have given equivocal results, as reported in Erickson *et al.* (*10*).
15. Intraventricular injection volumes were 5 μl. Cations alone lowered the body temperature 1.0° to 4.2°C. Ethanol alone lowered the body temperature 2.5°C ($N = 7$ for each determination).
16. Also called the "moving belt apparatus" [R. J. Gibbins, H. Kalant, A. E. LeBlanc, *J. Pharmacol. Exp. Ther.* **159**, 236 (1968)].
17. The rats were injected intraventricularly according to the method of F. R. Popick [*Life Sci.* **18**, 197 (1976)]. The intraventricular calcium dose had no obvious behavioral effect when administered alone.
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* Present address: College of Pharmacy, University of Texas, Austin 78712.

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Cat Color Vision: The Effect of Stimulus Size

Abstract. *Adult cats were trained to discriminate blue from green and gray. Although the cats could discriminate the intensity of stimuli whose areas ranged from 33 to 0.36 square centimeters they could not discriminate color when the stimulus was 0.36 square centimeter (< 20° visual angle). This influence of stimulus size may account for both positive and negative results of previous studies.*

The color vision capability of the cat has been an issue of debate for over half a century. Early investigators (*1–3*) concluded that cats were color blind, but more recent studies (*4–6*) have clearly indicated that cats can discriminate some differences in wavelength distribution. What remains unclear is why the positive findings were difficult to obtain.

We were encouraged to address this question because of certain improvements in the behavioral testing procedures for the cat (*7*) and a simplification of procedures necessary to demonstrate color discrimination (*8*). The testing procedures proved to be effective for training normal adult cats to discriminate colors, and stimulus size emerged as a variable of critical importance.

Four untrained adult cats were maintained at 80 percent of their weight when given free access to food, and were trained to press either of two clear Plexiglas response panels with their noses for a reward of diluted beef baby food (*7, 9*). Visual stimuli were then rear projected onto a viewing screen positioned 1.5 cm behind the response panels. The cats were first trained to press the panel in front of the brighter of two blue (Kodak CC50 B) patches of light [area 4.5 cm^2 (*10*)]. Only responses to the correct (brighter) stimulus were reinforced, and the position of the brighter stimulus was varied in a haphazard fashion. After they had mastered the intensity discrimination (200 trials per day, four consecutive days at ≥ 80 percent correct or two consecutive days at ≥ 90 percent correct), cats A and B were trained to discriminate blue from gray [Kodak CC50 B versus neutral densities (ND) of log 0.0, 0.5, or 1.2] and cats C and D were trained to discriminate blue from green (Kodak CC50 B versus CC50 G). In the blue versus gray discrimination the blue stimulus transmitted more light at all wavelengths from 400 to 700 nm, 400 nm for blue versus ND 0.0 and less light at all wavelengths for blue versus ND 1.2. In the blue versus green discrimination, on half of the trials blue transmitted more light than green at every wavelength, and on the other half of the trials the reverse was true (*8*). For both discriminations and all brightness relations the blue stimulus was correct and its position was varied in a haphazard fashion. Thus, a cat could not reliably select the blue stimulus on the basis of brightness or position (*10*).

Although all four cats rapidly learned the intensity discrimination (mean, 13.25 sessions) after 50 sessions of testing on either color discrimination, none of the cats had reached the 80 percent correct level of performance; they all gave a stable performance of 65 to 70 percent correct, that is, better than chance but less than criterion. This mediocre performance was maintained through changes in stimulus intensity, contrast, and pupil dilation. However, when the size of the stimuli was increased to an area of 32.5 cm^2 there was a dramatic improvement in the performance of cats A, B, and C (*11*), two of them quickly reaching the 90 percent correct level and the third stabilizing around 75 to 80 percent correct. Cat C, which was learning the blue versus green discrimination, was then transferred to blue versus gray, and cats A and B, which were discriminating blue from gray, were transferred to blue versus green. All three cats maintained high levels of performance (> 80 percent correct) that indicated their ability to discriminate blue from green or gray (*8*).

We then returned cats A, B, and C to the blue versus green discrimination and began a systematic manipulation of stimulus size. A method of limits was employed so that each day a cat was given 70 trials at each of four stimulus sizes. Ascending and descending series were conducted on alternate days. After 5 days (1400 trials) of color and stimulus-size testing the cats were retrained on the originally learned intensity discrimination until criterion performance was reached (mean, 4.3 sessions). Stimulus size was then manipulated as for the color discrimination for an additional five testing sessions. As shown in Fig. 1, these cats were able to discriminate color (80 to 90 percent correct) when the stimuli were 84 to 32.5 cm^2, but their performance became progressively worse as stimulus size decreased (around 55 percent correct when the stimuli were 0.36 cm^2). These changes in stimulus size had no effect upon our ability to discriminate between the blue and green stimulus. Manipulation of stimulus size over the same range had no effect upon the cats' ability to perform the intensity discrimi-

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