Ceramide and sphingosine have an antagonistic effect on the plasma-membrane Ca^{2+}-ATPase from human erythrocytes

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The plasma-membrane Ca^{2+}-ATPase is a key enzyme in the regulation of the intracellular Ca^{2+} concentration. On the other hand, sphingolipids have been recognized recently as important second messengers, acting in many systems in combination with Ca^{2+}. In view of the fact that the Ca^{2+}-ATPase is stimulated by ethanol, and since sphingolipids possess free hydroxy groups, we decided to study the possible effect of ceramide and sphingosine on this calcium pump. Here we show that ceramide stimulates the Ca^{2+}-ATPase in a dose-dependent manner and additively to the activation observed in the presence of calmodulin or ethanol, when compared with any of these effectors added alone. Ceramide affects both the affinity for Ca^{2+} and the V_{max} of the enzyme. Furthermore, this second messenger also stimulates Ca^{2+} transport in inside–out plasma-membrane vesicles from erythrocytes. Conversely, sphingosine, which is reported to act in many systems antagonistically with ceramide, showed an inhibitory effect on Ca^{2+}-ATPase activity. This inhibition was also observed on the calmodulin-stimulated enzyme. These results, taken together, suggest that ceramide and sphingosine act antagonistically on the plasma-membrane Ca^{2+}-ATPase. This is in accordance with the frequently reported opposite effect of these sphingolipids on intracellular Ca^{2+} concentration.

Key words: calcium, calcium pump, calmodulin, ethanol, sphingolipid.

INTRODUCTION

The plasma-membrane Ca^{2+}-ATPase has been recognized as a fundamental enzyme in the regulation of the cytoplasmic basal concentration of Ca^{2+}, essential for its role as a messenger [1,2]. An interesting property of this Ca^{2+}-ATPase that distinguishes it from other P-type ionic pumps is the multiplicity of regulatory mechanisms [3]. Thus this ionic pump is regulated by calmodulin (CaM) [4,5], protein kinases A [6] and C [7], acidic phospholipids [8], controlled proteolysis [9,10], auto-aggregation [11], organic solvents [12] and phosphatidylethanol [13]. We have shown previously that ethanol is able to stimulate this pump to a greater extent than all the effectors mentioned above [14,15]. Interestingly, this effect is additive to that of CaM, which indicates that CaM and ethanol stimulate the enzyme by different mechanisms [14,15]. However, ethanol is not a physiological effector. Thus it is plausible that another compound exists in the cell, capable of reproducing the activation of the Ca^{2+}-ATPase by this alcohol. Since the natural effector could be an amphiphilic lipid with free hydroxy groups, we decided to study the effect of sphingolipids, which besides possessing the mentioned biochemical structure, were reported as being important second messengers involved in signal transduction, frequently acting in conjunction with Ca^{2+} [16–19]. Sphingolipids are ubiquitous among eukaryotic organisms and they have been implicated in cellular growth regulation, differentiation and apoptosis [17–19].

The precursor of these second messengers is sphingomyelin, which generates ceramide via a sphingomyelinase. Subsequently, sphingosine is derived from ceramide, by action of a ceramidase [16,19]. Ceramide and sphingosine may be phosphorylated by means of specific kinases, generating ceramide 1-phosphate and sphingosine 1-phosphate, respectively [16,19]. It have been reported that ceramide and sphingosine act antagonistically in many systems [19–21].

In the present study, using purified Ca^{2+}-ATPase derived from human erythrocytes, we show that the activity of the enzyme is stimulated by ceramide. The stimulatory effect observed is additive to that obtained when the enzyme is stimulated by CaM and ethanol. Conversely, sphingosine inhibits the activity of the Ca^{2+}-ATPase, in both the presence and absence of CaM. We also show that sphingomyelin, ceramide 1-phosphate and sphingosine 1-phosphate do not show any discernible effect on the enzyme.

MATERIALS AND METHODS

Chemicals

All the reagents were of the highest purity available. Ceramide (C_2 and C_8), ceramide 1-phosphate and sphingosine 1-phosphate were purchased from Sigma or Calbiochem. Sphingosine and sphingomyelin were purchased from Avanti Polar Lipids. All lipids were microdispersed by sonication at 4 °C under N_2 before use. Stocks of concentrated lipids were dissolved in DMSO. The final concentration of DMSO in the reaction mixture was always below 1 % (v/v). Other reagents were from Sigma. CaM was purified from bovine brain according to the method of Guerini et al. [22], with the modifications introduced in [23].

Purification of erythrocyte Ca^{2+}-ATPase

Human erythrocyte ghosts that were free of CaM were prepared by the method of Niggli et al. [24]. The plasma-membrane Ca^{2+}-ATPase was purified by using a CaM affinity column as described before [10]. A coupled-enzyme assay system was used to determine Ca^{2+}-ATPase activity during purification of the enzyme, as described in [25]. The medium contained 1 unit/ml pyruvate kinase, 1 unit/ml lactate dehydrogenase, 100 mM KCl, 30 mM Hepes/KOH (pH 7.4), 2.5 mM $MgCl_2$, 0.2 mM NADH, 0.5 mM phosphoenolpyruvate, 1 mM ATP and 50 μM $CaCl_2$, 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 against time using a dual-wavelength spectrophotometer (Amin-

Abbreviations used: CaM, calmodulin; IOV, inside–out plasma-membrane vesicle.

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co DW-2a). The purified enzyme was stored under N_2 at -70 °C at a concentration of 100–200 μg/ml, in a buffer containing 130 mM KCl, 20 mM Hepes/KOH (pH 7.2), 2 mM $MgCl_2$, 2 mM EDTA, 2 mM dithiothreitol, 0.05% Triton X-100, 0.5 mg/ml phosphatidylcholine, 50 μM $CaCl_2$ and 5% (v/v) glycerol.

Determination of the 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 7.4), 1 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP and the appropriate quantity of $CaCl_2$, to obtain the desired free calcium concentrations. The final concentration of ionic calcium was calculated using an iterative computer program modified from that described by Fabiato and Fabiato [26], as described previously [27]. The reaction was carried out for 45 min at 37 °C and was stopped by the addition of cold trichloroacetic acid at 8% (v/v; final concentration). The phosphate produced by ATP hydrolysis was determined according to the method of Fiske and SubbaRow [28], but using $FeSO_4$ as the reducing agent. Appropriate blanks were included to correct any interference with the colorimetric method.

Ca^{2+} transport by inside–out plasma-membrane vesicles (IOVs) from human erythrocytes

IOVs from erythrocytes were prepared as described by Sarkadi et al. [29]. Ca^{2+} transport was determined by the use of arsenazo III as a calcium indicator using the wavelength pair 675–685 nm, following the methodology described in [23]. To determine Ca^{2+} transport, aliquots of IOVs was diluted in a final volume of 1 ml of a buffer containing 160 mM KCl, 50 μM arsenazo III, 0.5 mM $MgCl_2$, 25 μM $CaCl_2$ and 10 mM Tris/HCl (pH 7.4) at 37 °C. The Ca^{2+} transport was initiated by the addition of 0.5 mM ATP. The system was calibrated by successive additions of known concentrations of $CaCl_2$.

Protein determination

The protein concentration of the plasma-membrane fraction was determined by the Biuret assay [30] in the presence of deoxycholate, using BSA as a standard. The method of Lowry et al. [31] was used for the purified protein. To avoid interference from Triton X-100, the protein was precipitated with trichloroacetic acid in the presence of deoxycholate [32].

Analysis of 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 Figures and Table 1 are means $\pm$ S.D. for n different experiments, using different enzyme preparations. Statistical significance was determined by Student's t test. Significance was considered for $P < 0.05$.

RESULTS

In order to investigate the effect of ceramide on Ca^{2+}-ATPase activity, aliquots of purified enzyme from human erythrocytes were incubated with different concentrations of C_2-ceramide. This was done also in the presence of CaM, ethanol and both effectors simultaneously. As can be seen in Figure 1, addition of ceramide induced a 2-fold stimulation of the Ca^{2+}-ATPase activity. The maximal stimulation was observed at a concentration of 10 μM C_2-ceramide. It can also be seen in the same Figure that an additive effect was obtained for Ca^{2+}-ATPase activity when C_2-ceramide was added to the assay medium in the presence of CaM. When ethanol was added together with the sphingolipid, an additive effect was again observed (Figure 1). Furthermore, when CaM and ethanol were both present simultaneously, a further increase in the Ca^{2+}-ATPase activity was observed. On the other hand, the enzyme activity was not affected by ceramide 1-phosphate under the same conditions (results not shown).

The effect of C_2-ceramide on the affinity of Ca^{2+}-ATPase for Ca^{2+} was determined by incubating the enzyme with different concentrations of free Ca^{2+} at 10 μM C_2-ceramide, in the absence or presence of CaM (Figure 2). It may be observed that C_2-ceramide increased the affinity of the enzyme for Ca^{2+} (K_m, 0.14 ± 0.02 μM) with respect to the control (K_m, 0.99 ± 0.04 μM), and to a larger extent to that obtained in the presence of CaM (K_m, 0.39 ± 0.03 μM). When both effectors were present, although an additive effect was observed for V_{max} (Figure 1), the K_m value was the same as when C_2-ceramide was added alone (K_m, 0.15 ± 0.04 μM).

Since ethanol also has a marked effect on the affinity of the Ca^{2+}-ATPase for Ca^{2+}, we determined the K_m of the enzyme for Ca^{2+} in its presence, together with ceramide. This effect is shown in Figure 3, where it may be observed that C_2-ceramide increased the affinity of the enzyme for Ca^{2+} to a larger extent than obtained in the presence of ethanol (K_m, 0.4 ± 0.02 μM). Interestingly, in contrast with CaM, when ceramide was added together with ethanol, an additive effect on the affinity of the enzyme for Ca^{2+} was obtained (K_m, 0.08 ± 0.02 μM).

There are different types of ceramides, which can be distinguished by the length of the acyl chain, and it has been reported that short-chain and long-chain ceramides affect several enzymes in different ways [33]. The effect of C_8-ceramide, compared with C_2-ceramide is shown in Table 1. It can be seen that both types of ceramide affect the V_{max} of the Ca^{2+}-ATPase in an identical manner. However, C_2-ceramide had a more pronounced effect on the affinity of the enzyme for Ca^{2+} (K_m, 0.14 ± 0.02 μM) than C_8-ceramide (K_m, 0.47 ± 0.06 μM). Nevertheless, the latter is still able to lower the K_m of the Ca^{2+}-ATPase for Ca^{2+} when compared with the control (K_m, 0.99 ± 0.04 μM). Thus it seems that the short-chain ceramide is more potent than the long-chain ceramide with respect to its effect on the enzyme's affinity for the cation.

The effect of ceramide on the Ca^{2+}-ATPase could be attributable to non-specific action of the sphingolipid on the solubilized purified form of the enzyme. Thus we studied the effect of this sphingolipid on Ca^{2+} transport in IOVs from erythrocytes. The time course of the changes in free calcium concentration in the extravesicular medium was registered as the change in absorbance of the indicator arsenazo III. The base line was obtained in the absence of ATP (Figure 4). After ATP addition, the vesicles began to capture Ca^{2+}, decreasing the absorbance. Ca^{2+} transport increased in the presence of ceramide by 2.6-fold (Figure 4) with respect to the control. After addition of CaM to the same preparation, a more pronounced increase in Ca^{2+} transport was observed (4.6-fold). This additive increase in transport upon addition of both effectors added simultaneously was in accordance with the results obtained for ATPase activity described above. Addition of the calcium ionophore A23187 induced the exit of Ca^{2+} from the vesicles, which demonstrates that this cation was accumulated actively inside the vesicles. Taken together, these results strongly support a possible direct stimulatory effect of ceramide on the plasma-membrane Ca^{2+} pump.

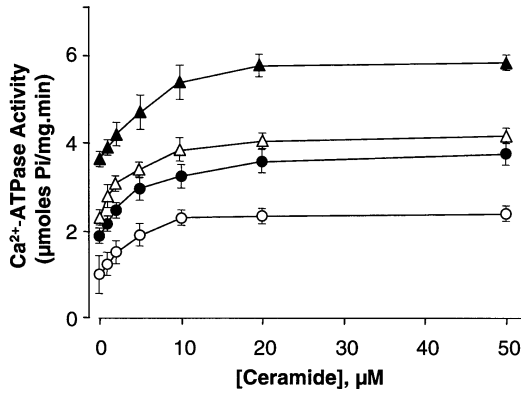

Figure 1 Stimulation of plasma-membrane Ca^{2+}-ATPase activity by ceramide

The reaction medium contained 130 mM KCl, 20 mM Hepes/KOH (pH 7.4), 1 mM ATP, 1 mM $MgCl_2$, 1 mM EGTA, the amount of $CaCl_2$ to give a final Ca^{2+} concentration of 10 μM and the indicated concentrations of C_2-ceramide. The reaction was started by the addition of 1 μg/ml purified Ca^{2+}-ATPase in a final volume of 0.5 ml at 37 °C with continuous stirring. ○, Control; ●, 5 μg/ml CaM; △, 5% ethanol; ▲, 5 μg/ml CaM plus 5% ethanol. The reaction was incubated for 45 min and arrested by the addition of cold trichloroacetic acid (8%, final concentration) and P_i was determined as explained in the Materials and methods section. Values represent the means ± S.D. from at least five independent experiments. Other experimental details are given in the Materials and methods section.

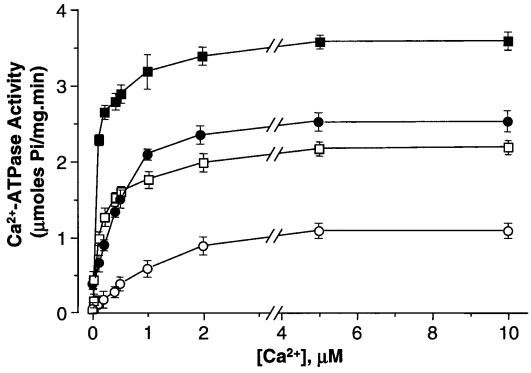

Figure 2 Effects of ceramide and CaM on Ca^{2+}-ATPase's affinity for Ca^{2+}

Experimental conditions were as in Figure 1, except that the final free Ca^{2+} concentration was obtained upon addition of 1 mM EGTA and the calculated quantity of $CaCl_2$. ○, Control; □, 10 μM C_2-ceramide; ●, 5 μg/ml CaM; ■, 10 μM C_2-ceramide plus 5 μg/ml CaM. Values represent the means ± S.D. from at least five independent experiments. Other experimental details are given in the Materials and methods section.

Since it has been reported that ceramide in many biological systems acts antagonistically with sphingosine [19,21,33,34], we next studied the effect of this ceramide derivative on the Ca^{2+}-ATPase activity. Interestingly, enzyme activity was inhibited by sphingosine in a dose-dependent manner (Figure 5). It was also found that this inhibition was maintained after CaM stimulation. The IC_{50} for sphingosine was 4.4 ± 0.3 μM, whereas it was 3.9 ± 0.4 μM in the presence of CaM. The effect of sphingosine on the enzyme's affinity for Ca^{2+} was studied in either the presence or absence of CaM (Figure 6), using the IC_{50} for sphingosine obtained in the experiments shown in Figure 5. The results obtained demonstrated that sphingosine, under both sets of conditions, increased the K_m value of the Ca^{2+}-ATPase for Ca^{2+} when compared with the control conditions (Table 1).

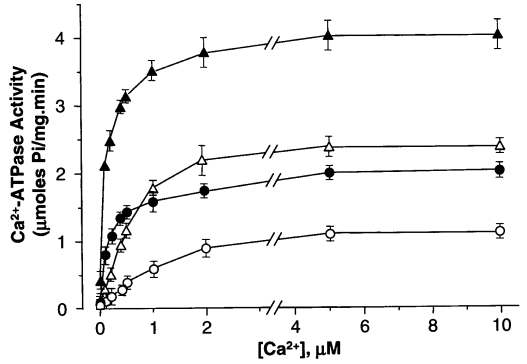

Figure 3 Effects of ceramide and ethanol on Ca^{2+}-ATPase's affinity for Ca^{2+}

Experimental conditions were as in Figure 1. ○, Control; ●, 10 μM C_2-ceramide; △, 5% ethanol; ▲, 10 μM C_2-ceramide plus 5% ethanol. Values represent the means ± S.D. from at least five independent experiments. Other experimental details are given in the Materials and methods section.

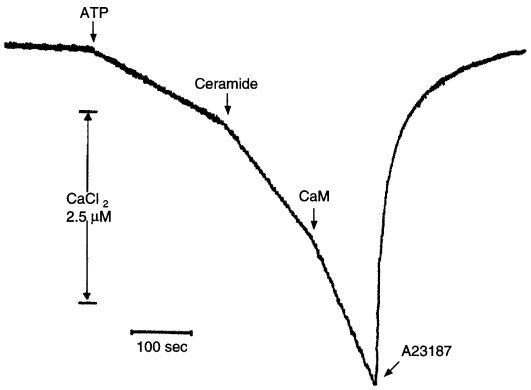

Figure 4 Stimulation of Ca^{2+} transport by ceramide in IOVs from erythrocytes

The reaction medium (1 ml) contained 130 mM KCl, 30 mM Hepes/KOH (pH 7.4), 0.5 mM $MgCl_2$, 50 μM arsenazo III, 10 μM $CaCl_2$ and 0.5 mg/ml IOVs. Additions were MgATP (0.5 mM), C_2-ceramide (10 μM), CaM (5 μg/ml) and A23187 (1 μM). The reaction was carried out at 37 °C. Other experimental details are given in the Materials and methods section.

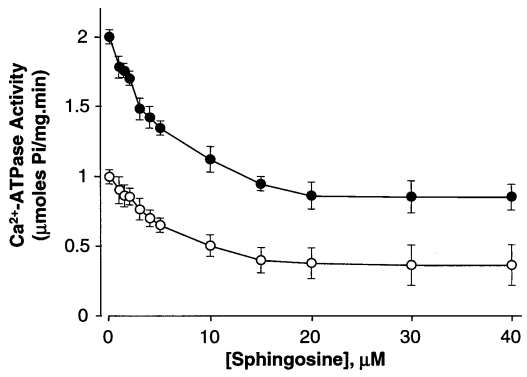

Figure 5 Inhibition of plasma-membrane Ca^{2+}-ATPase activity by sphingosine

Experimental conditions were as in Figure 1. Final free Ca^{2+} concentration was 10 μM. ○, Control; ●, 5 μg/ml CaM. Values represent the means ± S.D. from at least five independent experiments. Other experimental details are given in the Materials and methods section.

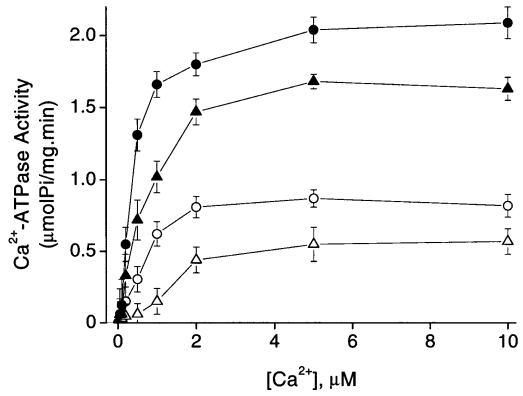

Figure 6 Effects of sphingosine and CaM on Ca^{2+}-ATPase's affinity for Ca^{2+}

Experimental conditions were as in Figure 1, except that the final free Ca^{2+} concentration was obtained upon addition of 1 mM EGTA and the calculated quantity of $CaCl_2$. ○, Control; △, 4.4 μM sphingosine; ●, 5 μg/ml CaM; ▲, 4.4 μM sphingosine plus 5 μg/ml CaM. Values represent the means ± S.D. from at least four independent experiments. Other experimental details are given in the Materials and methods section.

Table 1 Effect of short- and long-chain ceramides and sphingosine on the K_m for Ca^{2+} and the V_{max} of the plasma-membrane Ca^{2+}-ATPase

The experimental values from Figures 2, 3 and 6, and data from experiments done with C_8-ceramide (results not shown) were replotted linearly in order to calculate the respective parameters. Values are means ± S.D. of the number of experiments indicated in parentheses under V_{max}. Different superscript letters indicate that differences between values are significant; other statistical details are given in the Materials and methods section.

Condition	K_m (Ca^{2+}; μM)	V_{max} (μmol of P_i/min per mg)
Control	0.99 ± 0.04^a	1.1 ± 0.05^a (5)
CaM	0.39 ± 0.03^b	2.7 ± 0.03^b (5)
Ethanol	0.4 ± 0.02^b	2.9 ± 0.05^b (5)
C_2-Ceramide	0.14 ± 0.02^c	2.2 ± 0.04^c (5)
C_2-Ceramide + CaM	0.15 ± 0.04^c	3.6 ± 0.08^d (5)
C_2-Ceramide + ethanol	0.08 ± 0.02^d	4.2 ± 0.09^d (5)
C_8-Ceramide	0.47 ± 0.06^b	2.5 ± 0.04^d (4)
C_8-Ceramide + CaM	0.41 ± 0.09^b	4.2 ± 0.06^d (4)
C_8-Ceramide + ethanol	0.6 ± 0.08^e	4.3 ± 0.07^d (4)
Sphingosine	2.43 ± 0.09^f	0.8 ± 0.1^e (4)
Sphingosine + CaM	0.82 ± 0.18^g	1.8 ± 0.11^f (4)

In order to study the possible effect of other directly related sphingolipids on Ca^{2+}-ATPase activity, experiments with ceramide 1-phosphate, sphingosine 1-phosphate and sphingomyelin were performed. The results indicated that none of these sphingolipids had any discernible effect on enzyme activity. Nevertheless, when these effectors were present together with the tested lipid they did not prevent stimulation of the Ca^{2+}-ATPase by CaM or ethanol (results not shown).

DISCUSSION

Sphingolipids have been recognized recently as important second messengers. These compounds have been implicated in signal transduction, directly interacting with calcium as a messenger. Thus ceramide decreases the intracellular Ca^{2+} concentration in rat pinealocytes [35], whereas sphingosine induces Ca^{2+} mobilization in human fibroblasts [36]. Since the Ca^{2+}-ATPase is one of the main regulators of intracellular Ca^{2+} concentration, and since this enzyme is highly regulated, here we studied the effects of different sphingolipids on the purified form of the Ca^{2+}-ATPase. Here we show that ceramide stimulates Ca^{2+}-ATPase activity from human erythrocytes in a dose-dependent manner, with an additive effect in the presence of CaM.

The effect of ceramide on Ca^{2+}-ATPase activity was also observed in the functional expression of the enzyme, Ca^{2+} transport, as was the additive effect observed upon addition of CaM. These results support the notion that the effect of ceramide could occur in intact cells. In this respect, it was interesting to observe that ceramide increases the affinity for Ca^{2+} to a larger extent than and additively with CaM. It may be hypothesized that, after an increment in the ceramide concentration due to a specific cell signal, Ca^{2+}-ATPase is able to reduce the intracellular Ca^{2+} concentration to a lower level and more rapidly than CaM alone, with predictable consequences for the signal-transduction functions carried out by this messenger.

Concerning the mode of interaction of ceramide with the calcium pump, it is noteworthy that since the stimulatory effect of this sphingolipid is additive to that of CaM, these two effectors should interact, at least partially, through different mechanisms. The locus of interaction of the Ca^{2+}-ATPase with CaM is well documented [3], whereas we have reported that ethanol interacts with a section of the enzyme in the C-terminal domain that is comprised of at least 95 amino acids [15]. Taking into account the presence of two hydroxy groups in the ceramide molecule, we expected that the mechanism of action of this lipid to be the same as that of ethanol. The results obtained in the present work do not support this suggestion, since an additive response for these effectors was clearly observed, not only for V_{max} but also for the affinity of the enzyme for Ca^{2+}. Thus ceramide's mechanism of action on this enzyme remains to be elucidated.

Regarding the length of the carbon chain, C_2-ceramide has been used more frequently because of its higher solubility. Although some authors claim that C_8-ceramide is found more frequently in biological membranes [33], the presence of C_2-ceramides in membranes is also documented [37]. In this study we show that long- and short-chain ceramides stimulate the enzyme to the same V_{max}, whereas the effect on the enzyme's affinity for Ca^{2+} was more pronounced when the short-chain ceramide was used.

Sphingosine is derived directly from ceramide and in many systems acts antagonistically with ceramide [21–23]. For example, ceramide induces apoptosis, whereas sphingosine and sphingosine 1-phosphate stimulate mitogenic pathways [21,23]. The results presented in this work demonstrate that this sphingolipid inhibits the Ca^{2+}-ATPase in a dose-dependent manner, affecting also its affinity for Ca^{2+}. Supporting this finding, a report by Grossman [38] indicates that sphingosine inhibits the activity of the plasma-membrane Ca^{2+}-ATPase in rat synaptosomes and leucocyte membranes. However, as the author states, these results do not permit a distinction between a direct action of this lipid with the Ca^{2+}-ATPase itself and interference with the environment of the enzyme, since the work was performed on membrane preparations [38]. Our results, using purified enzyme, indicate that a direct interaction of sphingosine with the Ca^{2+}-ATPase indeed occurs.

It has been reported that sphingosine could act as a CaM antagonist in view of the fact that this lipid is able to inhibit some CaM-regulated enzymes [39]. The results obtained in the present work do not support this view, at least with respect to the Ca^{2+}-ATPase, since the observed inhibitory effect of sphingosine was essentially the same, either in the presence or in the absence of CaM.

It is interesting to note that sphingosine is a potent inhibitor of protein kinase C [19,20,39], whereas this kinase stimulates the plasma-membrane Ca^{2+}-ATPase [3,7]. It is tempting to speculate

that sphingosine, after being formed from ceramide, could have a double inhibitory role on Ca^{2+}-ATPase activity, indirectly through protein kinase C and directly on the enzyme, which would result in a rise in the intracellular Ca^{2+} concentration.

Curiously, neither ceramide 1-phosphate nor sphingosine 1-phosphate affected Ca^{2+}-ATPase activity. This means that the hydroxy group that is removed after phosphorylation of the precursors is essential for recognition of the enzyme by the sphingolipids. This suggestion is also supported by the lack of effect of sphingomyelin on this enzyme, as this precursor possesses only one hydroxy group. On the other hand, the antagonistic effects of ceramide and sphingosine also indicate that at least one of the two hydrocarbonated chains is essential for the stimulatory effect on the Ca^{2+}-ATPase, in view of the fact that when this chain is substituted by an amine group upon conversion of ceramide in sphingosine, the product inhibits the enzyme.

Taken together, the results obtained in this study suggest that the plasma-membrane Ca^{2+}-ATPase can be regulated antagonistically by ceramide and sphingosine. Thus these two second messengers could influence intracellular Ca^{2+} concentration directly after an appropriate signal. It is worth mentioning in this context that, in some systems, ceramide acts concomitantly with a lowering of intracellular Ca^{2+} [35]. Conversely, the rise of sphingosine is usually accompanied by an increase in the intracellular Ca^{2+} concentration [20,34,40,41]. These two facts are in accordance with the dual role, reported here, that these sphingolipids have with respect to the Ca^{2+}-ATPase.

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