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ETHANOL AND SYNAPTOSOMAL CALCIUM HOMEOSTASIS

MARK DAVIDSON, PETER WILCE and BRIAN SHANLEY*
Alcohol Research Unit, Department of Biochemistry, University of Queensland, St Lucia, Qld, 4067, Australia

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Abstract—The effect of ethanol on synaptosomal calcium homeostasis was studied in the rat using the fluorescent dye, fura-2, and ^{45}Ca uptake. The mitochondrial poison, cyanide, caused a substantial rise in intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$, over that of control synaptosomes. This rise was enhanced by ethanol. Ethanol also augmented the rise in $[Ca^{2+}]_i$ induced by ouabain, indicating that modulation of Na^+–Ca^{2+} exchange is probably not the underlying mechanism. The Ca^{2+} channel blockers, verapamil and La^{3+}, also failed to inhibit the rise in $[Ca^{2+}]_i$ caused by ethanol. Preincubation of synaptosomes with caffeine, however, caused a significant decrease in the rise of $[Ca^{2+}]_i$ due to ethanol, suggesting that ethanol exerts effects on Ca^{2+} homeostasis at the level of the endoplasmic reticulum.

Ethanol has been shown to increase Ca^{2+}-dependent K^+ conductance [1, 2], to inhibit voltage-dependent ^{45}Ca uptake [3, 4], and to cause a rise in intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$†, [5–7] in various cell types. The first two findings are probably secondary to the elevation of $[Ca^{2+}]_i$. Maintenance of $[Ca^{2+}]_i$ at approximately 10^{-8} M is regulated by calcium-binding proteins, non-specific intracellular anions, uptake and release of Ca^{2+} by subcellular organelles, an ATP-dependent Ca^{2+} pump, and Na^+–Ca^{2+} exchange. Calcium enters neurons via voltage-dependent or receptor-operated Ca^{2+} channels, through "leakage" pathways or reverse Na^+–Ca^{2+} exchange [8]. Interference with the activity of any of the regulatory mechanisms or entry pathways can lead to a rise in neuronal $[Ca^{2+}]_i$, thereby causing alterations in neurotransmitter release and Ca^{2+}-regulated metabolic activities. It is possible to manipulate $[Ca^{2+}]_i$ in synaptosomes by (i) interference by metabolic inhibitors with the sequestration of Ca^{2+}; (ii) blockade of Ca^{2+} channels; and (iii) inhibition of Na^+–Ca^{2+} exchange. The purpose of this study was to examine the effect of ethanol on homeostatic processes for $[Ca^{2+}]_i$ within synaptosomes and to define a mechanism for the rise in $[Ca^{2+}]_i$ caused by ethanol.

MATERIALS AND METHODS

Synaptosome preparation. Adult male Wistar rats (180–250 g) were decapitated and the anterior part of the cerebrum was quickly dissected out and homogenized in 10 volumes of 0.32 M sucrose at 4°. Synaptosomes were prepared according to the method of Hajos [9]. Briefly, the homogenate was centrifuged at 1500 *g* for 10 min and the pellet washed with an equal volume of 0.32 M sucrose. Supernatants were combined and centrifuged at 9000 *g* for 20 min. The pellet was resuspended in 0.32 M sucrose (5 mL per 2 g of brain) and 5 mL layered over 20 mL 0.8 M sucrose. This was centrifuged at 9000 *g* for 25 min. The 0.8 M sucrose layer was diluted slowly to 0.32 M with ice-cold distilled water and centrifuged at 11,000 *g* for 15 min. The pellet was resuspended to a protein concentration of 5 mg/mL in HEPES buffer A comprising, NaCl 125 mM, KCl 5 mM, Na_2HPO_4 1.2 mM, $MgCl_2$ 1.2 mM, $NaHCO_3$ 5 mM, glucose 10 mM, $CaCl_2$ 1 mM and HEPES 25 mM. The final pH was adjusted to 7.4 with HCl.

$[Ca^{2+}]_i$ *measurements.* $[Ca^{2+}]_i$ was measured using the fluorescent Ca^{2+} indicator dye, fura-2 [10], according to the method of Komulainen and Bondy [11]. To 1 mL-aliquots of synaptosomes (adjusted to 2 mg protein/mL) was added a solution of fura-2/AM (Calbiochem, La Jolla, CA) in DMSO (final concentration 5 μM) and the mixture incubated for 20 min at 37°. Warm HEPES buffer A (9 mL) was then added and incubation continued for 25 min. Synaptosomes were centrifuged at 9000 *g* for 10 min and resuspended in 5 mL of ice-cold HEPES buffer A. A control aliquot of synaptosomes was treated in exactly the same manner, except that it was incubated in buffer + DMSO prior to autofluorescence measurements. Aliquots of fura-2- and DMSO-loaded synaptosomes (equivalent to 200 μg protein) were centrifuged in a Beckmann Microfuge B for 1 min and resuspended in 2 mL of HEPES buffer B (HEPES A minus Na_2HPO_4 and $NaHCO_3$). After 20 min incubation at 37°, including 10 min in the presence of ethanol, fluorescence was measured in an Aminco SPF-500 spectrofluorimeter operated in the ratio mode. Excitation wavelengths were 340 and 380 nm (bandpass 2 nm) and the emission wavelength was set at 510 nm (bandpass 8 nm). For each

* To whom correspondence should be addressed.

† Abbreviations: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid; DTPA, diethylenetriamine pentaacetic acid; SDS, sodium dodecyl sulphate; BSA, bovine serum albumin; and fura-2/AM, fura-2 acetoxymethylester.

resting measurement extrasynaptosomal fura-2 was quenched by addition of $MnCl_2$ (40 μM) and R (340/380 ratio) determined within 15 sec. Background autofluorescence was subtracted from each measurement. The synaptosomes were then treated with 50 mM KCl and R determined after 30 sec. $[Ca^{2+}]_i$ was calculated using the formula:

$$[Ca^{2+}]_i = K_d \times (R - R_{min}/R_{max} - R) \times (Sf_2/Sb_2) \quad [10].$$

R_{min} and R_{max} were calculated for each batch of synaptosomes. R_{min} was the 340/380 ratio upon addition of 5 mM EGTA, 0.1% SDS, 10 μM DTPA and excess Tris base to raise the pH above 8. R_{max} was recorded after addition of 12.5 mM $CaCl_2$. Sf_2 and Sb_2 were the fluorescence at excitation wavelength 380 nm at zero and saturated $[Ca^{2+}]$, respectively.

Determination of Ca^{2+} uptake. Synaptosomal protein concentration was adjusted to about 1 mg/mL. Aliquots (0.2 mL) of the preparation were preincubated for 10 min at 37° with gentle agitation before addition of ethanol or distilled water (control) and incubation was continued for a further 10 min. ^{45}Ca uptake was initiated by addition of either 0.2 mL of HEPES buffer A (5 mM KCl) or a high potassium buffer (110 mM KCl) in each case containing 1 μCi/μmol of ^{45}Ca (Amersham, U.K.). Samples were incubated for a further 30 sec before being stopped by addition of 4 mL of ice-cold Tris–EGTA buffer (NaCl 132 mM, KCl 5 mM, Na_2HPO_4 1.2 mM, $NaHCO_3$ 5 mM, Tris 20 mM, $MgCl_2$ 1.2 mM, EGTA 3 mM, pH 7.4). This suspension was immediately filtered through premoistened GF/C filters (Whatman) and rapidly washed thrice with 4 mL of buffer. The filters were placed in vials with 5 mL of Filtercount (Packard) and radioactivity determined by scintillation spectrometry.

Protein assay. The protein content of synaptosomal preparations was determined according to Lowry *et al.* [12] using BSA as the standard and incorporating appropriate controls for the interference caused by HEPES.

Statistics. Statistical significance of results was determined using Student's t-test.

RESULTS

Ouabain

In studies on Na^+–Ca^{2+} exchange ouabain (1 mM) was added 10 min before addition of ethanol and incubation continued for 10 min before measurement of $[Ca^{2+}]_i$. Table 1 shows that ouabain caused a significant increase in $[Ca^{2+}]_i$ in resting (5 mM KCl) and depolarized (50 mM KCl) synaptosomes, presumably through enhancement of Na^+–Ca^{2+} exchange following inhibition of Na^+,K^+-ATPase. Ethanol augmented the increase produced by ouabain suggesting that the mechanism underlying the effect of ethanol does not involve Na^+–Ca^{2+} exchange. These results complement those by Daniell *et al.* [5] who demonstrated that the elevation of $[Ca^{2+}]_i$ by ethanol was only slightly enhanced at lower ethanol concentrations in a Na^+-free buffer.

Cyanide

Cyanide acts by inactivation of the electron transport chain, thereby decreasing ATP production and lowering the energy charge of the cell. The cessation of energy-dependent Ca^{2+} regulatory pathways within the nerve terminal results in cytoplasmic accumulation of Ca^{2+} [13]. The effect of ethanol on cyanide-treated synaptosomes is shown in Figs 1 and 2. Figure 1 shows the well known inhibition by ethanol of ^{45}Ca uptake upon depolarization. Measurements under resting conditions showed no inhibition by ethanol, although cyanide-treated synaptosomes demonstrated greater ^{45}Ca uptake ($P < 0.001$) for all ethanol concentrations tested. This effect is reflected in the resting $[Ca^{2+}]_i$ values shown in Fig. 2. The data provide further evidence for $[Ca^{2+}]_i$ being involved in the inhibition of Ca^{2+} uptake through voltage-operated channels as $^{45}Ca^{2+}$ uptake in the depolarized cyanide-treated samples was lower than in control synaptosomes (Fig. 1). Ethanol plus cyanide caused a substantial increase over control values in the resting and depolarized levels of $[Ca^{2+}]_i$. This is probably indicative of the lower Ca^{2+}-buffering capacity of cyanide-treated synaptosomes [14].

Verapamil and lanthanum

Attention has recently been focused on the increase in sensitivity to Ca^{2+} of neurons previously exposed to ethanol [15]. This has led to studies on the ability of Ca^{2+} channel blockers to alleviate ethanol withdrawal symptoms [16]. Since the dihydropyridine class of Ca^{2+} channel blockers does not block synaptosomal Ca^{2+} channels [17, 18] we examined the effects of verapamil (a phenylalkylamine that acts on the slow Ca^{2+} channel) and La^{3+} (a non-specific Ca^{2+} channel blocker) on $[Ca^{2+}]_i$ in response to ethanol treatment. The effects of ethanol on verapamil-treated synaptosomes are shown in Fig. 3. Verapamil (300 μM) reduced resting $[Ca^{2+}]_i$ to 87% of the control level and inhibited the KCl-induced rise in $[Ca^{2+}]_i$ to 9% of the increase in control (data not shown). As ethanol still caused a rise in resting $[Ca^{2+}]_i$ in the presence of verapamil, it can be concluded that this did not occur via a verapamil-sensitive mechanism, such as leakage through verapamil-sensitive Ca^{2+} channels or blockade of α-adrenergic receptors. The effect of La^{3+} on $[Ca^{2+}]_i$ (Fig. 4) was quite marked at the concentration used (100 μM), increasing resting $[Ca^{2+}]_i$ from 259 ± 9 to 734 ± 75 nM (mean ± SE). This suggests that care should be taken in interpreting the results obtained with La^{3+}. Although it effectively blocks the KCl-induced rise in $[Ca^{2+}]_i$ (data not shown) it markedly elevates basal $[Ca^{2+}]_i$. The effect of ethanol may well be enhanced by action of La^{3+} on an intracellular Ca^{2+} regulatory process.

Caffeine

It has been shown that caffeine releases Ca^{2+} from microsomal stores in rat dorsal root ganglion neurons [19], and Ca^{2+} sequestered by endoplasmic reticulum elements of saponin-treated neuroblast cells [20]. Depending on concentration, caffeine can stimulate or inhibit ATP-dependent Ca^{2+} uptake by lysed

Table 1. Effect of ouabain and ethanol on synaptosomal $[Ca^{2+}]_i$

Treatment	Increase in $[Ca^{2+}]_i$ (nM) Resting −Ouabain	Resting +Ouabain	Depolarized −Ouabain	Depolarized +Ouabain
Control	—	143 ± 8	—	243 ± 40
Ethanol (100 mM)	26 ± 5	164 ± 10	17 ± 8	331 ± 41
Ethanol (200 mM)	32 ± 6*	164 ± 10	18 ± 10	322 ± 38
Ethanol (500 mM)	95 ± 14‡	236 ± 20†	50 ± 13*	511 ± 62†

Synaptosomes were incubated with ouabain (1 mM) for 10 min before addition of ethanol and a further 10 min before measurement of $[Ca^{2+}]_i$. Values are mean ± SE increase over mean value for resting (257 nM) and depolarized (447 nM) synaptosomes in the absence of ouabain or ethanol. N = 9 determinations from three separate experiments.

*$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ compared with zero ethanol concentration.

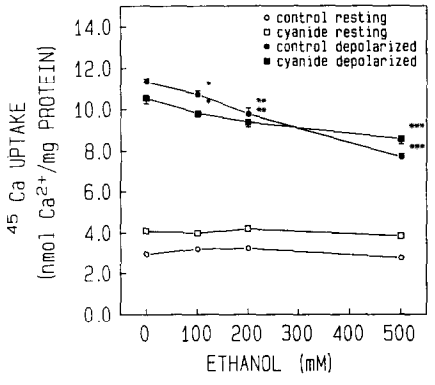

Fig. 1. ^{45}Ca uptake in control and cyanide-treated synaptosomes. Synaptosomes were incubated at 37° in the absence of 2 mM KCN for 10 min before addition of ethanol (0–500 mM). After a further 10 min uptake was measured as described in Materials and Methods. Results are mean ± SE of 10–12 determinations from four separate experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with zero ethanol concentration.

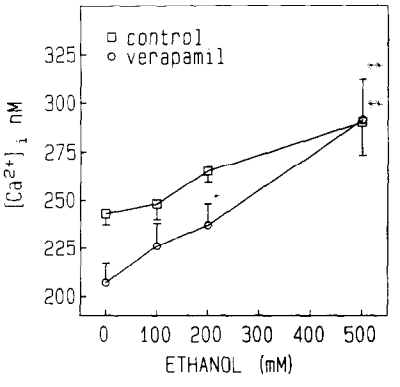

Fig. 3. Effect of ethanol on $[Ca^{2+}]_i$ in control and verapamil-treated synaptosomes. Synaptosomes were treated with verapamil (300 μM) for 10 min before addition of ethanol (0–500 mM). Fluorescence readings were taken after a further 10 min incubation as described in Materials and Methods. Data are expressed as mean ± SE of five determinations from three separate experiments. P values as for Fig. 1.

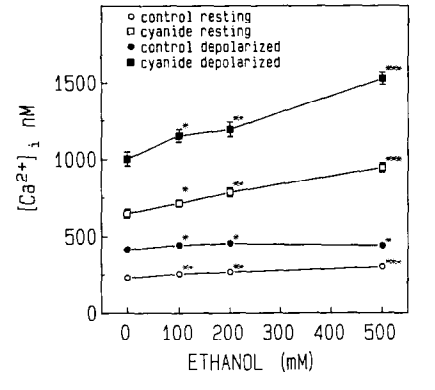

Fig. 2. Effect of ethanol on $[Ca^{2+}]_i$ in control and cyanide-treated synaptosomes. Synaptosomes were treated with KCN (0 or 2 mM) for 10 min before addition of ethanol (0–500 mM). Fluorescence readings were taken after a further 10 min incubation as described in Materials and Methods. Data represent mean ± SE of 8–9 determinations from four separate experiments. P values as for Fig. 1.

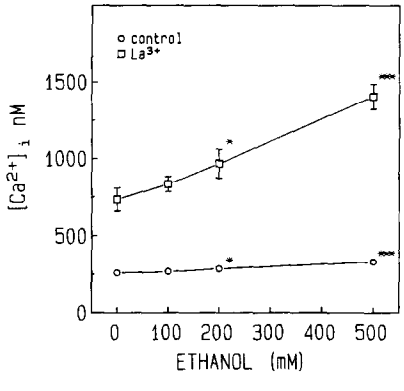

Fig. 4. Effect of ethanol on $[Ca^{2+}]_i$ in control and La^{3+}-treated synaptosomes. Synaptosomes were treated with La^{3+} (100 μM) for 10 min before addition of ethanol (0–500 mM). Fluorescence readings were taken after a further 10 min incubation as described in Materials and Methods. Results are mean ± SE of six determinations from three separate experiments. P values as for Fig. 1.

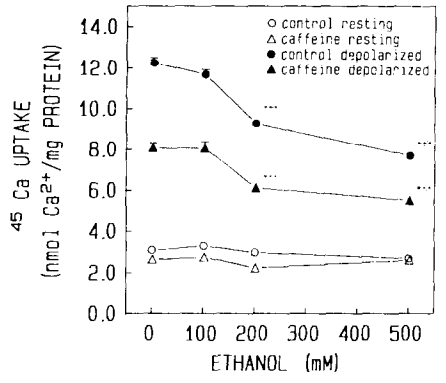

Fig. 5. $^{45}Ca^{2+}$ uptake in control and caffeine-treated synaptosomes. Synaptosomes were incubated at 37° in the absence or presence of caffeine (25 mM) for 10 min before addition of ethanol (0–500 mM). After a further 10 min uptake was measured as described in Materials and Methods. Results are mean ± SE of 4–6 determinations from two separate experiments. P values as for Fig. 1.

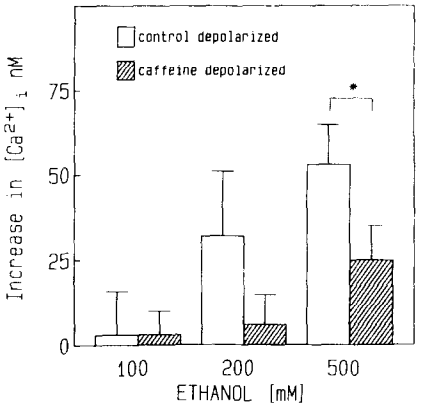

Fig. 7. Effect of caffeine on the rise in depolarized $[Ca^{2+}]_i$ produced by ethanol. Synaptosomes were treated with caffeine (25 mM) for 10 min before addition of ethanol (0–500 mM). Fluorescence readings were taken after a further 10 min incubation as described in Materials and Methods. Increase in $[Ca^{2+}]_i$ is expressed as that over the depolarized $[Ca^{2+}]_i$ which was 558 ± 40 nM for control synaptosomes and 354 ± 19 nM for caffeine-treated synaptosomes (mean ± SE, N = 8). P values as for Fig. 1.

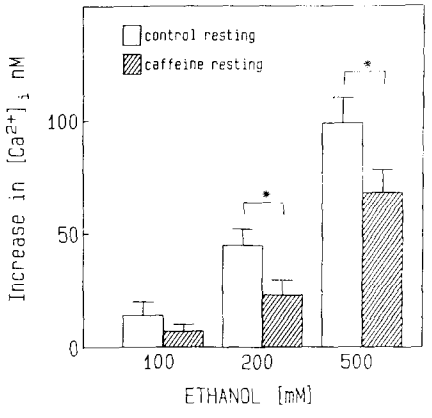

Fig. 6. Effect of caffeine on the rise in $[Ca^{2+}]_i$ produced by ethanol. Synaptosomes were treated with caffeine (25 mM) for 10 min before addition of ethanol (0–500 mM). Fluorescence readings were taken after a further 10 min incubation as described in Materials and Methods. Increase in $[Ca^{2+}]_i$ is expressed as that over the resting $[Ca^{2+}]_i$, which was 307 ± 20 nM for controls and 251 ± 13 nM for the caffeine-treated synaptosomes (mean ± SE, N = 8). P values as for Fig. 1.

synaptosomes [21]. Caffeine also increases intracellular accumulation of cAMP through inhibition of phosphodiesterases, and is capable of blocking adenosine receptors, but neither of these effects has been shown to influence calcium homeostasis. Our results show that caffeine inhibited ^{45}Ca uptake in intact synaptosomes (Fig. 5) and reduced $[Ca^{2+}]_i$ levels in resting and depolarized synaptosomes (Figs 6 and 7). Ethanol caused inhibition of ^{45}Ca uptake in depolarized synaptosomes, while caffeine treatment apparently attenuated this effect (Fig. 5). In the studies of $[Ca^{2+}]_i$ (Figs 6 and 7) caffeine caused a significant reduction in the rise in $[Ca^{2+}]_i$ produced by ethanol. This result implies that part of the effect of ethanol on Ca^{2+} homeostasis within synaptosomes may be exerted at the level of the endoplasmic reticulum.

DISCUSSION

To maintain the normal 10^4-fold Ca^{2+} gradient across the plasma membrane neurons possess a complex system for regulating $[Ca^{2+}]_i$. Ethanol has been shown by various workers using different systems to cause a rise in $[Ca^{2+}]_i$ [5–7]. How ethanol produces this effect, and whether the effect is specific or nonspecific for a given regulatory pathway(s), is open to conjecture. We have attempted to address these questions using the Ca^{2+} fluorescent indicator, fura-2, in freshly isolated rat brain synaptosomes.

Our previous work has shown that the rise in synaptosomal $[Ca^{2+}]_i$ following exposure to ethanol is probably due to a reduction in cytosolic buffering capacity for Ca^{2+} [7] and that ethanol causes an increase in synaptosomal $[Ca^{2+}]_i$ after acute or chronic treatment of animals [22, unpublished data]. It may well be that the results of the present study reflect the action of ethanol on neuronal calcium homeostasis *in vivo*, although caution must be exercised in extrapolating results obtained with relatively high concentrations of ethanol *in vitro*.

Quenching with 40 μM $MnCl_2$ (data not shown) showed that ethanol at the highest concentration used did not cause significant leakage of fura-2 compared to control preparations, thereby excluding damage to the plasma membrane as a cause for ethanol's modulation of $[Ca^{2+}]_i$.

Ca^{2+} entry into the neuronal cytoplasm occurs via several routes of which voltage-sensitive calcium channels is the major one [23]. In synaptosomes dihydropyridine drugs do not block voltage-sensitive calcium channels [17, 18] implying that the L type

channel [24] has no role or is masked in this preparation. However, we were able to inhibit (90%) the depolarization-induced increase in $[Ca^{2+}]_i$ using the inorganic cation, La^{3+}, or the phenylalkylamine, verapamil. Ethanol was still able to elevate $[Ca^{2+}]_i$ under these circumstances, suggesting that the source of Ca^{2+} was not entry through plasma membrane Ca^{2+} channels.

Specific inhibition of Na^+,K^+-ATPase by ouabain (1 mM) caused a slow rise in $[Ca^{2+}]_i$, which was augmented by ethanol. Inhibition of the Na^+,K^+-ATPase causes increased intrasynaptosomal $[Na^+]$, thereby increasing $[Ca^{2+}]_i$ by reverse Na^+–Ca^{2+} exchange. Our results indicate that ethanol does not inhibit neuronal Na^+–Ca^{2+} exchange significantly.

Mitochondria have long been regarded as playing an important part in regulating $[Ca^{2+}]_i$ in neurons. Recent work, however, has shown that mitochondria do not sequester much Ca^{2+} from the cytosol, unless the other buffer systems are saturated, or the cell is subject to pathological conditions of Ca^{2+} overload [8]. Since ethanol increased $[Ca^{2+}]_i$ in the face of cyanide inhibition of mitochondrial calcium sequestration, it is probable that mitochondria play no part in the effect of ethanol on calcium homeostasis.

Recent experimental evidence suggests that ethanol may elevate $[Ca^{2+}]_i$ by stimulating release of Ca^{2+} from intracellular storage sites [5]. In particular Garret and Ross [25] have shown that an acute dose of ethanol inhibited the sequestration of Ca^{2+} into endoplasmic reticulum-like organelles in synaptosomal membranes. Shah and Pant [26] demonstrated that ethanol releases intracellular Ca^{2+} in a temperature-dependent manner from microsomes, suggesting that ethanol may diffuse through the plasma membrane and act directly on the endoplasmic reticulum. As this organelle is also now considered the primary site for sequestration of intracellular Ca^{2+} in nerve terminals (within the physiological range for $[Ca^{2+}]_i$ of 100 to 5000 nM), we studied the effect of ethanol on caffeine-treated synaptosomes. If the effect of prolonged incubation with caffeine is to deplete microsomal stores of Ca^{2+}, then subsequent addition of ethanol could be expected to cause much less of a rise in $[Ca^{2+}]_i$, presuming that a primary site of action of ethanol is also at the level of the endoplasmic reticulum. Our results show that caffeine causes a reduction in KCl-induced ^{45}Ca uptake and in resting and depolarized $[Ca^{2+}]_i$ levels within synaptosomes. Subsequent addition of ethanol provokes a much smaller rise in $[Ca^{2+}]_i$ than that caused by ethanol alone. This suggests that ethanol acts at the level of the endoplasmic reticulum in influencing Ca^{2+} homeostasis in synaptosomes.

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