Phosphatidylethanol stimulates the plasma-membrane calcium pump from human erythrocytes

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Phosphatidylethanol is formed by 'transphosphatidylation' of phospholipids with ethanol catalysed by phospholipase D and can be accumulated in the plasma membrane of mammalian cells after treatment of animals with ethanol. In the present work we show that phosphatidylalcohols, such as phosphatidylethanol and phosphatidylbutanol, produced a twofold stimulation of the Ca^{2+}-ATPase activity of human erythrocytes. This stimulation occurs with the purified, solubilized enzyme as well as with ghost preparations, where the enzyme is in its natural lipidic environment and is different to that obtained with other acidic phospholipids such as phosphatidylserine. Addition of either phosphatidylserine, phosphatidylethanol or phosphatidylbutanol to the purified Ca^{2+}-ATPase, or to ghosts preparations, increased the affinity of the enzyme for Ca^{2+} and the maximal velocity of the reaction as compared with controls in the absence of acidic phospholipids. However, in contrast with what occurs with phosphatidylserine, simultaneous addition of phosphatidylalcohols and calmodulin increased the affinity of the enzyme for Ca^{2+} to a greater extent than each added separately. When ethanol was added to either the purified erythrocyte Ca^{2+}-ATPase or to erythrocyte-ghost preparations in the presence of acidic phospholipids, an additive effect was observed. There was an increase in the affinity for Ca^{2+} and in the maximal velocity of the reaction, well above the values obtained with ethanol or with the acidic phospholipids tested separately. These findings could have pharmacological importance. It is conceivable that the decrease in the intracellular Ca^{2+} concentration that has been reported in erythrocytes as a result of ethanol intoxication could be due to the stimulation of the Ca^{2+}-ATPase by the accumulated phosphatidylethanol, to a direct effect of ethanol on the enzyme or to an additive combination of both.

INTRODUCTION

The existence of mammalian phospholipase D catalysing the hydrolysis of phosphatidylcholine to phosphatidic acid and choline was first detected in 1975 using a microsomal preparation from rat brain [1]. Subsequent studies have demonstrated phosphatidylcholine-preferring phospholipase D in homogenates and membranes from various tissues and cells, including lung, liver, adipose tissue, endothelial cells, erythrocytes and spermatozoa, with lung and brain being the richest sources [2–8]. Ethanol and several other short-chain primary alcohols (methanol, propan-1-ol, butan-1-ol, glycerol) can act as alternative substrate to water in the reaction, leading to formation of the corresponding phosphatidylalcohol, a process named 'transphosphatidylation' [9].

Once it is formed, the rate of degradation of phosphatidylethanol is slow compared with its rate of synthesis, even after ethanol is removed; hence the prolonged presence of ethanol can result in a significant accumulation of this phospholipid in some cells (up to 1–2 % of the total cellular phospholipid pool) [10]. There are reports of a significant accumulation of phosphatidylethanol in tissues of animals treated with ethanol *in vivo* [11,12]. Phosphatidylethanol accumulation has also been reported in lymphocytes from human alcoholics [13]. Even though accumulation of phosphatidylethanol after alcohol intoxication is well documented, the potential implication of this effect is not known. Phosphatidylethanol is a negatively charged phospholipid, and if it is generated from the neutral phosphatidylcholine, it may affect structural parameters of the phospholipid bilayer [14]. The consequences of phosphatidylethanol accumulation for the activity of membrane-bound enzymes are equally unknown. It has been reported that phosphatidylethanol prevents activation by ethanol of the Na^+/K^+-ATPase activity in a crude rat brain membrane fraction [14] and that a specific protein kinase C isoenzyme could be stimulated by phosphatidylethanol, which would bind to the phospholipid-binding site of the enzyme [15]. It is conceivable that some of the chronic toxic effects of ethanol are the consequence of an accumulation of phosphatidylethanol in specific membrane areas.

The plasma-membrane Ca^{2+}-ATPase is responsible for the maintenance of the intracellular Ca^{2+} concentration at the resting level [16,17]. The activity of this enzyme is highly regulated. Thus it can be stimulated by calmodulin, acidic phospholipids, polyunsaturated fatty acids [18] and phosphorylation by cAMP-dependent protein kinase [19] and by protein kinase C [20]. Besides, controlled proteolysis with trypsin [21–23] and other proteolytic enzymes [24] also stimulates the Ca^{2+}-ATPase. Hydrophobic interactions promoted by the presence of organic solvents as DMSO and polyalcohols (i.e. ethylene glycol) also mimics calmodulin [25,26]. Auto-aggregation of the enzyme [27] through a monomer $\rightleftharpoons$ dimer transition [28] is also translated in an increase of the ATPase activity. Interestingly, this enzyme has been shown to be stimulated by different alcohols [29]. The stimulation of this enzyme by ethanol is additive to that of calmodulin [29].

In the present study, using purified enzyme as well as membrane preparations, we show that the activity of the Ca^{2+}-ATPase is stimulated by phosphatidylethanol and phosphatidylbutanol,

Abbreviation used: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

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which have an additive effect with calmodulin on the stimulation of the affinity of the enzyme for calcium. The stimulatory effect observed is also additive to that obtained when the enzyme is stimulated by ethanol.

MATERIALS AND METHODS

Chemicals

All reagents were of the highest purity available. ATP, EGTA, NADH, phosphatidylcholine, dithiothreitol, pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate, phosphatidylserine (1,2-diacyl-*sn*-glycero-3-phospho-L-serine, from bovine brain) and calmodulin–Sepharose were from Sigma. Phosphatidylethanol (1,2-dimyristoyl-*sn*-glycero-3-phosphoethanol) and phosphatidylbutanol (1,2-dimyristoyl-*sn*-glycero-3-phosphoethanol) were from Avanti Polar Lipids, Inc., Alabaster, AL, U.S.A. All other reagents were analytical grade.

Purification of the erythrocyte Ca^{2+}-ATPase

Erythrocyte ghosts deficient in calmodulin were prepared as described by [18], from recently outdated human blood. Purified Ca^{2+}-ATPase was obtained using a calmodulin affinity column as described previously [23]. Routinely, 0.5–0.6 mg of ATPase were obtained from 500–600 mg of ghost protein. The purified ATPase was stored under N_2 at -70 °C at a concentration of 100–200 μg/ml, in a buffer containing 0.05 % Triton X-100, 130 mM KCl, 20 mM Hepes/KOH (pH 7.2), 2 mM EDTA, 2 mM $MgCl_2$, 50 μM $CaCl_2$, 2 mM dithiothreitol, 0.5 mg/ml phosphatidylcholine and 5 % glycerol (v/v). Bovine brain calmodulin was obtained as described by Guerini et al. [30].

Determination of ATPase activity

Aliquots of purified Ca^{2+}-ATPase (about 1–2 μg of protein/ml) were incubated in a medium containing 130 mM KCl, 20 mM Hepes/KOH, pH 7.2, 1 mM ATP, 1 mM $MgCl_2$, 1 mM EGTA and the appropriate concentrations of $CaCl_2$ to obtain the desired free calcium concentration. The final concentration of calcium ions was calculated by using an iterative computer program as described previously [31]. Since the rates of ATPase activity were linear over 45 min incubation at 37 °C, the reaction was arrested at 45 min by the addition of 8 % (final concn.) cold trichloroacetic acid. When ghost preparations were used, the mixture was centrifuged and the supernatant was kept for inorganic phosphate determination. The latter was carried out according the colorimetric method of Fiske and SubbaRow [32], modified by the use of $FeSO_4$ as reducing agent. When necessary, appropriate blanks were made to correct for the slight interference of ethanol or phospholipids with the colorimetric method. A coupled-enzyme assay system was used to measure the Ca^{2+}-ATPase activity during purification of the enzyme, as described previously [33]. The medium contained 10 μM free Ca^{2+}, 120 mM KCl, 30 mM Hepes/KOH, pH 7.4, 2.5 mM $MgCl_2$, 1 mM ATP, 0.2 mM NADH, 0.5 mM phosphoenolpyruvate, 1 unit of pyruvate kinase and 1 unit of lactic dehydrogenase, and the reaction was monitored at 37 °C in a final volume of 1 ml. The difference in absorbance between 366 and 550 nm was plotted versus time using a dual-wavelength spectrophotometer (SLM Aminco DW-2000). The phospholipids were microdispersed in 10 mM Hepes, pH 7.4, by sonication at 0 °C (Branson model B-30 sonifier, in the pulse mode, 50 %) under a stream of nitrogen (2–5 mg of phospholipid/ml).

Determination of the protein concentration

The protein concentration was determined by the method of Lowry et al. [34]. To avoid interferences with Triton X-100, the protein was precipitated by trichloroacetic acid in the presence of deoxycholate [35].

Analysis of the results

The different values of K_m and V_{max} were determined using Eadie–Hofstee plots and the computer program Enzfitter (version 1.03, Elsevier Biosoft). The values shown in the different Figures and Tables are, unless indicated, means $\pm$ S.D. for six independent experiments using different enzyme preparations. Statistical significance was determined using the StatGraphics Program, version 4.0, by variance analysis ($P < 0.01$).

RESULTS

Effect of acidic phospholipids on the activity of the purified Ca^{2+}-ATPase or on the Ca^{2+}-ATPase from erythrocyte ghosts

The Ca^{2+}-ATPase of the erythrocyte plasma membrane has been shown to be stimulated by acidic phospholipids such as phosphatidylserine [18]. Since phosphatidylethanol is also an acidic phospholipid, we investigated if addition of this phospholipid stimulated the activity of the purified enzyme or ghost preparations obtained from human erythrocytes. The effect of phosphatidylbutanol was also investigated. Figure 1 shows that, as reported previously [18], addition of phosphatidylserine induced a 2-fold stimulation of the enzyme at a phospholipid concentration of 50–100 μg/ml. At higher concentrations of phosphatidylserine, the activation of the enzyme became less evident. Comparable results were obtained after addition of phosphatidylethanol or phosphatidylbutanol to the purified, solubilized enzyme (Figure 1) or to erythrocyte ghosts (not shown), i.e. maximal stimulation at a concentration of 50–100 μg

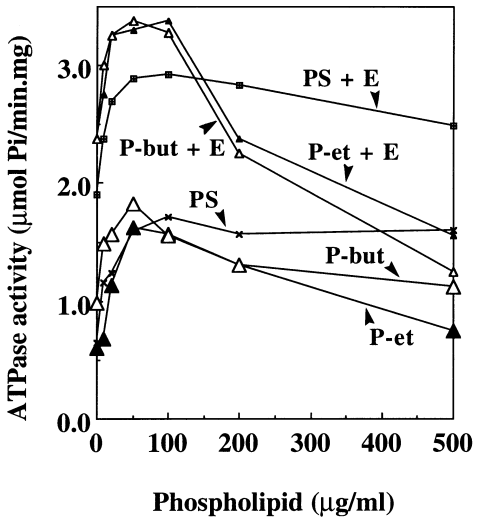

Figure 1 Effect of acidic phospholipids and ethanol on the activity of purified Ca^{2+}-ATPase

The reaction medium (0.5 ml, 37 °C) contained 1 μg/ml purified Ca^{2+}-ATPase, 130 mM KCl, 20 mM Hepes/KOH, pH 7.2, 1 mM ATP, 1 mM $MgCl_2$, 1 mM EGTA and the amount of $CaCl_2$ to give a final Ca^{2+} concentration of 10 μM and the indicated concentration (μg/ml) of the respective phospholipid. Phosphatidylserine (PS, $\times$); phosphatidylethanol (P-et, $\blacktriangle$); phosphatidylbutanol (P-but, $\triangle$). Where indicated 5% ethanol (E) was included. Similar results were observed in five additional experiments.

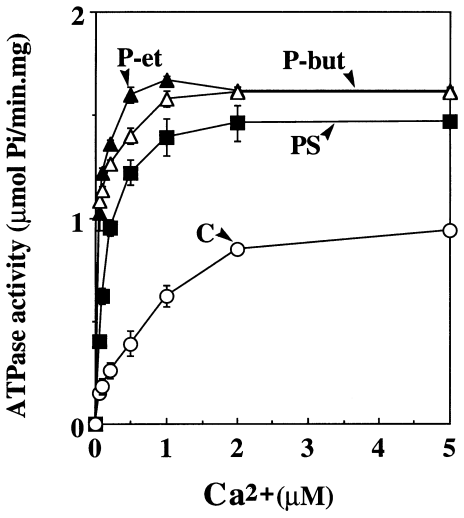

Figure 2 Effect of acidic phospholipids on the Ca^{2+}-ATPase activity

Experimental conditions were as in Figure 1. ○, Control (C); ■, 100 μg/ml phosphatidylserine (PS); ▲, 100 μg/ml phosphatidylethanol (P-et); △, 100 μg/ml phosphatidylbutanol (P-but).

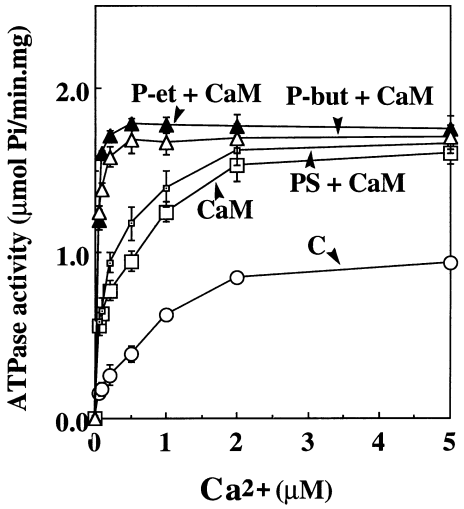

Figure 3 Effect of acidic phospholipids and calmodulin on the Ca^{2+}-ATPase affinity for Ca^{2+}

Experimental conditions were as in Figure 1. ○, Control (C); □, 5 μg/ml calmodulin (CaM); □, 100 μg/ml phosphatidylserine plus 5 μg/ml calmodulin (PS + CaM); ▲, 100 μg/ml phosphatidylethanol plus 5 μg/ml calmodulin (P-et + CaM); △, 100 μg/ml phosphatidylbutanol plus 5 μg/ml calmodulin (P-but + CaM).

phospholipid/ml and less evident stimulation at higher concentrations.

Effect of acidic phospholipids on calmodulin-stimulated Ca^{2+}-ATPase activity

As has been demonstrated for other acidic phospholipids [36], addition of either phosphatidylserine, phosphatidylethanol or phosphatidylbutanol to purified, solubilized erythrocyte Ca^{2+}-ATPase, increased the affinity of the enzyme for Ca^{2+} and the maximal velocity of the reaction as compared with controls in the absence of acidic phospholipids (Figure 2). However, in contrast with what occurs with phosphatidylserine, simultaneous addition of phosphatidylalcohols and calmodulin increased the affinity of the enzyme for Ca^{2+} to a greater extent than each added separately (Figure 3). In all cases the maximal velocity was similar to the maximal velocity obtained with calmodulin alone, independently of the phospholipid added (Table 1). Comparable results were obtained when erythrocyte ghosts were used instead of the purified, solubilized Ca^{2+}-ATPase, and the values of K_m and V_{max} obtained are shown in Table 2.

Table 1 Effect of acidic phospholipids on the K_m and V_{max} of purified, solubilized Ca^{2+}-ATPase

Experimental conditions were as in Figure 1. Different letters indicate that differences between values were significant as indicated in the Materials and methods section. Abbreviations: Ptd, phosphatidyl; Ser, serine; Eth, ethanol; But, butanol; CaM, calmodulin.

Addition	$K_m(Ca^{2+})$ (μM)	V_{max} (μmol of P_i/min per mg)
Control	0.889 ± 0.033^a	0.916 ± 0.051^a
CaM	0.412 ± 0.018^b	1.536 ± 0.056^b
Ethanol	0.360 ± 0.023^c	2.022 ± 0.063^c
PtdSer	0.200 ± 0.026^d	1.524 ± 0.042^b
PtdEth	0.210 ± 0.020^d	1.548 ± 0.032^b
PtdBut	0.200 ± 0.018^d	1.643 ± 0.051^b
PtdSer + CaM	0.217 ± 0.030^d	1.568 ± 0.022^b
PtdEth + CaM	0.157 ± 0.010^e	1.610 ± 0.063^b
PtdBut + CaM	0.144 ± 0.007^e	1.690 ± 0.091^b
PtdSer + Eth	0.144 ± 0.015^e	3.126 ± 0.062^d
PtdEth + Eth	0.137 ± 0.024^e	3.002 ± 0.136^d
PtdBut + Eth	0.152 ± 0.010^e	2.980 ± 0.062^d

Table 2 Effect of acidic phospholipids on the K_m and V_{max} on Ca^{2+}-ATPase activity from erythrocyte ghosts

Experimental conditions were as in Figure 1. Different letters indicate that differences between values were significant as indicated in the Materials and methods section. For abbreviations, see Table 1.

Addition	K_m (Ca^{2+}) (μM)	V_{max} (nmol of P_i /min per mg)
Control	0.796 ± 0.062^a	14 ± 3^a
CaM	0.421 ± 0.008^b	27 ± 2^b
Ethanol	0.381 ± 0.009^c	34 ± 3^c
PtdEth	0.267 ± 0.040^d	29 ± 3^b
PtdBut	0.262 ± 0.017^d	28 ± 2^b
PtdEth + CaM	0.191 ± 0.018^e	30 ± 2^b
PtdBut + CaM	0.218 ± 0.002^e	31 ± 3^b
PtdEth + Eth	0.190 ± 0.009^e	41 ± 3^d
PtdBut + Eth	0.172 ± 0.024^e	42 ± 1^d

Effect of ethanol on acidic phospholipids-stimulated Ca^{2+}-ATPase

Since phosphatidylethanol has been reported to induce tolerance to fluidification by ethanol in artificial lipid bilayers [14], and ethanol, by itself, has been shown to stimulate the activity of the erythrocyte Ca^{2+}-ATPase [29], we investigated whether ethanol had any additive effect on the phosphatidylalcohol- or the phosphatidylserine-stimulated Ca^{2+}-ATPase. Figure 4 shows that, as has been described previously [29], addition of ethanol increased the maximal velocity of the reaction catalysed by the purified, solubilized erythrocyte Ca^{2+}-ATPase above the values obtained with calmodulin (Figure 3) or with any acidic phospholipid tested separately (Table 1). The increase in the affinity for Ca^{2+} produced by ethanol, however, was lower than that obtained

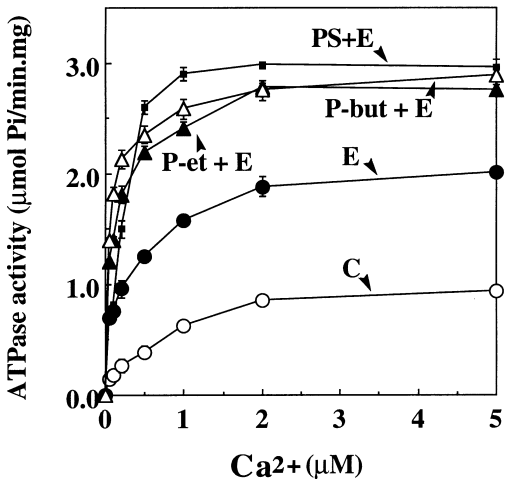

Figure 4 Effect of acidic phospholipids and ethanol on the Ca^{2+}-ATPase affinity for Ca^{2+}

Experimental conditions were as in Figure 1. ○, Control (C); ●, 5% ethanol (E); ■, 100 μg/ml phosphatidylserine plus 5% ethanol (PS + E); ▲, 100 μg/ml phosphatidylethanol plus 5% ethanol (P-et + E); △, 100 μg/ml phosphatidylbutanol plus 5% ethanol (P-but + E).

with addition of phosphatidylethanol, phosphatidylbutanol, or phosphatidylserine, separately (Table 1). When ethanol was added in the presence of acidic phospholipids (Figure 4) an additive effect was observed. There was an increase in the affinity of the enzyme for Ca^{2+} and in the maximal velocity of the reaction, well above the values obtained with ethanol or with the acidic phospholipids tested separately (Table 1). Similar results were obtained when erythrocyte ghosts were used instead of the purified, solubilized Ca^{2+}-ATPase, and the values of K_m and V_{max} obtained are shown in Table 2. Maximal effects of ethanol on the phospholipid-activated Ca^{2+}-ATPase activity were observed at a phospholipid concentration of 50–100 μg/ml (Figure 1 and results not shown).

DISCUSSION

In the present study we show that phosphatidylalcohols stimulate the Ca^{2+}-ATPase activity of human erythrocytes. This stimulation occurs with the purified, solubilized enzyme as well as with ghost preparations, and is different from that obtained with other acidic phospholipids such as phosphatidylserine, in that both phosphatidylethanol and phosphatidylbutanol increased the affinity of the calmodulin-stimulated enzyme for Ca^{2+}. In addition, all the acidic phospholipids tested had an additive effect on the stimulation of the Ca^{2+}-ATPase activity by ethanol.

It is noteworthy that no significant differences were observed between the effect of phosphatidylethanol or phosphatidylbutanol on the Ca^{2+}-ATPase activity. This is in contrast with the more pronounced effect of butanol than ethanol on this activity [29] and could be attributed to the loss of the hydroxy group of the alcohols by transphosphatidylation. The different effect of the phosphatidylalcohols on the Ca^{2+}-ATPase activity with respect to phosphatidylserine, i.e. their additive effect on its affinity for Ca^{2+} in the presence of calmodulin, could be explained by the complex nature of the interactions between acidic phospholipids and the Ca^{2+}-ATPase. In this regard, studies performed with peptides obtained by trypsin treatment of the enzyme shed some light on these complex interactions. Peptides with different affinities for Ca^{2+} could be obtained depending on the conditions used for trypsin proteolysis. One of the peptides (81 kDa) had higher affinity for Ca^{2+} than the native enzyme and was not activated by calmodulin [22,37], but its affinity for Ca^{2+} could be further increased by acidic phospholipids [38]. The 76 kDa tryptic fragment had a higher affinity for Ca^{2+} than either the native enzyme or the 81 kDa fragment and was no longer activated by calmodulin or acidic phospholipids [38]. On the other hand, it has been shown more recently [39] that acidic phospholipids, besides interacting with the N-terminal domain of the Ca^{2+}-ATPase, also interact with the calmodulin-binding domain, located toward the C-terminal end of the enzyme. Thus a more complex picture emerges, as reviewed previously [40,41], possibly accounting for the differential effect of phosphatidylserine and both phosphatidylalcohols. In addition, it is noteworthy that, although the three phospholipids possess a net negative charge, phosphatidylserine has two negative charges and one positive, while the phosphatidylalcohols possess only one negative charge.

Several effectors besides calmodulin have been reported which are able to stimulate the plasma-membrane Ca^{2+}-ATPase activity. Most of them, however, only increase the affinity of the enzyme for Ca^{2+} [42] and/or increase its maximal velocity to the same extent obtained in the presence of calmodulin [17,42,43]. By contrast, ethanol stimulates the Ca^{2+}-ATPase to a larger extent than that obtained when this enzyme is activated by calmodulin [29]. The increase in the degree of activation is observed in the affinity of the enzyme for Ca^{2+} as well on its maximal velocity. Interestingly, the effect of ethanol besides being additive to that of calmodulin [29] is also additive to the effect of acidic phospholipids (the present work). This additive response was observed on the affinity of the Ca^{2+}-ATPase for its substrates, and also on the maximal velocity of the enzyme, thus suggesting that these effectors interact with the Ca^{2+}-ATPase through different mechanisms.

The mechanism of stimulation of the Ca^{2+}-ATPase by calmodulin is well established [40,42]: an autoinhibitory domain of about 9 kDa, located at the C-terminus of the enzyme is removed upon calmodulin binding, leaving the substrates free access to its active site. By contrast, the mechanism of stimulation by acidic phospholipids is less well understood, although since the stimulation of the V_{max} by these compounds is not additive to that of calmodulin, it could be stated that they partially mimic the action of calmodulin. This could be achieved by bringing the enzyme to an 'open conformation' such that the substrate gains access to the catalytic pocket of the Ca^{2+}-ATPase. This interpretation is supported by experiments performed after trypsin proteolysis of the Ca^{2+}-ATPase in the presence of different ligands. Thus, similarly to what occurs in the presence of calmodulin, the presence of linoleic acid, at concentrations that stimulate the Ca^{2+}-ATPase in the absence of calmodulin, greatly accelerates the digestion of the enzyme [23], which can be interpreted as if upon binding of this ligand to the enzyme there is an increase in the number of cleavage sites accessible to trypsin. Calmodulin, besides increasing the rate of trypsin proteolysis, protects some peptides from further digestion [23]. Thus, the mechanism of interaction of calmodulin and stimulatory lipids with the Ca^{2+}-ATPase is somehow similar in nature. This is also supported by studies undertaken in order to follow the different conformations of the enzyme by CD [44]. In these studies it was shown that both calmodulin and phosphatidylserine decreased the α-helical content of the Ca^{2+}-ATPase in a similar manner. However, acidic phospholipids increased the affinity of the enzyme for Ca^{2+} to a larger extent than calmodulin, but again, the induced increase in the affinity of the enzyme for Ca^{2+}

was not additive with that obtained in the presence of calmodulin alone [44]. In the present work we show for the first time that phosphatidylalcohols are able to increase the affinity of the enzyme for Ca^{2+} to a higher level, even when calmodulin is present in the assay medium.

Concerning the mechanism of action of ethanol, the fact that its stimulation of the Ca^{2+}-ATPase is additive to that obtained with calmodulin and acidic phospholipids even when these effectors are present at optimal levels, indicates that it exerts its effect through a different mechanism. One possible interpretation, based on the postulated mechanism of interaction of calmodulin with the Ca^{2+} pump, is that ethanol acts on another putative autoinhibitory domain which is not able to be removed by proteolysis of the enzyme. It should be mentioned that trypsin proteolysis of the Ca^{2+}-ATPase under conditions which originates a stimulated and calmodulin-insensitive enzyme renders a form which is still able to be stimulated by ethanol to the same extent to that obtained when the alcohol is added to the intact enzyme in the presence of calmodulin [29]. It is noteworthy that while ethanol additively increases the affinity of the enzyme for Ca^{2+} when added together with calmodulin [29] or acidic phospholipids (Table 1), the decrease of the K_m for Ca^{2+} is higher when added simultaneously with acidic phospholipids (Table 1) than when added with calmodulin [29]. These results indicate a complex pattern of interaction between ethanol, acidic phospholipids, and the enzyme.

It has been reported that the relative potency of different acidic phospholipids in increasing the Ca^{2+}-ATPase affinity for Ca^{2+} is a function of the number of negative charges at physiological pH (phosphatidylinositol 4,5-bisphosphate > phosphatidylinositol 4-phosphate ≱ phosphatidylinositol = phosphatidic acid = phosphatidylserine) [45] and that the stimulation of the enzyme by negatively charged phospholipids is based in the direct binding of the lipids to the enzyme [46]. As mentioned above, all the acidic phospholipids used in this work possess only one negative net charge and therefore they were not expected to show differences in their relative potency to increase the affinity of the enzyme for Ca^{2+}. This indeed appears to be the case, except when calmodulin is present, in which case phosphatidylalcohols were more potent than phosphatidylserine. This indicates a different interaction of the former with the calmodulin-activated enzyme, probably as a consequence of their simpler molecular headgroup structures. It could also be possible that the distinct effect observed between phosphatidylserine and phosphatidylalcohols could be related to their different hydrophobic acyl chains. It should be mentioned it this respect that organic solvents such as DMSO mimic the stimulatory effect of calmodulin and acidic phospholipids on the Ca^{2+}-ATPase activity [25], suggesting that a hydrophobic interaction could be partially involved in the enzyme stimulation. However, this does not appear to be the explanation, since it has been reported [41] that different phosphatidylserines isolated from bovine brain and from chicken egg are similar with respect to their effect on the Ca^{2+}-ATPase activity.

Ethanol has been demonstrated to affect the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of different cells [10]. Interestingly, Ca^{2+} efflux is stimulated in human erythrocytes after their exposure to concentrations of ethanol (0.25–0.5%) achieved in the blood after its human consumption [47], and it has been demonstrated that, at these concentrations, ethanol increases Ca^{2+} transport by inside-out erythrocyte vesicles [29]. Accordingly, addition of similar concentrations of ethanol to vascular smooth muscle [48] or skeletal-muscle cells [49] produced a decrease in their $[Ca^{2+}]_i$. In addition, although an increase in the $[Ca^{2+}]_i$ of hepatocytes was observed after addition of similar concentrations of ethanol, this change was transitory and paralleled the stimulation of the inositol-lipid-specific phospholipase C, and the concomitant increase in the levels of inositol 1,4,5-trisphosphate [10,50].

Ethanol affects a wide variety of membrane-bound enzyme activities,and many of its effects may be associated with its fluidification action on the membrane bilayer [14]. However, it cannot be excluded that, in some cases, ethanol-induced perturbations could be due to accumulation of phosphatidylethanol [12,14]. It is generally agreed that the phospholipid composition of membranes is important for their biological activity. Properties such as K_m and V_{max} [51,52] or the degree of cooperativity [53] of some enzymes are sensitive to their lipid environment. It has been shown that phosphatidylethanol causes an increase of fluidity of artificial and natural bilayers and that is able to confer membrane 'tolerance' to the effects of ethanol [14]. Membrane tolerance has been detected in membrane preparations isolated from rats intoxicated with ethanol for prolonged periods. Ethanol concentrations causing significant membrane disordering in control preparations have little effect on membranes prepared from ethanol-treated rats [54–56]. Despite the considerable body of work that has been carried out in the last few years, the molecular basis of the membrane tolerance phenomenon is still debated. It is conceivable that the additive effect of ethanol and phosphatidylethanol on the activation of some membrane-bound enzymes could be involved in this phenomenon. Synergism of the effects of ethanol in human alcoholics or ethanol-treated animals that have previously accumulated phosphatidylethanol in their cell plasma membranes should also be taken into account.

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REFERENCES

1 Saito, M., Burque, E., and Kanfer, J. N. (1975) Arch. Biochem. Biophys. **164**, 420–428
2 Chalifour, R. J. and Kanfer, J. N. (1980) Biochem. Biophys. Res. Commun. **96**, 742–747
3 Bocckino, S. P., Blackmore, P. F., Wilson, P. B. and Exton, J. H. (1987) J. Biol. Chem. **262**, 15309–15315
4 Martin, T. W. (1988) Biochim. Biophys. Acta **962**, 282–296
5 Tettenborn, C. S. and Mueller, G. C. (1988) Biochem. Biophys. Res. Commun. **155**, 249–255
6 Anthes, J. C., Eckel, S., Siegel, M. I., Egan, R. W. and Billah, M. M. (1989) Biochem. Biophys. Res. Commun. **163**, 657–664
7 Domino, S. E., Bocckino, S. B. and Garbers, D. L. (1989) J. Biol. Chem. **264**, 9412–9419
8 Musch, M. W. and Goldstein, L. (1990) J. Biol. Chem. **265**, 13055–13059
9 Billah, M. M. and Anthes, J. C. (1990) Biochem. J. **269**, 281–291
10 Hoek, J. B., Thomas, A. P., Rooney, T. A., Higashi, K. and Rubin, E. (1992) FASEB J. **6**, 2386–2395
11 Alling, C., Gustavsson, L., Mansson, J.-E., Benthin, G. and Angard, E. (1984) Biochim. Biophys. Acta **793**, 119–122
12 Gustavsson, L. (1995) Alcohol Alcohol. **30**, 391–406
13 Mueller, G. C., Fleming, M. F., LeMahieu, M. A., Lybrand, G. S. and Barry, K. J. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 9778–9782
14 Omodeo-Sale, F., Lindi, C., Palestini, P. and Masserini, M. (1991) Biochemistry **30**, 2477–2482
15 Asaoka, Y., Kikkawa, U., Sekiguchi, K., Shearman, M. S., Kosaka, Y., Nakano, Y., Satoh, T. and Nishizuka, Y. (1988) FEBS Lett. **231**, 221–224
16 Carafoli, E. (1987) Annu. Rev. Biochem. **56**, 395–433
17 Penniston, J. T. (1983) in Calcium and Cell Function (Cheung, W. V., ed), pp. 99–149, Academic Press, New York
18 Niggli, V., Adunyah, E. S. and Carafoli, E. (1981) J. Biol. Chem. **256**, 8588–8592
19 Neyses, L., Rieinlib, L. and Carafoli, E. (1985) J. Biol. Chem. **260**, 10283–10287
20 Smallwood, J. I., Gigi, B. and Rasmussen, H. (1988) J. Biol. Chem. **263**, 2195–2202
21 Sarkadi, B., Enyedi, A. and Gardos, G. (1980) Cell Calcium **1,** 287–297

22 Zurini, M., Krebs, J., Penniston, J. T. and Carafoli, E. (1986) J. Biol. Chem. **259**, 618–627
23 Benaim, G., Zurini, M. and Carafoli, E. (1984) J. Biol. Chem. **259**, 8471–8477
24 Wang, K. K. W., Villalobo, A. and Roufogalis, B. D. (1988) Arch. Biochem. Biophys. **260**, 696–704
25 Benaim, G. and de Meis, L. (1989) FEBS Lett. **244**, 484–486
26 Benaim, G. and de Meis, L. (1990) Biochim. Biophys. Acta **1026**, 87–92
27 Kosk-Kosicka, D. and Bzdega, T. (1988) J. Biol. Chem. **263**, 18184–18189
28 Coelho-Sampaio, T., Ferreira, S. T., Benaim, G. and Vieyra, A. (1991). J. Biol. Chem. **266**, 22266–22272
29 Benaim, G., Cervino, V., Lopez-Estraño, C. and Weitzman, C. (1994) Biochim. Biophys. Acta **1195**, 141–148
30 Guerini, D., Krebs, J. and Carafoli, E. (1984) J. Biol. Chem. **259**, 15172–15177
31 Benaim, G., Losada, S., Gadelha, F. R. and Docampo, R. (1991) Biochem. J. **280**, 715–720
32 Fiske, C. H. and SubbaRow, Y. (1925) J. Biol. Chem. **66**, 375–400
33 Niggli, V., Penniston, J. T. and Carafoli, E. (1979) J. Biol. Chem. **254**, 9955–9958
34 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193,** 265–275
35 Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. **70**, 241–250
36 Choquette, D., Hakim, G., Filoteo, A., Plishker, G., Bostwick, R. and Penniston, J. (1984) Biochem. Biophys. Res. Commun. **125**, 908–915
37 Benaim, G., Clark, A. and Carafoli, E. (1986) Cell Calcium **7**, 175–186
38 Enyedi, A., Flura, M., Sarkadi, B., Gardos, G. and Carafoli, E. (1987) J. Biol. Chem. **262**, 6425–6430
39 Filoteo, A. G., Enyedi, A. and Penniston, J. T. (1992) J. Biol. Chem. **267**, 11800–11805
40 Wuytack, F. and Raeymaekers, L. (1992) J. Bioenerg. Biomemb. **24**, 285–300
41 Lehotsky, J., Raeymaekers, L., Missiaen, L., Wuytack, F., De Smedt, H. and Casteels, R. (1992) Biochim. Biophys. Acta **1105**, 118–124
42 Carafoli, E. (1991) Physiol. Rev. **71**, 129–153
43 Rega, A. F. and Garrahan, P. J. (1986) The Ca^{2+} Pump of Plasma Membranes (Rega, A. F. and Garrahan, P. J., eds.), CRC Press, Boca Raton, FL
44 Wrzosek, A., Famulski, K. S., Lehotsky, J. and Pikula, S. (1969) Biochim. Biophys. Acta **986**, 263–270
45 Missiaen, L., Raeymaaekers, L., Wuytack, F., Vrolix, M., De Smedt, H and Cateels, R. (1989) Biochem. J. **263**, 687–694
46 Verbist, J., Gadella, T. W. J., Raeymaekers, L., Wuytack, F., Wirtz, K. W. A. and Casteels, R. (1991) Biochim. Biophys. Acta **1063**, 1–6
47 Yamamoto, H. A. and Harris, R. A. (1983) Biochem. Pharmacol. **32**, 2787–2791
48 Zhang, A., Cheng, T. P. O. and Altura, B. M. (1992) Alcohol Clin. Exp. Res. **16**, 55–57
49 Cofan, M., Fernandez-Sola, J., Nicolás, J. M., Poch, E. and Urbano-Marquez, A. (1995) Alcohol Alcohol. **30**, 617–621
50 Hoek, J. B., Thomas, A. P., Rubin, R. and Rubin, E. (1987) J. Biol. Chem. **268**, 682–691
51 Demel, R. A., Geurts, W. S. and Van Deenen, L. L. (1972) Biochim. Biophys. Acta **266**, 26–34
52 Zachim, D. and Vessey, D. A. (1976) in The Enzymes of Biological Membranes (Martonosi, A., ed.), vol. 2, pp. 443–469, Plenum Press, New York
53 Esfahani, M., Rudkin, B. B., Cutler, C. J. and Waldron, P. (1977) J. Biol. Chem. **252**, 3194–3202
54 Harris, R. A., Baxter, D. M., Mitchell, M. A. and Hiltzemann, R. J. (1984) Mol. Pharmacol. **25**, 401–409
55 Goldstein, D. B. and Chin, J. H. (1981) Fed. Proc. Fed. Am. Soc. Exp. Biol. **40**, 2073–2080
56 Lyon, R. and Goldstein, D. B. (1982) Mol. Phamacol. **23**, 86–92

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