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Effect of Ethanol on Intracellular Ionized Calcium Concentrations in Synaptosomes and Hepatocytes

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SUMMARY

The effect of ethanol on intracellular ionized calcium concentrations (Ca_i) was studied in synaptosomes isolated from mouse whole brain and in hepatocytes isolated from rat liver. The fluorescent calcium chelator, fura-2, was used to quantitate Ca_i. Incubation of synaptosomes with ethanol (350–700 mM) increased resting Ca_i and decreased the effectiveness of KCl to raise Ca_i in a concentration-dependent manner. Ethanol produced an initial rapid (<10 sec) increase in resting Ca_i that remained elevated for at least 14 min in the presence of the drug. The increase in resting Ca_i was correlated with the inhibitory effect of ethanol on depolarization-induced increases in Ca_i. Resting Ca_i was dependent on the external calcium concentration (0–1 mM). However, the ethanol-induced increase in resting Ca_i (expressed as percent of control) did not differ in the presence of several extracellular calcium concentrations (0.01, 0.1, and 1 mM). Incubation of synaptosomes in a Na-free buffer resulted in a higher resting Ca_i and slightly enhanced the effect of ethanol to increase resting Ca_i. In contrast to these results in brain tissue, ethanol (30–600 mM) did not alter resting Ca_i or vasopressin-stimulated increases in Ca_i in hepatocytes. Our results suggest that the anesthetic effects of alcohols may be mediated, in part, by increased resting Ca_i and by decreased calcium influx through voltage-sensitive calcium channels. In addition, our findings suggest possible mechanisms by which ethanol increases resting Ca_i in neuronal tissue.

Ethanol is a widely abused drug that produces sedation, hypnosis, and anesthesia as the dose increases. These effects are presumed to be due to changes in synaptic transmission, but the exact nature of these changes remains to be defined. Numerous studies have reported on the effect of *in vitro* addition of ethanol on neurotransmitter release from brain slices and synaptosomes. Several studies have shown that ethanol stimulates resting neurotransmitter release (1–6), but reports of the effect of ethanol on depolarization-induced neurotransmitter release are conflicting. Although reports have shown that ethanol increases (7) or decreases KCl-stimulated neurotransmitter release (8), ethanol is usually reported to have no effect on KCl-evoked neurotransmitter release unless very large concentrations of ethanol are tested (9–11).

A rise in Ca_i is a direct stimulus for neurosecretion (12). Therefore, any alteration in Ca_i produced by ethanol may have effects on neurotransmission. Several studies have shown that ethanol alters basic homeostatic mechanisms for maintenance of stable resting Ca_i. Intoxicant-anesthetic concentrations of ethanol decrease ATP-dependent calcium uptake into subcellular organelles (13, 14) and inhibit Na exchange (15). As a result of any one or all of these effects, ethanol would be expected to increase resting Ca_i, resulting in increased resting neurotransmitter release.

Intoxicating concentrations of ethanol also inhibit voltage-dependent calcium uptake into synaptosomes (16–19) and depress neuronal calcium currents (20). The fast phase of calcium uptake in synaptosomes is associated with neurotransmitter release and is most sensitive to the inhibitory effect of ethanol on KCl-stimulated calcium uptake (18). One interpretation of these data is that the intoxicating effects of ethanol may be due, in part, to inhibition of voltage-sensitive calcium influx into presynaptic nerve terminals and consequent effects on neurotransmission. However, this interpretation is not consistent with numerous studies demonstrating that ethanol often increases (7) or does not alter (9–11) neurotransmitter release. These diverse findings could be reconciled by postulating that inhibition of depolarization-induced calcium influx by ethanol is secondary to elevation of Ca_i. Thus, the effect on neurotransmitter release would depend on the balance between the stimulatory and inhibitory actions of ethanol on processes regulating Ca_i. This balance is difficult to determine from studies of ^{45}Ca fluxes because these may not accurately reflect movement

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ABBREVIATIONS: Ca_i, intracellular ionized calcium concentration; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; quin 2, 2-{[2-bis-(carboxymethyl)-amino-5-methylphenoxy]-methyl}-6-methoxy-8-bis-(carboxymethyl)-aminoquinoline.

of unlabeled calcium. However, the recent introduction of new intracellular fluorescent indicators for measurement of Ca_i (21) makes it possible to study changes in Ca_i.

The effect of ethanol on Ca_i in synaptosomes has not been previously determined. In this study, the fluorescent calcium chelator, fura-2, was used to measure Ca_i in synaptosomes. The effect of ethanol on Ca_i was also examined in hepatocytes. Whereas synaptosomes exhibit voltage-sensitive calcium channels, calcium channels in hepatocytes are opened by receptor-bound agonists such as vasopressin. It was therefore of interest to contrast the effects of ethanol on Ca_i in synaptosomes and hepatocytes. The results show that ethanol does not alter Ca_i in hepatocytes but increases resting Ca_i and reduces depolarization-induced increases in Ca_i in synaptosomes. These findings may provide an explanation for the various effects of ethanol on neurotransmitter release.

Materials and Methods

Preparation of Synaptosomes

Synaptosomes were prepared by the method of Fontaine *et al.* (22), with slight modification. Whole mouse brains obtained from male ICR mice (60–100 days old) were placed in ice-cold sucrose-HEPES (0.32 M sucrose, 3 mM HEPES, and 0.1 mM EDTA, pH 7.4 with Tris base) and homogenized in a Thomas size C Teflon to glass homogenizing tube at 1000 rpm. The resulting homogenate was centrifuged at 2000 × *g* for 6 min. The supernatant was centrifuged for 20 min at 17,000 × *g*. Preliminary studies showed that fura-2-AM, the cell-permeant ester form of fura-2, does not undergo complete hydrolysis when incorporated after purification of synaptosomes. Crude P_2 synaptosomes, however, hydrolyze 90–95% of the fura-2-AM. Therefore, in all experiments, fura-2 was incorporated into crude synaptosomes (P_2 fraction). The crude synaptosomal pellet was resuspended to 8–9 mg protein/ml in ice-cold sucrose HEPES and incubated with 10 μM fura-2-AM (Molecular Probes, Inc.; dissolved in dimethyl sulfoxide) or 1% dimethyl sulfoxide (control samples) for 45 min at 35° in a shaking water bath. At the end of the incubation period, crude synaptosomes were centrifuged at 17,000 × *g* for 20 min. The resulting pellets were resuspended in ice-cold sucrose-HEPES, layered onto a discontinuous Ficoll-sucrose gradient (13 and 7.5%), and centrifuged for 35 min at 64,000 × *g*. The synaptosomal band was removed and diluted with Low calcium buffer (in mM: 145 NaCl, 5 KCl, 1.2 $MgCl_2$, .07 $CaCl_2$, 10 glucose, 10 HEPES; pH 7.5 with Tris base). The synaptosomes were centrifuged at 17,000 × *g* for 20 min and resuspended to 1–1.5 mg protein/ml in Low calcium buffer. Synaptosomes were maintained on ice until used. Protein concentrations were assayed by the method of Lowry *et al.* (23), using bovine serum albumin as standard.

Preparation of Hepatocytes

Hepatocytes were obtained from the livers of fed male Sprague-Dawley rats, as previously described (24). Rat body weights averaged 294 ± 24 g (mean ± SE, $n = 5$). The protein concentration of cells was 2.43 ± 0.14 mg protein/10^6 cells (mean ± SE, $n = 5$). Viability was tested before, during, and at the end of each experiment by trypan blue exclusion. Viability averaged 80–95% during each experiment. Hepatocytes (10^6 cells/ml) were loaded with fura-2 by incubation for 30 min at 35° with 3 μM fura-2-AM in Low calcium buffer. Hepatocytes hydrolyzed 90–95% of incorporated fura-2-AM. After incorporation of fura-2, the cells were centrifuged at 500 × *g* for 2 min, resuspended in Low calcium buffer, and centrifuged again at 500 × *g* for 2 min. After final resuspension in Low calcium buffer, hepatocytes remained on ice and were continuously gassed with 95% O_2/5% CO_2 before measurement of Ca_i.

Determination of Ca_i

Synaptosomes. Fluorescence measurements were made using a Farrand Mark I fluorometer at excitation wavelengths of 350 and 380 nm and an emission wavelength of 498 nm. Immediately before fluorescence reading, aliquots of the synaptosomal suspension were removed and centrifuged at 13,000 × *g* for 30 sec. The pellet was resuspended in warm (35°) Low calcium buffer containing heavy metal chelator, diethylenetriaminepentaacetic acid (5 μM). A calibration was performed on each synaptosomal preparation, according to a modification of the procedure of Komulainen and Bondy (25), to determine R_{max}, R_{min}, and Sf_2/Sb_2. R_{max} was determined in the presence of sodium dodecyl sulfate (0.1%) and a saturating concentration of calcium, whereas R_{min} was measured in the presence of a 100-fold excess of EGTA to reduce the calcium concentration to <10 nM. In addition to correction for autofluorescence, a correction for leak of fura-2 from synaptosomes was computed and applied separately for each excitation wavelength for all experimental samples. Leak was measured by addition of $MnCl_2$ (40 μM final concentration) to a fresh sample. The sample reading was taken immediately after mixing (<10 sec) to prevent erroneous reading due to quenching of intracellular fura-2. Leak of fura-2 over the following 10-min incubation period was small (<1% of total fluorescence). The reduction in fluorescence at 350 and 380 nm was applied to each experimental sample to correct for extracellular fura-2. In addition, the amount of fluorescence in the supernatant of a fresh sample was determined. Many experiments showed that these two methods of determining leak of fura-2 produce the same result. After calculation of ratios of total fluorescence at excitation wavelengths of 350 and 380 nm with the above correction, Ca_i was calculated according to the method of Grynkiewicz *et al.* (21) using 224 nM as k_D.

Hepatocytes. Measurement of Ca in hepatocytes was determined in an analogous manner to that of synaptosomes. Aliquots of hepatocytes were diluted to 0.25 × 10^6 cells/ml with warm (37°C) Low calcium buffer. Resting Ca was measured after incubation for 3 min at 37°C in a water-jacketed sample compartment. Vasopressin-stimulated increases in Ca_i were measured immediately (approximately 10 sec) after addition of 1 μM vasopressin. The time-dependent leak of fura-2 was greater in hepatocytes than in synaptosomes and was measured by $MnCl_2$ quenching for each sample with and without addition of vasopressin (see above). A calibration for R_{max} and R_{min} was performed on at least one sample for each experiment.

Drug additions. Alcohols or H_2O (controls) were added in equal volumes to synaptosomes after resuspension in Low calcium buffer. The synaptosomes were incubated for 10 min at 35° in a shaking water bath. Synaptosomes were then transferred from test tubes to cuvettes and resting Ca_i was measured, followed immediately by the addition of KCl and measurement of depolarization-induced increases in Ca_i. For calcium dependence experiments, synaptosomes were suspended in Low calcium buffer and resuspended in a buffer of the appropriate calcium concentration after a final wash step (13,000 × *g* for 30 sec) immediately before incubation with or without ethanol. For sodium dependence experiments, synaptosomes obtained from the Ficoll-sucrose gradient were suspended in sodium-free

or sodium-containing buffer and maintained in the same buffer throughout the course of the experiment.

Calculations and Statistics

The stimulation-induced increase in Ca_i in synaptosomes and hepatocytes was calculated by subtraction of the resting Ca_i value from the value of Ca_i obtained after addition of KCl (synaptosomes) or vasopressin (hepatocytes). Data were analyzed by one-way analysis of variance followed by paired *t* test where appropriate. Correlations were calculated by least squares linear regression analysis.

Results

Synaptosomes. Fig. 1 shows the fluorescence excitation spectrum for fura-2 in synaptosomes. Synaptosomes were resuspended in Low calcium buffer containing approximately 70 μM calcium. Ca_i was determined using the ratio of total fluorescence at an excitation wavelength of 350 nm to that at 380 nm. After lysis of synaptosomes, addition of a 100-fold excess of EGTA shifted the spectrum to a peak at 370 nm. Addition of a saturating amount of calcium (final concentration approximately 5 mM) shifted the fura-2 excitation spectrum, revealing a peak at 345–350 nm. These spectra are similar to those

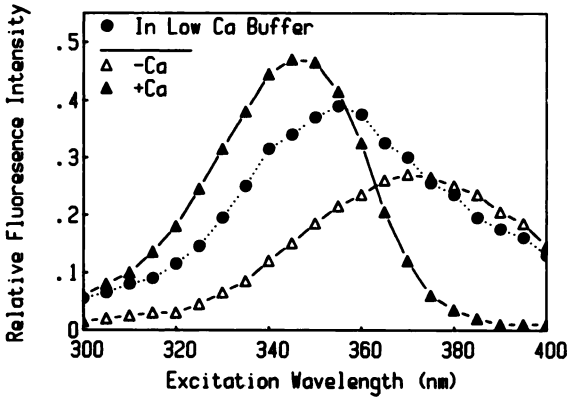

Fig. 1. Fura-2 excitation spectrum in synaptosomes. Synaptosomes were resuspended in Low calcium buffer and the excitation spectrum was recorded. The synaptosomal spectrum was corrected for autofluorescence and fura-2 leak (see Materials and Methods). Synaptosomes were then lysed with 1% sodium dodecyl sulfate and a 100-fold excess of EGTA was added (−calcium spectrum). $CaCl_2$ was added to yield a final concentration of approximately 4 mM (+calcium spectrum).

TABLE 1

Effect of ethanol on resting Ca_i and KCl-dependent increases in Ca_i in synaptosomes

Whole brain synaptosomes were incubated with various ethanol concentrations for 10 min. After measurement of resting Ca_i, KCl was added to synaptosomes. The increase in Ca_i produced by 50 mM KCl was measured immediately (<10 sec after mixing). Data are means ± SE of six experiments performed in duplicate. Data were analyzed by paired *t* test: [a] $p < 0.01$, with respect to the appropriate control; [b] $p < 0.05$.

	Resting Ca_i	Ca_i after addition of (50 mM) KCl	KCl-dependent increase in Ca_i
		nM	
Control	332 ± 35	401 ± 44	69 ± 13
Ethanol (mM)			
50	344 ± 36	398 ± 49	54 ± 14
100	337 ± 33	399 ± 45	63 ± 14
350	393 ± 45[a]	432 ± 52	39 ± 8[b]
700	504 ± 79[b]	536 ± 87[b]	32 ± 12[a]

observed previously for fura-2 in physiological intracellular buffer (21).

In the following experiments, synaptosomes were resuspended in Low calcium buffer containing approximately 70 μM calcium and incubated for 10 min at 35° before measurement of resting Ca_i. After incubation for 10 min, Ca_i was 332 ± 35 nM (Table 1). This value of resting Ca_i is somewhat higher than that measured using quin-2 (26–28). However, this value is similar to values of resting Ca_i measured previously in synaptosomes using fura-2 (25). Addition of 50 mM KCl significantly increased Ca_i to 401 ± 44 nM (Table 1), yielding a KCl-induced change of 69 ± 13 nM. The magnitude of the response observed after addition of KCl is smaller than previously reported measurements in synaptosomes using quin-2 (26–28). However, a recent study in synaptosomes using fura-2 showed that the increase in Ca_i following addition of KCl is dependent on the extracellular calcium concentration, and larger increases were observed with a higher calcium concentration than that used in these experiments (29) (see also calcium dependence below). In addition, because measurement was not continuous and required approximately 10 sec, a large transient increase in Ca_i may not have been detectable. The response to KCl was completely inhibited by 100 μM $LaCl_3$ or 1 mM $NiCl_2$ (data not shown), demonstrating that the increase in Ca_i produced by KCl occurs as a result of calcium entry through voltage-sensitive calcium channels.

Table 1 shows the effect of various ethanol concentrations on Ca_i. Resting Ca_i was increased by 350 and 700 mM ethanol in a concentration dependent manner. The Ca_i values measured after KCl addition were larger in samples incubated with the highest concentration of ethanol tested, 700 mM (Table 1). This effect of ethanol, however, was preceded by an increase in resting Ca_i. The KCl-induced increase in Ca_i was actually decreased in the presence of ethanol. The ability of ethanol to increase resting Ca_i was correlated with the inhibitory effect of ethanol on KCl-stimulated increases in Ca_i ($r = 0.88$). Similarly, the n-alkanols, methanol, ethanol, butanol, and pentanol, increased resting Ca_i and reduced increases in Ca_i after addition of KCl, and their potency was in accord with chain length (pentanol > butanol > ethanol > methanol) (data not shown), demonstrating that effects of ethanol on Ca_i were not due to osmotic effects. Comparison of the quenching ability of $MnCl_2$ for external fura-2 in 100 mM butanol and in control samples demonstrated that the rise in resting Ca_i produced by alcohols is not an artifact due to leakage of fura-2 (data not shown). The ethanol-induced increase in resting Ca_i was reversed after washing by centrifugation. Resting Ca values were 242 ± 24 and 349 ± 34 nM (mean ± SE, $n = 3$) after 10 min incubation at 35° in the absence or presence of 700 mM ethanol, respectively. After centrifugation and resuspension in Low calcium buffer, synaptosomal Ca_i was 200 ± 34 nM for control samples and 227 ± 34 nM for 700 mM ethanol samples.

Measurement of the time course of the effect of a large concentration of ethanol (700 mM) on resting Ca_i showed that ethanol produced a rapid increase in Ca_i (within approximately 10 sec of addition) that was sustained over the next 14 min of measurement (Fig. 2). Incubation of synaptosomes with ethanol for longer time intervals did not result in a significant increase in resting Ca_i above that measured after 10 sec ($F_{8,32} = 2.01$, $p = 0.08$). Measurement of resting Ca_i at 4°C showed that resting Ca_i rises to a high level (>1 μM) (data not shown), possibly due

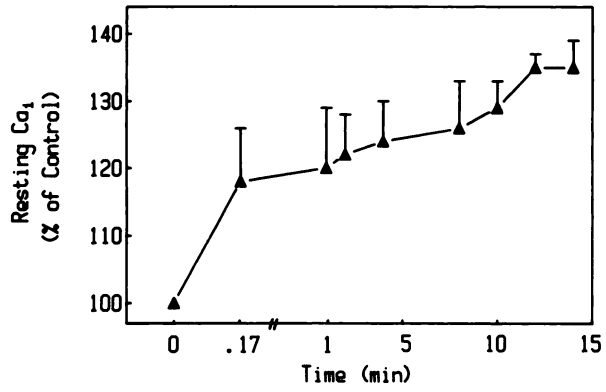

Fig. 2. Time course of the effect of ethanol on resting Ca_i. Whole brain synaptosomes were incubated with 700 mM ethanol. Resting Ca_i was followed for 14 min in each synaptosomal sample. Data are expressed as means ± SE of the per cent of the appropriate control for each time interval (n = 5).

to inhibition of enzymes that maintain stable resting Ca_i. Therefore, no conclusions could be drawn regarding the effect of ethanol on resting Ca_i at lowered temperature.

The dependence of resting Ca_i on the extracellular calcium concentration is shown in Fig. 3. Resting Ca_i was 72 ± 4 nM after 10 min incubation in a buffer containing 100 μM EGTA and no added calcium. Resting Ca_i levels were 172 ± 13, 336 ± 21, and 506 ± 34 nM in the presence of 0.01, 0.1, and 1 mM external concentrations of calcium, respectively. Incubation with 700 mM ethanol increased resting Ca_i in the presence of 0.01, 0.1, and 1 mM calcium but did not alter resting Ca_i when incubated in a buffer containing 100 μM EGTA (Fig. 3). When expressed as a per cent of control resting Ca_i, the ethanol-induced increase in resting Ca_i did not differ when synaptosomes were incubated in the presence of various external calcium concentrations (0.01, 0.1, and 1 mM).

The effect of varying the external calcium concentration on Ca_i values measured following addition of KCl is shown in Fig. 4. Addition of KCl (50 mM) significantly increased Ca_i above resting levels with 0.01, 0.1, and 1 mM concentrations of extracellular calcium but not in the absence of extracellular calcium. Ca_i was increased by 12, 30, and 40% above resting Ca_i levels in the presence of 0.01, 0.1, and 1 mM external calcium concentrations, respectively. Ethanol did not alter values of Ca_i measured after KCl addition at any of the calcium concentrations examined ($F_{1,8}$ = 3.20; p = 0.11). Depolarization-dependent increases in Ca_i were reduced by ethanol (700 mM) in the presence of the highest external calcium concentration tested (1 mM) (Fig. 5).

To determine whether ethanol-induced inhibition of Na/Ca exchange contributes to the effect of ethanol on resting synaptosomal Ca_i, NaCl was isosmotically replaced by choline chloride. Resting Ca_i increased by 104 nM in Na-free buffer (Table 2, Fig. 3). Addition of ethanol increased Ca_i in both the presence and absence of Na. However, in a Na-free buffer, the effect of low concentrations of ethanol (50 and 100 mM) on resting Ca_i was slightly enhanced (% of control values, Table 2).

Hepatocytes. Because ethanol altered Ca_i levels in synaptosomes, it was of interest to determine whether ethanol also altered Ca_i in non-neuronal tissue. The effect of ethanol on Ca_i in hepatocytes was examined after incubation for 30 sec and 3 min. After incubation for 3 min at 37°, resting Ca_i in hepatocytes was 159 ± 45 nM (Table 3). This value is similar to resting Ca_i levels obtained previously in hepatocytes using quin-2 or aequorin (29–32). After addition of 1 μM vasopressin, Ca_i increased to 252 ± 51 nM. Ethanol (30–600 mM) did not alter resting Ca_i or vasopressin-stimulated increases in Ca_i (Table 3). In addition, ethanol (30–600 mM) failed to alter resting Ca_i in hepatocytes after a 30-sec incubation (data not shown; $F_{3,12}$ = 2.46, p = 0.11).

TABLE 2

Effect of Na on ethanol-stimulated increase in resting Ca_i in synaptosomes

Mouse whole brain synaptosomes were incubated with either Na-free Low calcium buffer or Low calcium buffer containing 145 mM Na. After 10 min, resting Ca_i was measured. Data are means ± SE of four separate determinations performed in duplicate. Data were analyzed by paired t test: [a] $p < 0.01$ with respect to +Na control value of Ca_i; [b] $p < 0.01$ with respect to +Na control value expressed as % control; [c] $p < 0.01$ with respect to appropriate control value of Ca_i.

	Resting Ca_i	
	nM	*% control*
Control		
+Na	311 ± 15	
−Na	415 ± 4[a]	
Ethanol (mM)		
50		
+Na	291 ± 19	94 ± 3
−Na	431 ± 21	104 ± 5[b]
100		
+Na	310 ± 15	100 ± 5
−Na	443 ± 10	107 ± 1[b]
350		
+Na	333 ± 26	107 ± 5
−Na	484 ± 36**	117 ± 8
700		
+Na	413 ± 27**	133 ± 6
−Na	599 ± 20**	144 ± 4

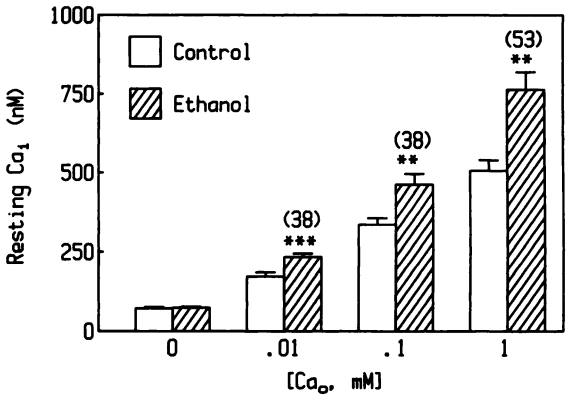

Fig. 3. Dependence of resting synaptosomal Ca_i on external calcium concentration. Synaptosomes were resuspended in buffers containing various calcium concentrations. The zero calcium buffer contained 100 μM EGTA. Synaptosomes were incubated for 10 min in the presence or absence of 700 mM ethanol before measurement of resting Ca_i. Data are means ± SE of five separate experiments. *Numbers in parentheses*, ethanol-induced increases in resting Ca_i calculated as per cent of control resting Ca_i values. Data were analyzed by paired t test with the following results: **, $p < 0.01$; ***, $p < 0.001$, with respect to controls incubated under same conditions.

Discussion

Our results show that alcohols increase resting Ca_i in synaptosomes. These findings provide a potential mechanism to explain previous observations that anesthetic concentrations of ethanol increase the resting release of dopamine (2, 6) and norepinephrine (3, 5) from brain slices and synaptosomes and

TABLE 3
Effect of ethanol on Ca_i in hepatocytes

	Resting Ca_i	Ca_i after addition of vasopressin	Vasopressin-dependent increase in Ca_i
	nM		
Control	159 ± 45	252 ± 51	94 ± 9
Ethanol (mM)			
30	156 ± 34	239 ± 45	83 ± 14
100	154 ± 40	225 ± 43	71 ± 10
600	143 ± 36	214 ± 38	71 ± 4

Resting Ca_i was measured after 3-min incubation with various concentrations of ethanol. The effect of vasopressin (1 μM) on Ca_i was measured immediately after addition of vasopressin to the cuvette. Data are means ± SE of five experiments. Data were analyzed by one-way analysis of variance and paired *t* test. *F* values obtained from one-way analysis of variance for repeated measures follow: for resting Ca_i, $F_{3,12} = 1.23$, $p = 0.34$; for Ca_i after vasopressin addition, $F_{3,12} = 4.44$, $p = 0.03$; for values of vasopressin-induced increases in Ca_i, $F_{3,12} = 1.89$, $p = 0.19$. Post hoc analysis by paired *t* test showed that none of the concentrations of ethanol tested altered values of Ca_i measured after vasopressin addition.

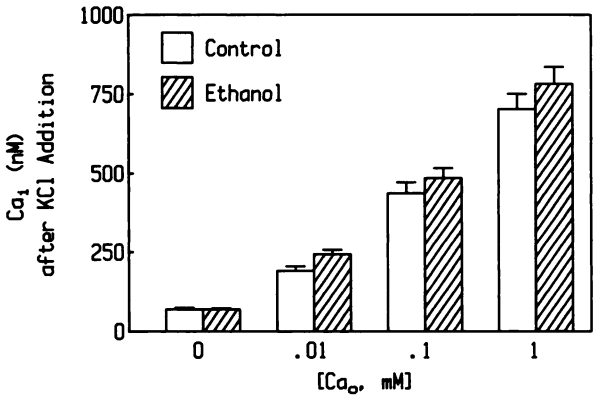

Fig. 4. Synaptosomal Ca_i after KCl addition in the presence of various external calcium concentrations. KCl (50 mM final concentration) was added to synaptosomes after incubation for 10 min with or without added ethanol (700 mM). Data are means ± SE of five experiments. EGTA (100 μM) was included in the zero calcium buffer.

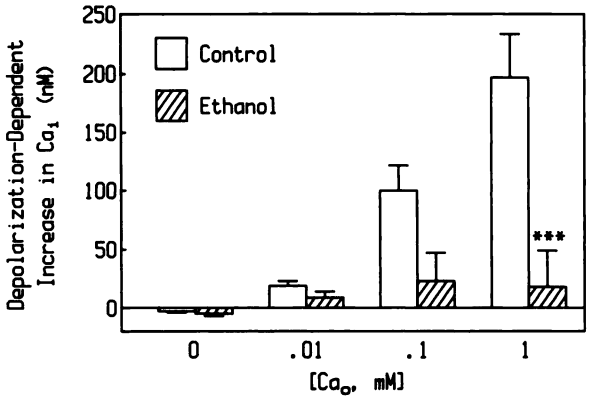

Fig. 5. KCl-dependent increases in Ca_i measured in the presence of various extracellular calcium concentrations. Synaptosomes were incubated for 10 min in buffers containing various calcium concentrations with or without ethanol (700 mM). KCl-dependent increases in Ca_i were determined by subtraction of resting Ca_i values from those measured after addition of KCl (50 mM final concentration). Data were analyzed by paired *t* test: *, $p < 0.05$ with respect to appropriate control. Values shown are means ± SE of five determinations.

that ethanol (40–1250 mM) increases the frequency of miniature endplate potentials at the neuromuscular junction (1, 4, 33, 34). This effect of ethanol has been attributed to enhancement of synaptic vesicle fusion produced by ethanol in a calcium-independent manner (1). However, our results indicate that the ethanol-mediated increases in resting neurotransmitter release and miniature endplate potentials reported by others may be secondary to increases in resting Ca_i. Electrophysiological work has shown that ethanol also increases calcium-dependent potassium conductances in mammalian neurons (35). Similarly, ethanol increases calcium-dependent ^{86}Rb efflux from synaptosomes (36). We suggest that a common mechanism for these diverse neurochemical actions of ethanol is increased intraneuronal calcium.

We also found that ethanol inhibits the increase in Ca_i observed after depolarization with KCl (Fig. 4). This finding agrees with previous work which showed that intoxicant-anesthetic concentrations of alcohols inhibit voltage-sensitive calcium uptake into synaptosomes (16–19) and is consistent with an electrophysiological study which showed that calcium currents in Aplysia are reduced by an anesthetic concentration of ethanol (20). Ethanol preferentially inhibits the fast phase of calcium uptake into synaptosomes (18). This phase of calcium uptake ends within 3 sec and is associated with the release of neurotransmitters (37–39). Based solely on these observations, ethanol would be expected to decrease evoked neurotransmitter release. Studies examining the effect of ethanol on stimulated neurotransmitter release, however, are conflicting. Addition of ethanol *in vitro* is generally reported to have no effect on KCl-stimulated neurotransmitter release (9–11), although studies have reported inhibition (8) or stimulation of neurotransmitter release by ethanol (7). Our results show that ethanol increases Ca_i and reduces calcium entry after depolarization. Because evoked neurotransmitter release reflects the sum total of the ionized cytoplasmic calcium concentration, the balance of these two effects of ethanol may lead to no change or a small decrease or increase in Ca_i after depolarization and attendant effects on depolarization-induced neurotransmitter release. These results may explain the contradictory findings regarding the inhibitory effect of *in vitro* ethanol addition on synaptosomal calcium uptake and the absent or weak effect of ethanol on KCl-stimulated neurotransmitter release.

Our findings show that Ca_i is dependent on extracellular calcium concentrations. Incubation in a buffer containing 100 μM EGTA and no added calcium appeared to deplete Ca_i and prevented KCl-dependent increases in Ca_i. As was shown previously (25, 26, 29), we found that resting Ca_i was greater at higher external calcium concentrations, indicating that Ca_i is partially regulated by external calcium. In addition, greater KCl-dependent increases in Ca_i were observed with higher extracellular calcium concentrations, in agreement with a previous study (29). Previous work also demonstrated that ^{45}Ca uptake (41) and neurotransmitter release (38, 40) increase with increased external calcium concentrations.

Ethanol did not increase resting Ca_i in the absence of extracellular calcium. As noted above, Ca_i may be depleted under these conditions. However, ethanol increased resting Ca_i to the same degree in the presence of 0.01, 0.1, and 1 mM calcium. These results indicate that ethanol does not produce an increase in plasma membrane permeability to calcium. Otherwise, a larger increase in resting Ca_i would be expected with higher extracellular calcium concentrations. Ethanol did not alter Ca_i values measured following the addition of KCl with any of the

external calcium concentrations examined, indicating, as discussed above, that ethanol would not be expected to alter KCl-evoked neurotransmitter release under these conditions.

The increase in resting Ca_i produced by ethanol developed rapidly (<10 sec, Fig.5). Several possibilities can be raised for the mechanisms by which ethanol increases resting Ca_i. One possible mechanism for the ethanol-induced increase in resting Ca_i observed in this study is increased release of calcium from intracellular calcium stores. The suggestion that ethanol increases release of calcium from intracellular sites is supported by both the rapid initial increase in Ca_i produced by ethanol and the independence of ethanol-mediated enhancement of miniature endplate frequency on extracellular calcium (1, 34). In addition, our results show that the degree of increase in resting Ca_i produced by ethanol is independent of the extracellular calcium concentration. Several studies have shown that the resting uptake of calcium into synaptosomes is not altered by ethanol (16–19), further supporting the suggestion that ethanol may increase Ca_i by release of calcium from intracellular sites.

Ethanol could also increase resting Ca_i by inhibition of calcium homeostatic processes. Previous work has shown that ethanol inhibits Na/calcium exchange activity in synaptic vesicles in concentrations within the anesthetic range (15). Our results, however, demonstrate that the elevation of resting Ca_i by ethanol is slightly enhanced at lower ethanol concentrations in a Na-free buffer. After equilibration in Na-free buffer, the Na gradient across the synaptosomal plasma membrane is abolished. Therefore, inhibition of Na/calcium exchange as a mechanism by which ethanol increases resting Ca_i is unlikely. The slight enhancement of ethanol effects on resting Ca_i observed in Na-free buffer suggests that Na/calcium exchange, by rapidly removing cytosolic ionized calcium, may partially obscure the effect of ethanol on Ca_i in the presence of Na. Our finding that ethanol increases resting Ca_i in the absence of Na also demonstrates that the effect of ethanol on resting Ca_i is not due to inhibition of Na/K-ATPase.

Ethanol also inhibits ATP-dependent calcium uptake into subcellular preparations and reduces calcium-ATPase activity in synaptosomal membranes (13, 14). This action of ethanol could raise resting Ca_i. Ethanol could also increase resting Ca_i by partially depolarizing or damaging synaptosomal membranes, but this possibility seems unlikely in view of our findings showing that the effect of ethanol on resting Ca_i is reversible. Another report has also shown that concentrations of ethanol as high as 800 mM do not affect resting membrane potential measured by distribution of tetraphenylphosphonium (17), indicating that the ethanol concentrations used in this study do not result in synaptosomal depolarization.

Significant increases in resting synaptosomal Ca_i produced by ethanol required higher concentrations than those achieved *in vivo* during anesthesia (100–150 mM in mice; A.M. Allan, personal communication). Several studies have shown brain regional differences in sensitivity to the inhibitory effect of ethanol on depolarization-stimulated calcium influx (17–19). Therefore, brain regional study of the effect of ethanol on resting Ca_i may reveal areas of greater sensitivity to the effect of ethanol on Ca_i than observed in this study. In addition, the inhibitory effect of ethanol on depolarization-induced calcium influx is greatest at time intervals of 0–3 sec (18), whereas measurements in this study required approximately 10 sec. Observable effects of anesthetic ethanol concentrations might be increased by improvement of the temporal sensitivity for measurement of Ca_i. It is also possible that various homeostatic mechanisms including Na exchange and calcium-ATPase activity may actively extrude or sequester cytosolic ionized calcium and reduce observable changes produced by anesthetic concentrations of ethanol. Finally, the calcium-buffering capacity of fura-2 could preclude measurement of small changes in Ca_i which may occur after incubation with lower ethanol concentrations.

Our results show that alcohol-induced increases in synaptosomal resting Ca_i are correlated with inhibition of KCl-stimulated increases in Ca_i, suggesting that increases in resting Ca_i may produce inhibition of depolarization-induced calcium entry. Recent electrophysiological work has shown that voltage-sensitive calcium channels exhibit a Ca_i-dependent inactivation in several different cell types (40–42). A quench flow study in synaptosomes has also shown that KCl-stimulated calcium uptake is depressed by prior calcium entry (43) supporting a Ca_i-dependent calcium channel inactivation process in mammalian brain. Further work is required to determine whether KCl-stimulated increases in Ca_i are directly inhibited by the increase in resting Ca_i produced by ethanol.

Ethanol did not produce any measurable changes in either resting Ca_i or in vasopressin-stimulated increases in Ca_i in hepatocytes. This result is not consistent with a recent report which showed that ethanol increased resting Ca_i in isolated hepatocytes (44). In that study, incubation with ethanol increased resting Ca_i transiently with a return of Ca_i to basal levels after approximately 3 min, even in the continued presence of the drug. Our findings show that ethanol does not increase resting Ca_i in hepatocytes even after incubation periods as short as 30 sec.

Our finding that ethanol does not alter Ca_i in hepatocytes differs from our observations of the effect of ethanol on resting Ca_i in synaptosomes. In that preparation, incubation with ethanol increased resting Ca_i for up to 14 min. At present, it is unclear what differences in calcium homeostatic mechanisms may exist between neuronal and non-neuronal tissues, but the differences in the effects of ethanol on Ca_i in synaptosomes and hepatocytes observed in this study suggest that Ca_i may be regulated differently in these two tissues.

In summary, our results show that ethanol increases resting Ca_i and inhibits KCl-induced increases in Ca_i in synaptosomes. This finding provides an explanation for the absent or weak effects of ethanol on stimulated neurotransmitter release and previous observations of ethanol enhancement of resting neurotransmitter release. Our results also show that ethanol effects on Ca_i differ between hepatic and neuronal tissue. Because Ca_i regulates certain enzymes and ion channels, alteration of Ca_i in neuronal tissue by ethanol, as demonstrated in this study, may have important consequences for many cellular processes.

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