Ethanol has different effects on Ca^{2+}-transport ATPases of muscle, brain and blood platelets

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The effects of ethanol on different sarco/endoplasmic reticulum Ca^{2+}-transport ATPases (SERCAs) were studied. In sarcoplasmic reticulum vesicles, ethanol concentrations varying from 5 to 20% promoted a progressive inhibition of Ca^{2+} uptake, enhancement of Ca^{2+} efflux, activation of the ATPase activity, increase of the enzyme phosphorylation by ATP and inhibition of enzyme phosphorylation by P_i. The effects of ethanol on Ca^{2+} uptake and Ca^{2+} efflux were antagonized by Mg^{2+}, P_i and spermine. The increased efflux promoted by ethanol was antagonized by Ca^{2+} and thapsigargin. In brain and platelet vesicles a biphasic effect of ethanol was observed, so that activation occurred at low concentrations (5–10%) and inhibition at higher concentrations. The activation was not observed with the use of n-propanol and n-butanol. Different from the situation in sarcoplasmic reticulum, the decrease of the Ca^{2+} uptake in brain and platelet vesicles was associated with an inhibition of the ATPase activity. Mg^{2+} and P_i antagonized the enhancement of Ca^{2+} efflux and the inhibition of Ca^{2+} uptake promoted by ethanol. However, thapsigargin and Ca^{2+} did not arrest the Ca^{2+} efflux promoted by ethanol in brain and platelet preparations. These results suggest that, in sarcoplasmic reticulum vesicles, ethanol uncouples the pump, promoting its activity as a Ca^{2+} channel. The SERCA isoform found in skeletal muscle has different properties from the isoforms found in brain and blood platelets.

INTRODUCTION

Different sarco/endoplasmic reticulum isoforms of the Ca^{2+}-transport ATPases (SERCAs) are found in skeletal muscle ($SERCA_1$), brain ($SERCA_{2b}$) and blood platelets ($SERCA_{2b}$ and $SERCA_3$) [1–7]. These membrane-bound proteins are able to use the energy derived from the hydrolysis of ATP to pump Ca^{2+} from the cytosol into membranous intracellular compartments, thus ensuring the maintenance of the cytosolic Ca^{2+} concentration in the submicromolar range [8–10]. At present, the physiological implications of isoform diversity are not clear. The catalytic cycle of the sarcoplasmic reticulum Ca^{2+}-ATPase has been described and discussed in previous reports [11–15].

Efflux measurements with sarcoplasmic reticulum vesicles previously loaded with Ca^{2+} have shown that Ca^{2+} leaks through the ATPase when the vesicles are incubated in a medium containing none of the enzyme ligands [14–17]. The rate of leakage is greatly increased by hydrophobic drugs such as phenothiazines, local anaesthetics, fatty acids and by heparin [18–23]. Thus, the Ca^{2+}-ATPase can operate either as a pump or as a Ca^{2+} channel. Leakage through this channel seems to be mediated by the dephosphoenzyme form E_2. A common feature of the drugs that increase Ca^{2+} efflux is that they act as competitive inhibitors of the phosphorylation of the enzyme by P_i [18,20,24,25]. The leakage is arrested when the concentrations of P_i and Mg^{2+} in the medium are increased so as to decrease the level of free E_2 by forming the phosphoenzyme E_2-P. The leakage of Ca^{2+} promoted by hydrophobic drugs is also antagonized by the addition of Ca^{2+} to the efflux medium so as to convert the enzyme form E_2 into $2Ca:E_1$. Additional antagonists are spermine, a polyamine, and thapsigargin, a specific inhibitor of the SERCAs [18–21].

Earlier reports [26–31] have shown that ethanol inhibits the Ca^{2+} uptake of sarcoplasmic reticulum vesicles without inhibiting the hydrolysis of ATP. The decrease of uptake was a result of leakage from the vesicles and was thought to be promoted by changes in the lipid structure leading to an increase in membrane permeability, rather than by a direct effect of alcohol on the ATPase.

In this report it is shown that, in contrast to what is observed with sarcoplasmic reticulum, low ethanol concentrations increase the uptake of Ca^{2+} in brain ($SERCA_{2b}$) and blood platelet ($SERCA_{2b}$ and $SERCA_3$) vesicle preparations. Evidence is presented that in sarcoplasmic reticulum vesicles ($SERCA_1$) the Ca^{2+} efflux promoted by ethanol is mediated by the ATPase.

MATERIALS AND METHODS

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle as described by Eletr and Inesi [32] and stored in liquid nitrogen. Protein concentration was determined according to Lowry et al. [33]. Vesicles derived from the dense tubular system of blood platelets were prepared as described by Le Peuch et al. [34]. Plasma enriched with platelets was obtained from a blood bank. The vesicles were resuspended in 20 mM Hepes buffer, pH 7.5, containing 5 mM $MgCl_2$, 50 μM $CaCl_2$, 0.5 mM sodium azide, 0.1 mM PMSF and 20% (v/v) glycerol, and stored in liquid nitrogen until required for use. Each sample was used only once. The Ca^{2+} uptake and Ca^{2+}-ATPase activity of the preparation did not vary after 2 months of storage in liquid nitrogen. Brain microsomes were prepared using adult rat brains frozen immediately after dissection. Brains (30 g) were homogenized in 200 ml of a medium containing 0.32 M sucrose, 10 mM Mops/Tris, pH 7.4, and 0.2 mM PMSF. The homogenate was centrifuged at 47000 *g* for 15 min. The pellet was discarded and the supernatant was centrifuged at 120000 *g* for 40 min to

Abbreviations used: SERCAs, sarco/endoplasmic reticulum Ca^{2+}-transport ATPases.

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yield the microsomal pellet. The pellet was resuspended and recentrifuged (120000 *g* for 40 min). The final pellet was resuspended in 0.32 M sucrose/10 mM Mops/Tris (pH 7.4) and frozen in liquid nitrogen.

[^{32}P]P_i was obtained from the Brazilian Institute of Atomic Energy and purified as previously described [35]. ATPase activity was assayed by measuring the release of P_i either colorimetrically [36] or using [γ-^{32}P]ATP [35]. Calcium uptake and efflux were measured at 35 °C by a filtration method using ^{45}Ca and Millipore filters [37]. After filtration, the filters were washed five times with 5 ml samples of 3 mM $La(NO_3)_3$, and the radioactivity remaining on the filter was counted in a scintillation counter.

For the Ca^{2+} efflux experiments, the vesicles were preloaded with ^{45}Ca in a medium containing 50 mM Mops/Tris, pH 7.0, 10 mM $MgCl_2$, 20 mM P_i, 0.3 mM $CaCl_2$, 3 mM ATP and 0.06 mg/ml of vesicle protein. After 30 min incubation at 35 °C, the loaded vesicles were sedimented by centrifugation at 30000 *g* for 30 min, the supernatant was discarded, and the walls of the tubes were blotted to minimize contamination by the residual loading medium. The pellet was kept on ice and resuspended in water immediately before use. All experiments were performed at pH 7.0 and at 35 °C.

Phosphorylation of the Ca^{2+}-ATPase by either [^{32}P]P_i or [γ-^{32}P]ATP was determined using Millipore filters and corrected for non-specific binding [35].

The experiments shown in the Figures were repeated at least three times.

RESULTS AND DISCUSSION

Effect of alcohols on Ca^{2+} uptake

Vesicles derived from the sarcoplasmic reticulum accumulate a larger amount of Ca^{2+} than vesicles derived from either brain or blood platelets [22,38]. The effect of ethanol on Ca^{2+} uptake was found to vary depending on the vesicle preparation used (Figure 1). In agreement with previous reports [26–31], it was found that the Ca^{2+} uptake measured with sarcoplasmic reticulum vesicles was progressively inhibited as the ethanol concentration in the medium was raised from 5 to 20%. Concentrations of ethanol varying from 1 to 4% failed either to activate or to inhibit the transport of Ca^{2+} (results not shown). In brain and platelet vesicles, a biphasic effect was observed with an activation in the presence of 5–10% ethanol, followed by an inhibition as the ethanol concentration was raised above 15% (Figure 1). The activation was not observed with the use of n-propanol or n-butanol. These two alcohols inhibited Ca^{2+} uptake in all three vesicle preparations, and the concentrations needed for inhibition decreased 5-fold (propanol) and 30-fold (butanol) as the length of the carbon chain increased. The concentrations of ethanol (Figure 1), n-propanol and n-butanol (results not shown) needed to inhibit sarcoplasmic reticulum vesicles were three times lower than the concentration needed to inhibit brain or blood platelet vesicles (Figure 1).

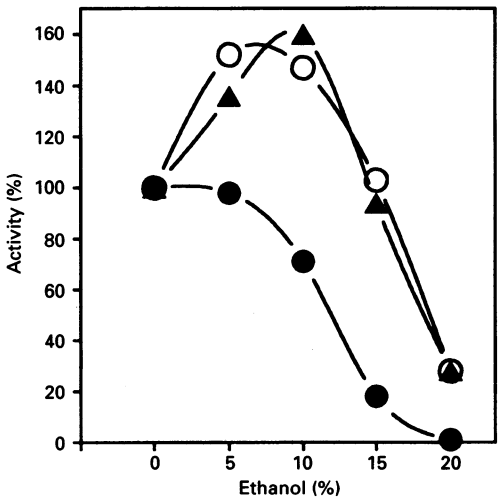

Figure 1 Inhibition of Ca^{2+} uptake by ethanol

For the experiments performed with the sarcoplasmic reticulum vesicles, the assay medium composition was 50 mM Mops/Tris buffer (pH 7.0), 2 mM $MgCl_2$, 2 mM ATP, 0.1 mM $^{45}CaCl_2$, 10 mM P_i and 0.040 mg/ml protein. For the experiments with vesicles of brain and blood platelets, the conditions were the same except that the concentrations of $MgCl_2$, P_i and vesicles used were 5 mM, 20 mM and 60 μg/ml respectively. The reaction time was 10 min and the temperature of the medium was 35 °C. ○, Brain microsomes; ▲, platelet vesicles; and ●, sarcoplasmic reticulum vesicles. In the Figure, 100% activity corresponds to 60 (○), 73 (▲) and 1900 (●) nmol of Ca^{2+}/mg per 10 min.

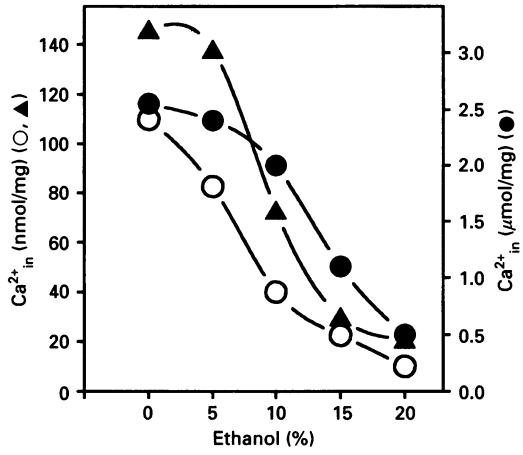

Figure 2 Stimulation of Ca^{2+} efflux by ethanol

Brain microsomes (○), platelet vesicles (▲) or sarcoplasmic reticulum vesicles (●) were loaded with Ca^{2+} as described in the Materials and methods section and diluted in a medium containing 50 mM Mops/Tris buffer (pH 7.0) and 0.5 mM EGTA. After either 20 min (○), 5 min (▲) or 20 s (●) incubation at 35 °C, samples of the efflux mixture were filtered using Millipore filters. The values in the Figure represent the amount of Ca^{2+} remaining in the vesicles.

Ca^{2+} efflux

Ethanol increased the rate of Ca^{2+} efflux in all three vesicle preparations (Figure 2). This increase did not seem to be promoted by a non-specific effect of ethanol as an organic solvent that solubilizes lipids, since there was no increase in Ca^{2+} efflux from sarcoplasmic reticulum vesicles when 60% of the water of the medium was replaced by DMSO (Figure 3), a non-denaturing solvent.

Role of Mg^{2+} and P_i in the rates of Ca^{2+} uptake and Ca^{2+} efflux

The amount of Ca^{2+} accumulated by the vesicles is determined by the ratio between the rates of Ca^{2+} influx and Ca^{2+} efflux. In the absence of ethanol, maximal accumulation of Ca^{2+} with the three vesicle preparations was obtained with $MgCl_2$ concentrations between 2 and 4 mM. For the sarcoplasmic reticulum Ca^{2+}-ATPase the effect of ethanol on both the amount of Ca^{2+} accumulated by the vesicles and the rate of Ca^{2+} efflux was

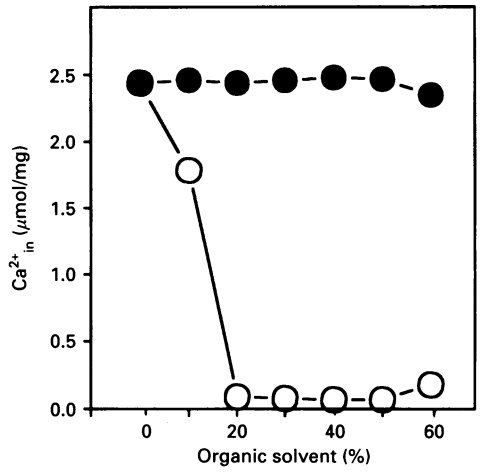

Figure 3 Effects of ethanol (○) and DMSO (●) on the rate of Ca^{2+} efflux

The composition of the efflux medium was 50 mM Mops/Tris buffer (pH 7.0), 0.1 mM P_i, 0.1 mM $MgCl_2$ and 0.5 mM EGTA. The reaction was started by the addition of sarcoplasmic reticulum vesicles (0.06 mg/ml) previously loaded with ^{45}Ca. The values in the Figure represent the amount of Ca^{2+} remaining in the vesicles after 2 min incubation at 35 °C.

antagonized by raising the concentration of Mg^{2+} and P_i (Figures 4 and 5). In brain and platelet vesicles, the activation of Ca^{2+} uptake promoted by ethanol did not depend on the Mg^{2+} or P_i concentrations (results not shown) in the medium. However, in these preparations both the enhancement of Ca^{2+} efflux, measured with different ethanol concentrations (Figure 6), and the inhibition of Ca^{2+} uptake observed with 15 and 20% ethanol were also antagonized by raising the concentration of Mg^{2+} and P_i.

Spermine, Ca^{2+} and thapsigargin

These substances antagonized the effect of ethanol on the sarcoplasmic reticulum Ca^{2+} pump, but had no effect on brain or

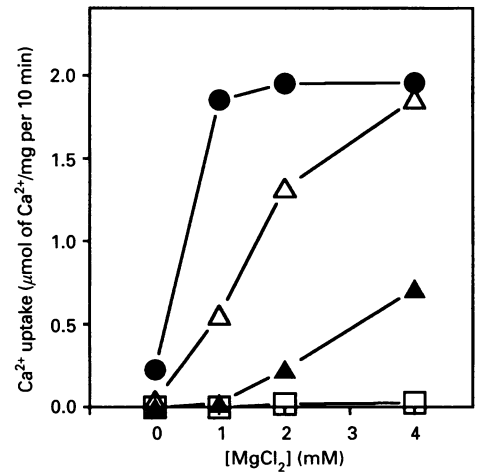

Figure 4 Antagonism by Mg^{2+} of the ethanol effect on sarcoplasmic reticulum Ca^{2+} uptake

The experimental conditions were as described in Figure 1, except that the concentration of $MgCl_2$ in the medium varied as shown in the abscissa. ●, Without ethanol; △, 10% ethanol; ▲, 15% ethanol; □, 20% ethanol.

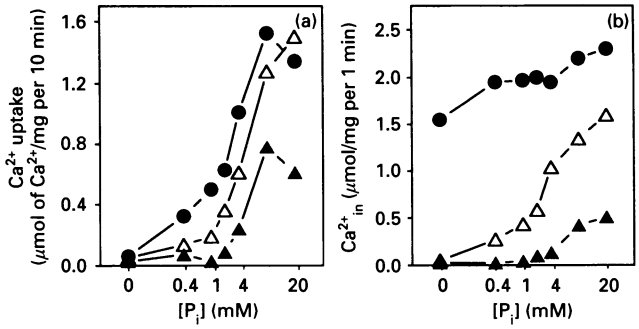

Figure 5 Antagonism by P_i of the ethanol effect on sarcoplasmic reticulum Ca^{2+} uptake (a) and Ca^{2+} efflux (b)

Uptake and efflux were measured as described in Figures 1 and 2, except that the concentration of P_i in the medium varied as shown in the Figure. ●, Without ethanol; △, 10% ethanol; or ▲, 15% ethanol.

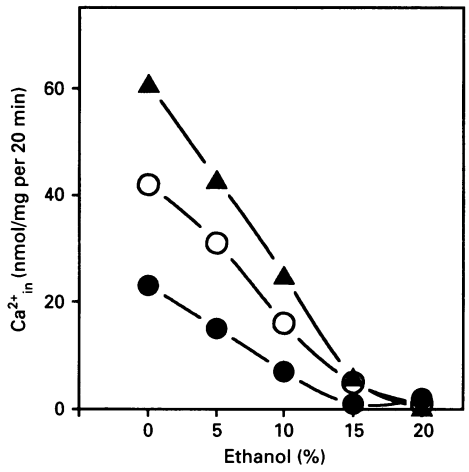

Figure 6 Brain microsomes: antagonism by Mg^{2+} of the ethanol effect on Ca^{2+} efflux

The efflux medium composition was 50 mM Mops/Tris buffer (pH 7.0), 0.5 mM EGTA and either no Mg^{2+} (●), 1 mM $MgCl_2$ (○), or 10 mM $MgCl_2$ (▲).

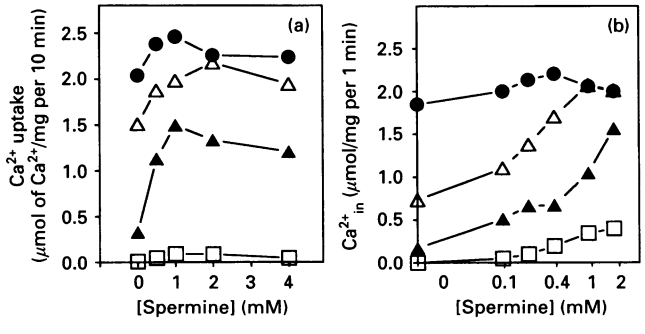

Figure 7 Antagonism of the ethanol effect by spermine in sarcoplasmic reticulum vesicles

(**a**) Ca^{2+} uptake: the assay medium composition was 50 mM Mops/Tris buffer (pH 7.0), 2 mM $MgCl_2$, 2 mM ATP, 0.1 mM ^{45}Ca, 10 mM P_i and 0.04 mg of vesicle protein. (**b**) ^{45}Ca efflux: the assay medium composition was 50 mM Mops/Tris buffer (pH 7.0) plus 0.5 mM EGTA. ●, Without ethanol; △, 10% ethanol; ▲, 15% ethanol; □, 20% ethanol.

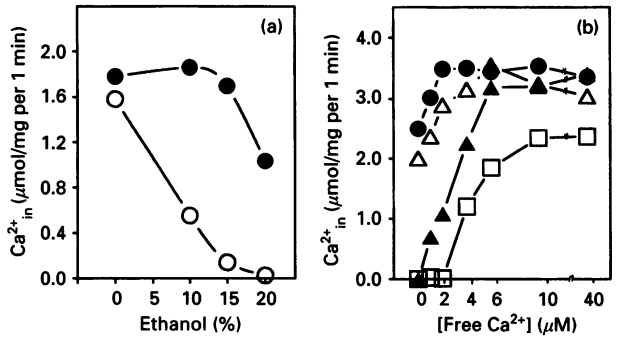

Figure 8 Antagonism of the ethanol effect on Ca^{2+} efflux by Ca^{2+} in sarcoplasmic reticulum vesicles

In (**a**) the efflux medium was 50 mM Mops/Tris buffer (pH 7.0) and either 0.5 mM EGTA (○) or 50 μM $CaCl_2$ (●). In (**b**) the efflux medium composition was 50 mM Mops/Tris buffer (pH 7.0), 0.5 mM EGTA and $CaCl_2$ concentrations varying from 0.10 to 0.50 mM to yield the free Ca^{2+} concentrations shown on the abscissa. These were calculated using a computer program, originally developed by Fabiato and Fabiato [39] and modified by Sorenson [40]. The apparent association constants for Ca/EGTA were provided by Schwartzenbach [41]. ●, Without ethanol; △, 10% ethanol; ▲, 15% ethanol; □, 20% ethanol.

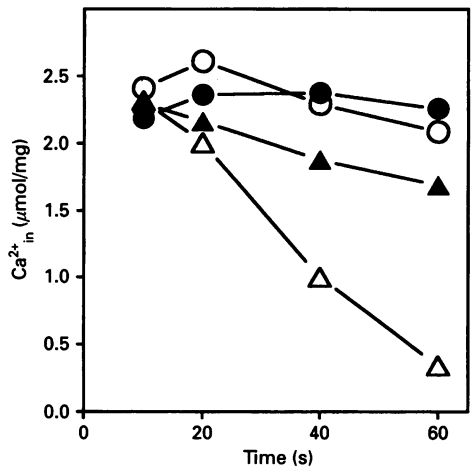

Figure 9 Antagonism of the ethanol effect on Ca^{2+} efflux by thapsigargin in sarcoplasmic reticulum vesicles

The efflux medium composition was 50 mM Mops/Tris buffer (pH 7.0), 0.5 mM EGTA and either no ethanol (○, ●) or 10% ethanol (△, ▲). Open symbols, without thapsigargin; closed symbols, with 1 μM thapsigargin.

platelet vesicles. For the sarcoplasmic reticulum vesicles, spermine antagonized both the decrease in Ca^{2+} uptake and the enhancement of Ca^{2+} efflux promoted by ethanol (Figure 7). In agreement with previous reports [17,18], the slow leakage of Ca^{2+}, observed when vesicles previously loaded with Ca^{2+} were incubated in a medium containing only buffer and EGTA, was decreased when the Ca^{2+} concentration in the efflux medium was raised to 2 μM (Figure 8b). The fast efflux promoted by ethanol was also arrested by Ca^{2+} (Figure 8), but the concentration needed to stop the leakage increased as more ethanol was added to the medium. With 20% ethanol, Ca^{2+} concentrations up to 40 μM decreased, but did not completely block, the leakage of Ca^{2+} from the vesicles (Figure 8). Similar results were observed with thapsigargin, a specific inhibitor of the SERCAs (Figure 9). The enhancement of Ca^{2+} efflux promoted by ethanol in brain

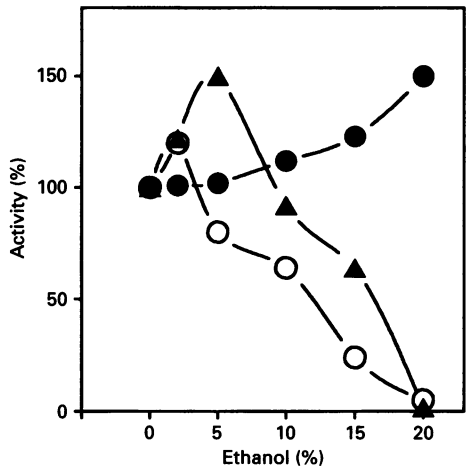

Figure 10 Effect of ethanol on the ATPase activity of sarcoplasmic reticulum (●), brain (○) and platelet vesicles (▲)

The assay medium composition was 50 mM Mops/Tris (pH 7.0), 0.1 mM $CaCl_2$, 5 mM $MgCl_2$, 2 mM ATP and 10 mM P_i. The reaction was started by the addition of protein: 0.025 mg/ml (sarcoplasmic reticulum), 0.05 mg/ml (brain) or 0.1 mg/ml (platelet vesicles). The reaction time at 35 °C was 10 min.

and platelet vesicles was not decreased by the addition to the efflux medium of either 1–6 mM spermine or 100 μM Ca^{2+}. Thapsigargin (1–5 μM) inhibited Ca^{2+} uptake in both brain and platelet vesicles, but did not alter the efflux of Ca^{2+} promoted by ethanol (results not shown).

ATP hydrolysis and enzyme phosphorylation

The effect of ethanol on the rate of ATP hydrolysis was found to vary depending on the vesicle preparation used. For the sarcoplasmic reticulum, an increase in the ATPase activity was observed as the ethanol concentration was raised from 5 to 20% (Figure 10). In brain and blood platelet vesicles, there was an initial activation followed by an inhibition of the ATPase activity. The phosphorylation experiments were performed only with the Ca^{2+}-ATPase of the sarcoplasmic reticulum. We were unable to obtain reproducible results with brain and blood platelet vesicles. The Ca^{2+}-ATPase of the sarcoplasmic reticulum can be phosphorylated by either ATP or P_i. In both reactions, an acylphosphate residue is formed at the catalytic site of the enzyme [8,10–13]. Phosphorylation by ATP was measured at 35 °C in the presence of 50 mM Mops/Tris buffer (pH 7.0), 2 mM $MgCl_2$, 1 mM P_i/Tris, 0.1 mM $CaCl_2$ and 0.2 mM [γ-^{32}P]ATP. For the phosphorylation by P_i, the assay medium composition was 50 mM Mops/Tris buffer (pH 6.2), 2 mM EGTA, 4 mM $MgCl_2$ and 4 mM [^{32}P]P_i. Ethanol (20%) increased the phosphorylation of the Ca^{2+}-ATPase by ATP from 2.1 to 3.6 and decreased the phosphorylation by P_i from 1.5 to 0.7 nmol of E ~ P/mg of protein. These values are the average of three experiments.

In conclusion, the data presented show that the Ca^{2+}-ATPase isoform found in skeletal muscle has different properties from the isoforms found in brain and blood platelets. The differences noted were: (i) low ethanol concentrations activated Ca^{2+} uptake in brain and platelet vesicles but not in sarcoplasmic reticulum vesicles; (ii) at high ethanol concentrations, the decrease in Ca^{2+} uptake by brain and platelet vesicles was associated with inhibition of the ATPase activity, whereas for the sarcoplasmic reticulum it was associated with enhancement of the ATPase

activity; (iii) spermine, Ca^{2+} and thapsigargin opposed the leakage of Ca^{2+} from sarcoplasmic reticulum vesicles but had no effect on the Ca^{2+} efflux from brain or platelet vesicles. Finally, ethanol seems to uncouple the sarcoplasmic reticulum pump by the same mechanism previously described for the phenothiazines and other hydrophobic drugs [18–22]. This involves a modification of the catalytic cycle of the transport ATPase and not an increase of the membrane permeability as previously proposed [26–31].

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