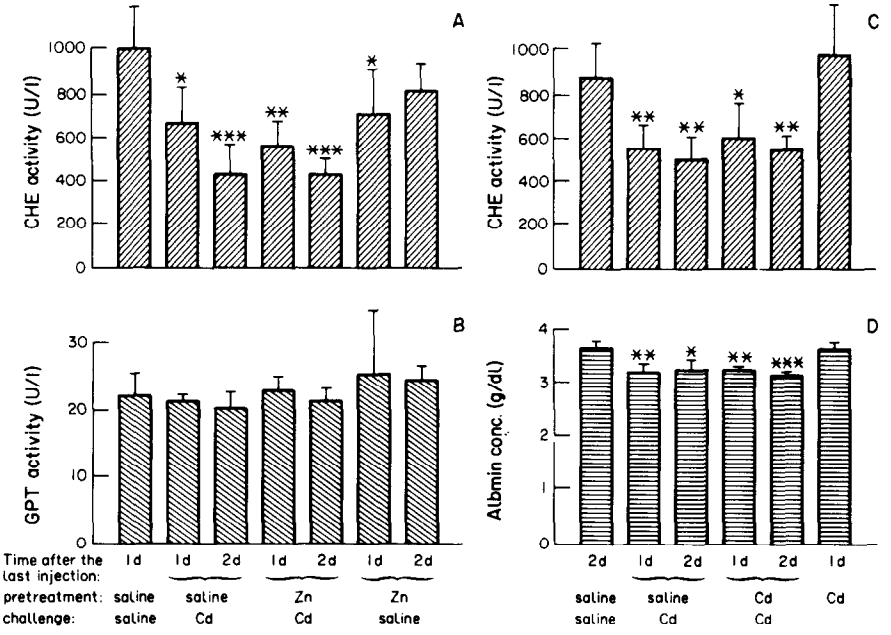

Fig. 1. Effects of pretreatment with Zn or Cd on Cd-induced alterations of serum levels of CHE, GPT and albumin. Rats received the following treatment 24 hr prior to the Cd challenge (1.5 mg/kg body wt, sc): A and B, a single i.p. injection of saline or Zn (8 mg/kg body wt); C and D, a single s.c. injection of saline or Cd (0.3 mg/kg body wt). For comparison two groups of rats pretreated with Zn were injected with saline instead of Cd (A, B) and one group of rats was given Cd pretreatment only (C, D). The control rats were injected with saline twice at 24 hr interval. Data are expressed as means ± SD of 5 samples in each group. *, ** and *** indicate the significant differences from the control at $P < 0.05$, 0.01 and 0.001, respectively.

sion was not accompanied by an enzyme leakage. Thus, the mechanism for the inhibitory action of Cd seems to be different from those of Zn and Cu.

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Effects of ethanol on $^{45}Ca^{2+}$ uptake in synaptosomes and in PC12 cells

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Depolarization-dependent calcium uptake is important in many neuronal functions, such as synaptic transmission [1], and also appears to play an important role in the effects of ethanol. Net $^{45}Ca^{2+}$ uptake into rodent synaptosomes is inhibited in a dose-dependent manner by ethanol *in vitro* [2–5], and after chronic ethanol exposure the potency of *in vitro* ethanol to inhibit synaptosomal Ca^{2+} uptake is reduced [2, 4]. Chronic exposure to ethanol was also reported to decrease net potassium-stimulated $^{45}Ca^{2+}$ uptake in mouse synaptosomes [2], but not in rat synaptosomes [4]. Potassium-stimulated $^{45}Ca^{2+}$ uptake into synaptosomes, however, occurs in a biphasic manner [6].

The fast first phase appears to represent uptake through a voltage-dependent Ca^{2+} channel [6], and the slow second phase, which reaches a steady-state rate of uptake at 15–30 sec, appears mainly due to Na^+/Ca^{2+} exchange [7]. Since the voltage-dependent calcium channel inactivates within the first second of uptake [6], most of the above studies investigated the combined effects of ethanol on both phases. Leslie and coworkers [4] using synaptosomes from rat cortex reported that $^{45}Ca^{2+}$ uptake at 1 sec is inhibited by *in vitro* ethanol; the specific effects of ethanol on the second phase of calcium uptake and the relative sensitivities of the two phases were not investigated. The present study was undertaken to investigate the effects of ethanol on the two phases of calcium uptake. In addition, the effects of chronic ethanol exposure on the calcium channel were investigated in PC12 pheochromocytoma cells. These cells have a number of neuronal characteristics [8], and calcium uptake occurs primarily through the voltage-dependent calcium channel [9, 10].

Methods

The cerebral cortices from male Holzman rats were dissected, and synaptosomes were prepared by the method of Hajos [11]. The synaptosome-enriched material in the 0.8 M sucrose layer was equilibrated with 2.5 vol. of buffer (buffer A) of the following composition (mM): NaCl, 132; KCl, 5; $CaCl_2$, 1.2; $MgCl_2$, 1.3; glucose, 10; Tris, 25, pH 7.4. The suspension was centrifuged at 10,000 *g* for 5 min, and the resulting pellet was resuspended in buffer A.

The fast phase of $^{45}Ca^{2+}$ uptake was determined using the method of Nachshen and Blaustein [6] by pre-equilibrating the synaptosomes for 10–12 min at 30° in choline-substituted buffer (equimolar substitution of NaCl in buffer A with choline chloride) in the presence of various concentrations of ethanol. Uptake was determined at 1 sec in choline-substituted buffer A containing $^{45}Ca^{2+}$ (1.2 μCi/μmole Ca^{2+}), various concentrations of ethanol, and either 5 mM K^+ or 68.5 mM K^+ (isotonic substitution by KCl) in a final volume of 0.5 ml at 30°. $^{45}Ca^{2+}$ uptake was terminated by the addition of 4 ml of ice-cold Ca^{2+}-free buffer A containing 3 mM ethyleneglycolbis(amino-ethyl-ether)tetra-acetate (EGTA). Samples were then rapidly filtered through Whatman GF/B filters, and the filters were rinsed four times with 4 ml of ice-cold buffer.

The slow phase of $^{45}Ca^{2+}$ uptake was determined using a predepolarization procedure [6]. Briefly, after equilibration of the synaptosomal suspension in buffer A at 30° for 10–12 min, aliquots of this suspension (100 μl containing 400–800 μg protein) were incubated for 15 sec at 30° with 100 μl of buffer A containing various concentrations of ethanol and either 132 mM KCl (isotonic substitution for NaCl) or 5 mM KCl. $^{45}Ca^{2+}$ uptake was determined by incubating 150 μl of this suspension for 10 sec at 30° as described above. Net calcium uptake was defined as the difference between uptake in the resting buffer (5 mM KCl) and that in depolarizing buffer (68.5 mM KCl).

PC12 cells were grown in 85% RPMI 1640 containing 10% heat-inactivated horse serum and 5% fetal calf serum in a humidified atmosphere of 95% air–5% CO_2 at 37°. In all experiments cells were plated onto polylysine-coated tissue culture plates. The effects of a 4-day exposure to 150 mM ethanol were determined by plating cells (2×10^6 cells/60 mm dish) in medium containing ethanol, and incubating the cells at 37° in plastic dessicators containing a 95% air–5% CO_2 atmosphere that was saturated with 150 mM ethanol. Medium was replaced after 2 days; loss of ethanol due to evaporation was 23.4 ± 3.88% over a 2-day period.

For the Ca^{2+} uptake studies PC12 cells were rinsed with buffer A and then were preincubated for 10 min at 30° in buffer A containing various concentrations of ethanol. $^{45}Ca^{2+}$ uptake into PC12 cells was determined in 2 ml of buffer A containing $^{45}Ca^{2+}$ (0.45 μCi/μmole Ca^{2+}), either 5 mM KCl or 68.5 mM KCl and various concentrations of ethanol. $^{45}Ca^{2+}$ uptake was terminated after 2.5 min at 30° by aspirating the medium and washing the plates four times with 2 ml of cold buffer A. The cells were digested in 1 ml of 0.5 N NaOH overnight, and radioactivity was determined by liquid scintillation spectrophotometry.

[^{3}H]PN 200-110 binding to PC12 cells was determined by homogenizing the cells in 50 mM Tris buffer (pH 7.4) using a loose fitting Teflon homogenizer. The homogenate was centrifuged at 48,000 *g* for 10 min at 4°, and aliquots of the resuspended pellet in Tris buffer (200–400 μg protein) were incubated at 25° for 1 hr in 1 ml of Tris buffer with [^{3}H]PN 200-110 (0.02 to 0.67 nM) in the absence and presence of 10^{-7} M nitrendipine to define nonspecific binding. Incubations were terminated by vacuum filtration through Whatman GF/B glass fiber filters, and the filters were rinsed three times with 4 ml of ice-cold buffer A. Protein content was determined by the method of Lowry *et al.* [12] for the uptake studies, and by the method of Bradford [13] for the binding studies.

Results and discussion

The *in vitro* addition of ethanol caused a dose-dependent inhibition of first phase calcium uptake with an IC_{50} of 220 ± 22.1 mM (Fig. 1 left). A similar dose-related inhibition of calcium uptake was observed with the PC12 cells; 800 mM ethanol completely abolished calcium uptake into PC12 cells and the IC_{50} value was 238 ± 25.2 mM (N = 4). The effects of *in vitro* ethanol on the slow second phase of synaptosomal calcium uptake were determined after inactivating the first phase by predepolarization [6]. Although ethanol elicited a dose-dependent reduction in net $^{45}Ca^{2+}$ uptake, significant inhibition was only observed with ethanol concentrations of 400 mM and above (Fig. 1 right). In addition, 800 mM ethanol only caused a 61% inhibition of the second phase. The inhibition of calcium uptake by ethanol involved an effect on potassium-stimulated, and not on resting, uptake.

The direct effects of chronic ethanol exposure on neuronal calcium uptake were investigated by growing PC12 cells for 4 days in the presence of medium containing 150 mM ethanol. The ethanol exposure increased both net calcium uptake (0.85 ± 0.04 and 1.22 ± 0.04 nmol $^{45}Ca^{2+}$/mg in control and ethanol-treated cells respectively, N = 5; $P < 0.001$) and sensitivity to *in vitro* ethanol (47 ± 2.8 and 69 ± 4.1% inhibition in control and chronic ethanol-treated cells, respectively, in the presence of 250 mM ethanol, N = 5; $P < 0.05$). The 4-day exposure to ethanol also increased the density of [^{3}H]PN 200-110 binding sites (B_{max}

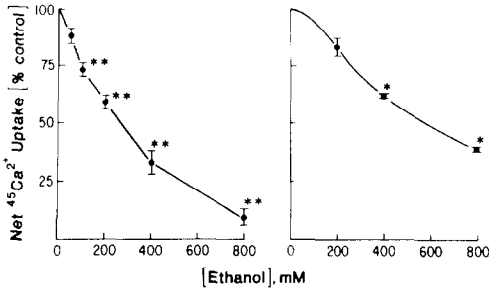

Fig. 1. Effects of *in vitro* ethanol on the first (left) and second (right) phase of net $^{45}Ca^{2+}$ uptake into synaptosomes. Each point represents the mean ± SEM of three to five separate experiments. Uptake in the absence of ethanol (i.e. control) was 2.74 ± 0.42 and 2.97 ± 0.03 nmol/mg protein for first phase and second phase respectively. Key: (*) $P < 0.05$, and (**) $P < 0.01$ compared to control (Dunnett's test for multiple comparisons).

was 63.8 ± 8.23 and 90.1 ± 10.46 fmol/mg protein for control and ethanol-treated cells respectively; $P < 0.05$, paired t-test) without a change in receptor affinity (K_D was 178 ± 16.2 and 200 ± 33.6 pM for control and ethanol-treated cells respectively).

The *in vitro* addition of ethanol inhibits net synaptosomal $^{45}Ca^{2+}$ uptake [2–5]. Of the two phases of synaptosomal calcium uptake, the first phase was more sensitive to ethanol. The *in vitro* addition of 200 mM ethanol significantly inhibited first phase uptake 41%, but only caused a non-significant (17%) reduction in second phase uptake. In addition, 800 mM ethanol abolished first phase uptake, while only causing a 61% inhibition of the second phase. Since the first phase of potassium-stimulated $^{45}Ca^{2+}$ uptake represents uptake through voltage-dependent calcium channels [6] and the second phase represents Na^+/Ca^{2+} exchange [7], the data indicate that the calcium channel is the primary site of action of ethanol on calcium uptake.

The importance of the calcium channel in the actions of ethanol is further indicated by the response of PC12 cells to chronic ethanol administration. In confirmation of a recent report [14], which appeared during preparation of this manuscript, exposure of PC12 cells to ethanol resulted in an increase in net $^{45}Ca^{2+}$ uptake that was due to an increase in the number of voltage-dependent calcium channels per cell as indicated by the increase in the density of binding sites for the calcium channel ligand [^{3}H]PN 200-110. Although Messing *et al.* [14] observed no difference in the potency of *in vitro* ethanol after chronic ethanol treatment, in the present study chronic exposure of PC12 cells to ethanol increased the sensitivity of the voltage-dependent calcium channels to *in vitro* ethanol. A similar increase in sensitivity of adenylate cyclase to *in vitro* ethanol was also observed in PC12 cells chronically exposed to ethanol (R. A. Rabin, manuscript submitted). The increased sensitivity was not due to residual ethanol from the chronic treatment since the inhibitory effects of *in vitro* ethanol on $^{45}Ca^{2+}$ were readily reversible by rinsing the cells (data not shown). The reason for this discrepancy is unclear but may be due to methodological differences or the use of different subclones of the PC12 cells especially since, in contrast to Messing *et al.*, we are unable to maintain viable cells in 200 mM ethanol.

The response of PC12 cells to chronic ethanol appears to be at odds with results obtained using animals. Although an increase in net calcium uptake after chronic ethanol administration was observed in liver microsomes [15], studies with synaptosomes showed either a decrease in net $^{45}Ca^{2+}$ uptake [2] or no change [4]. Results with PC12 cells represent the direct effects of ethanol, whereas ethanol has a wide variety of actions *in vivo* including changes in hormonal balance and systemic metabolism. Thus, the *in vivo* effects of chronic ethnaol exposure on calcium uptake may not be due to a direct neuronal action of ethanol, but rather the direct effects of ethanol on calcium uptake appear to be overridden by the other changes associated with ethanol administration.

In summary, the present results indicate that ethanol acts primarily on the voltage-dependent calcium channel, and that the direct neuronal effect of chronic ethanol exposure is an increase in both calcium channel density and sensitivity to *in vitro* ethanol.

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Catecholamine-metabolizing enzyme activity in the nigrostriatal system

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The nigrostriatal system is a well-defined model for the central catecholaminergic pathway [1]. This system has been studied extensively with respect to the synthesis, release, action, and degradation of dopamine. Lesion of either the presynaptic or postsynaptic elements has been employed to localize various enzymes related to catecholamine metabolism.

With respect to monoamine oxidase (MAO), several studies have shown that the high striatal activity of this enzyme remains unaffected after damage to the presynaptic