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EFFECTS OF ETHANOL ON CALCIUM TRANSPORT BY MICROSOMES PHOSPHORYLATED BY CYCLIC AMP-DEPENDENT PROTEIN KINASE*

JEFFREY N. RETIG, MADELEINE A. KIRCHBERGER,† EMANUEL RUBIN‡ and ARNOLD M. KATZ§

Division of Cardiology, Department of Medicine, and the Departments of Physiology and Biophysics, and Pathology, The Mount Sinai School of Medicine of the City University of New York, NY 10029, U.S.A.

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Abstract—The sensitivity of cardiac sarcoplasmic reticulum to the inhibitory effects of ethanol was significantly increased after phosphorylation of these membranes by cyclic AMP-dependent protein kinase. Ethanol concentrations needed to inhibit oxalate-supported calcium uptake were reduced approximately 4-fold after phosphorylation. Ethanol decreased the extent of stimulation of calcium uptake by cyclic AMP-dependent protein kinase, but protein kinase-catalyzed phosphorylation of these membranes was not affected by concentrations of ethanol used in these studies. These findings suggest that ethanol interferes with the ability of protein kinase-dependent phosphoprotein to stimulate the calcium pump of the sarcoplasmic reticulum.

Ethanol exerts a negative inotropic effect on the mammalian heart that is manifest as a decrease in the rate of tension development (dP/dt) and peak tension [1–3]. Such a depression of contractility may be due, in part, to a decrease in the amount of calcium available for release to the contractile proteins [4]. The role of the sarcoplasmic reticulum in regulating the availability of calcium for binding to the cardiac contractile proteins raises the possibility that ethanol may interfere with the ability of this intracellular membrane system to transport calcium. Studies of sarcoplasmic reticulum from the hearts of dogs which ingested large amounts of ethanol for several months have demonstrated significant impairment of calcium transport [5, 6]. Previous studies in our laboratory have shown that ethanol can inhibit calcium transport by sarcoplasmic reticulum from normal dog hearts, although significant effects were seen only at high ethanol concentrations [7]. The present study was undertaken in light of recent findings that calcium transport by the cardiac sarcoplasmic reticulum is stimulated when a 22,000 dalton protein component of these membranes (phospholamban) is phosphorylated by cyclic AMP-dependent protein kinase [8–12]. Evidence is presented that indicates that protein kinase-stimulated calcium transport by cardiac sarcoplasmic reticulum is more sensitive to ethanol than is basal calcium transport. The increased ethanol sensitivity of phosphorylated sarcoplasmic reticulum may reflect interference with the action of the phosphoprotein on the calcium pump.

METHODS

Canine cardiac microsomes enriched in sarcoplasmic reticulum were prepared by the method of Harigaya and Schwartz [13], modified in that sodium azide was omitted and homogenization was carried out in a Waring blendor. Microsomes were used on the same day as prepared. Protein concentration was measured by the biuret method with bovine serum albumin as standard.

Protein kinase was obtained from frozen bovine hearts and purified through the DEAE-cellulose chromatography step by the method of Miyamoto *et al.* [14]. The fractions containing protein kinase activity were dialyzed against 5 mM histidine–HCl buffer, pH 6.8, and stored in small aliquots at −12° prior to use. The specific activity of the enzyme, ranging from 1.4 to 5.9 nmoles P_i/mg of protein/min, was determined as described previously [9].

For measurements of calcium uptake, cardiac microsomes (0.02 mg/ml final concentration) were preincubated for 10 min at 25° in a partial reaction mixture consisting of 40 mM histidine–HCl buffer, pH 6.8, 120 mM KCl, 2.5 mM Tris–oxalate, and 5 mM MgATP. For measurements of protein kinase-stimulated calcium uptake, 0.1 mg/ml of protein kinase and 1 μM cyclic AMP were present during the 10-min preincubation prior to initiation of the calcium uptake reaction. Calcium uptake reactions were started by addition of a calcium–EGTA buffer (Ca^{2+} = 0.75 μM; $CaCl_2$ = 25 μM; EGTA = 84 μM)

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‡ Irene Heinz and John LaPorte Given Professor of Pathology.

§ Philip J. and Harriet L. Goodhart Professor of Medicine (Cardiology).

based on calculations described previously [15]. Samples were taken by the Millipore filtration method and analyzed by previously described methods [16].

The partial reaction mixture for the membrane phosphorylation assays consisted of 40 mM histidine–HCl buffer, pH 6.8, 120 mM KCl, 2.5 mM oxalate, calcium–EGTA buffer (Ca^{2+} = 0.75 μM; $CaCl_2$ = 1 mM; EGTA = 4.0 mM) and one or more of the following additions: 1 μM cyclic AMP plus varying concentrations of protein kinase, 1.0 M ethanol, or control solution. Cardiac microsomes (0.5 mg/ml final concentration) were added after a 10-min preincubation of the partial reaction mixture at 25°. The temperature equilibration was continued for an additional min, at which time the phosphorylation reactions were started by an addition of 5 mM Mg[γ-^{32}P]ATP (approximately 3.64 μCi/sample). The total sample volume was 0.2 ml. The reactions were stopped with a 10% (w/v) trichloroacetic acid solution and the resultant pellets were washed, centrifuged and counted, as described previously [9], except that the pellets were first washed twice with the trichloroacetic acid solution, then twice with 0.5 N NaOH, and finally twice more with trichloroacetic acid.

Ethanol was obtained from a constant boiling mixture of 95% ethanol in water. All studies on calcium uptake were performed with 1.0 M ethanol present in the preincubation medium except as otherwise noted.

Figures represent data from typical experiments. A minimum of three independent experiments was carried out in all cases except where indicated.

RESULTS

Effect of ethanol on microsomal calcium uptake. Ethanol inhibited oxalate-supported calcium uptake by cardiac microsomes that had previously been phosphorylated by a cyclic AMP-dependent protein kinase. As was found for control microsomes not exposed to the protein kinase [7], the inhibition by ethanol was time dependent and increased as the duration of exposure of the microsomes to ethanol increased (Fig. 1).

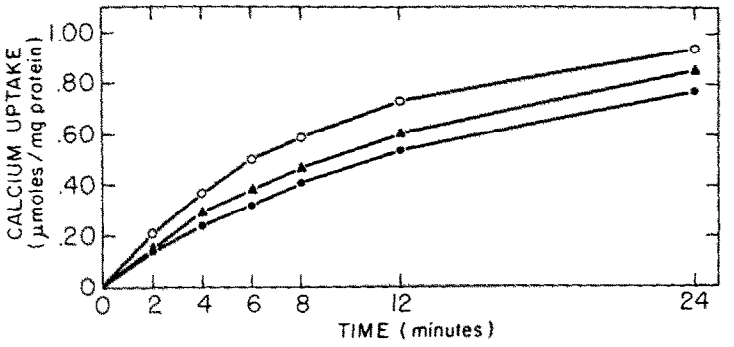

Fig. 1. Time dependence of ethanol-induced inhibition of protein kinase-stimulated calcium uptake. Microsomes were preincubated with protein kinase (0.15 mg/ml) and cyclic AMP (0.2 μM) for 10 min. During this preincubation period 1 M ethanol was present for 10 min (●), for only the last 25 sec (▲), or absent (○). Immediately after the 10-min preincubation, the calcium uptake reaction was started by addition of a calcium–EGTA buffer. Details of reaction conditions are described under Methods. Four independent experiments were performed; a typical experiment is shown.

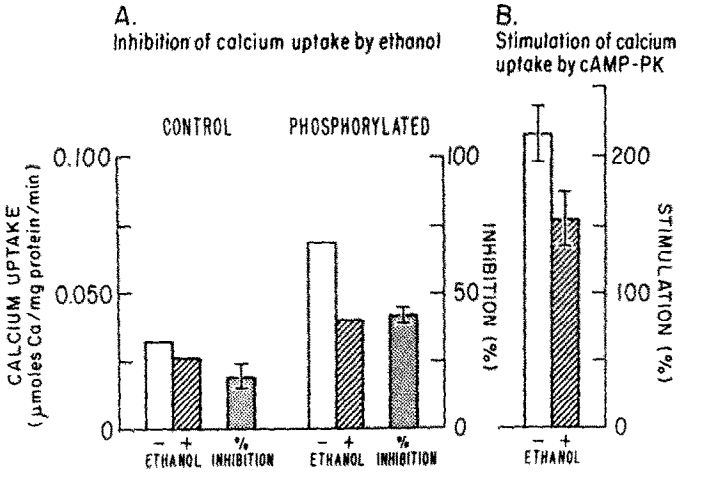

Fig. 2. Effect of a 10-min ethanol pretreatment on calcium uptake by control and phosphorylated cardiac microsomes (A) and on the stimulation of calcium uptake by cyclic AMP-dependent protein kinase (B). The per cent inhibition (A) and stimulation (B) are shown ±S. E. calculated from paired samples in four independent experiments.

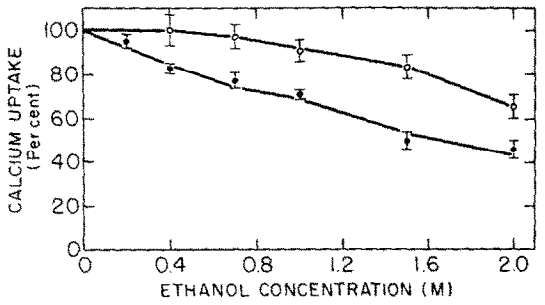

Fig. 3. Concentration dependence of ethanol-induced inhibition of control (○) and protein kinase-stimulated (●) calcium uptake by cardiac microsomes in 120 mM NaCl instead of 120 mM KCl. Other reaction conditions are described under Methods. Shown for each concentration of ethanol are averages of four independent experiments ± S. E.

The inhibitory effects of 1 M ethanol were significantly increased in microsomes previously exposed to the cyclic AMP-dependent protein kinase (Fig. 2A). Thus, ethanol reduced the extent to which the protein kinase stimulated calcium uptake velocity (Fig. 2B). Furthermore, a 4-fold enhancement in the sensitivity of protein kinase-treated microsomes to the inhibitory effects of ethanol on calcium transport was observed (Fig. 3). For example, 0.4 M ethanol caused 17 ± 2 per cent inhibition of calcium uptake in microsomes exposed to the protein kinase whereas 1.5 M ethanol was needed to produce 16 ± 5 per cent inhibition of control microsomes. The inhibitory effects of 0.4 M ethanol on calcium uptake by protein kinase-treated microsomes were highly significant ($P < 0.001$).

Effects of ethanol on membrane phosphorylation. To examine the possibility that ethanol sensitivity of cardiac microsomes previously exposed to the cyclic AMP-dependent protein kinase resulted from an effect of ethanol to inhibit protein kinase-catalyzed phosphorylation, the effects of ethanol on phosphorylation of cardiac microsomes were measured. Over a period of 20 min, 1 M ethanol caused no significant inhibition of phosphorylation catalyzed by either added or intrinsic [9, 17] protein kinase (Fig. 4). The possibility that an inhibitory effect of ethanol was masked by the use of saturating levels of protein kinase could be excluded by similar negative results obtained in experiments carried out at protein kinase concentrations well below those which catalyzed maximal phosphorylation (Table 1).

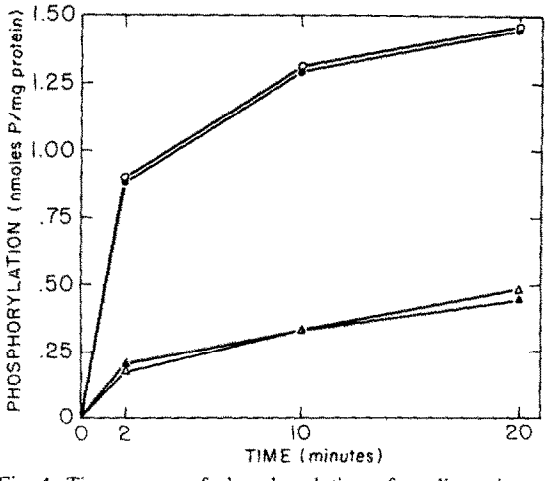

Fig. 4. Time course of phosphorylation of cardiac microsomes catalyzed by intrinsic (△, ▲) and 0.25 mg/ml added (○, ●) protein kinase and 1 μM cyclic AMP. Assays were carried out with 1 M ethanol (open symbols) or in its absence (closed symbols) under conditions described in Methods.

Table 1. Effect of ethanol on phosphorylation of cardiac microsomes*

	Phosphorylation (nmoles P/mg/2 min)
Control	0.18 ± 0.01
Control + ethanol (1 M)	0.23 ± 0.03
Protein kinase (25 μg/ml) + cyclic AMP (1 μM)	0.52 ± 0.01
Protein kinase (25 μg/ml) + cyclic AMP (1 μM) + ethanol (1 M)	0.53 ± 0.02
Protein kinase (100 μg/ml) + cyclic AMP (1 μM)	0.89 ± 0.02

* Effects of ethanol on phosphorylation were measured in control microsomes in the presence of low and high concentrations of protein kinase plus cyclic AMP. Assays were carried out as described in the legend to Fig. 4 except that the oxalate and calcium–EGTA buffers were omitted. Reactions were stopped with 10% trichloroacetic acid after a 2-min incubation. Data represent averages of triplicate determinations on a single preparation of microsomes ± S. E.

DISCUSSION

The present studies demonstrate that the sensitivity of cardiac microsomal calcium transport to ethanol is enhanced after exposure to a cyclic AMP-dependent protein kinase. Under the conditions employed in the present study, basal calcium transport was inhibited by 19 per cent by 1 M ethanol, a finding similar to that reported by Swartz *et al.* [7]. In contrast, protein kinase-stimulated calcium uptake was inhibited 42 per cent by the same concentration of ethanol (Fig. 2A) and phosphorylation of the microsomes was found to increase the sensitivity of calcium uptake to ethanol (Fig. 3). Because phosphorylation of the cardiac sarcoplasmic reticulum is closely correlated with the stimulation of calcium transport [9], the possibility that the greater ethanol sensitivity of protein kinase-stimulated calcium transport reflects inhibition of formation of this phosphoprotein was examined. No significant inhibition of membrane phosphorylation by 1 M ethanol was seen (Fig. 4). These findings suggest that ethanol interferes with the ability of the protein phosphorylated by the cyclic AMP-dependent protein kinase to stimulate the calcium pump of the cardiac sarcoplasmic reticulum. Evidence for such an effect is seen also at low ethanol concentrations where calcium transport by control microsomes is unaffected while that of phosphorylated microsomes is inhibited significantly.

The concentrations of ethanol necessary to produce inhibition of both control and protein kinase-stimulated calcium transport are above the lower level of

the lethal range of blood ethanol concentration in man. The relationship between the present findings and the impairment of calcium transport by sarcoplasmic reticulum from hearts of animals that have ingested ethanol for long periods [5, 6] is not clear.

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