Role of arginine residues in the stimulation of the smooth-muscle plasma-membrane Ca^{2+} pump by negatively charged phospholipids

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Negatively charged phospholipids strongly stimulate the purified plasma membrane Ca^{2+} pump of erythrocytes [Enyedi, Flura, Sarkadi, Gardos & Carafoli (1987) *J. Biol. Chem.* **262**, 6425–6430] and of smooth muscle [Missiaen, Raeymaekers, Wuytack, Vrolix, De Smedt & Casteels, (1989) *Biochem. J.* **263**, 687–694]. We have investigated the role of arginine residues in the interaction of these acidic phospholipids with the smooth-muscle Ca^{2+} transport ATPase. The arginine-modifying reagent phenylglyoxal inhibited the ATPase activity in a time-dependent fashion by decreasing the V_{max} of the Ca^{2+} -activation curve. Low concentrations of PtdIns, PtdIns4P, PtdIns(4,5) P_2 , phosphatidylserine and phosphatidic acid partially prevented this inactivation. This protective effect was however not apparent at higher concentrations of PtdIns4P, PtdIns(4,5) P_2 and phosphatidic acid, which may be related to the previously observed inhibition of the enzyme at higher concentrations of these phospholipids. These findings indicate that the functionally important interaction of the acidic lipids with the protein occurs at least partially via arginine residue(s).

INTRODUCTION

Negatively charged phospholipids strongly stimulate the purified plasma-membrane Ca^{2+} pump from erythrocytes and from smooth muscle by increasing the V_{max} , the affinity and the co-operativity for Ca^{2+} (Enyedi *et al.*, 1987; Missiaen *et al.* 1989a). The order of potency in stimulating the enzyme [PtdIns(4,5) P_2 > PtdIns4P > PtdIns $\approx$ phosphatidylserine (PS) $\approx$ phosphatidic acid (PA)] is proportional to the number of negative charges on these lipids (Missiaen *et al.*, 1989a). This stimulative effect could be reduced appreciably by complexing the negative charges with polyamines or neomycin (Missiaen *et al.*, 1989b). Because negative charges on the lipids seemed to be important for this stimulative effect, we addressed the question of whether the lipids interact with positive charges on the Ca^{2+} -transporting enzyme. It has been observed in many studies that arginine residues are involved in the interactions of enzymes with phosphate-containing ligands (see Schneider, 1978, for a review). This is inferred from studies on the effects of specific arginine-modifying α -carbonyl reagents such as phenylglyoxal (Takahashi, 1968). Inactivation by arginyl-residue-modifying reagents of the (Ca^{2+} + Mg^{2+})-ATPase in human erythrocyte membranes (Raess *et al.*, 1985) and in rabbit skeletal-muscle sarcoplasmic-reticulum vesicles (Murphy, 1976) has already been reported. In both of these studies, it was concluded that an arginine residue is critically involved in the binding of ATP to a low-affinity allosteric site. This paper describes a protective effect of negatively charged phospholipids on the inactivation of the Ca^{2+} -transporting ATPase by phenylglyoxal, indicating a role for arginine in the lipid-protein interaction.

EXPERIMENTAL

The plasma-membrane Ca^{2+} -transporting ATPase was purified from the antral part of pig stomach smooth muscle using calmodulin-affinity chromatography. Basically, the method of Kosk-Kosicka *et al.* (1986) was used, with minor modifications as described by Vrolix *et al.* (1988). The ATPase was re-activated by phospholipid suspensions as described previously (Missiaen *et al.*, 1989a). The phospholipid mixtures were added at a ratio of 1 mg of phospholipid/mg of protein and consisted of 100 % phosphatidylcholine (PC) or of 80 % PC and 20 % (w/w) negatively charged lipids (PtdIns, PtdIns4P, PtdIns(4,5) P_2 , PS or PA), unless indicated otherwise.

A 10 μl portion of the re-activated ATPase (corresponding to 2 μg of protein) was added to 20 μl of reaction buffer to obtain 30 μl of the following solution: 5 mM-phenylglyoxal, 0.1 M-N-ethylmorpholine, 40 mM-KCl, 6 mM-Hepes, 0.7 mM-MgCl₂ and 1.2 mM-EGTA, pH 7.7. As a control, the ATPase was preincubated in the same medium without phenylglyoxal. The ATPase was incubated with phenylglyoxal at 25 °C for 150 s, unless otherwise indicated.

A 20 μl portion of this ATPase-containing medium was then transferred to 580 μl of ATPase assay medium of the following composition (at 37 °C): phosphoenolpyruvate, 1.5 mM; pyruvate kinase, 40 units/ml; lactate dehydrogenase, 40 units/ml; imidazole/HCl (pH 6.9), 30 mM; MgCl₂, 5.4 mM; KCl, 100 mM; K-EGTA, 0.5 mM; NaN₃, 5 mM; ATP, 5 mM; CaCl₂, 0.5 mM (free Ca^{2+} concentration of 6 μM) and NADH, 0.2 mM. No calmodulin was added in these experiments. In the experiments shown in Fig. 2, Ca^{2+} was added in a cumulative way to obtain a sequence of free Ca^{2+}

Abbreviations used: (Ca^{2+} + Mg^{2+})-ATPase, (Ca^{2+} + Mg^{2+})-activated ATPase; PS, phosphatidylserine; PC, phosphatidylcholine; PA, phosphatidic acid.

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Fig. 1. Inactivation of the plasma-membrane Ca^{2+} pump by phenylglyoxal

The ATPase, purified by means of calmodulin-affinity chromatography, was re-activated by adding phospholipids (1 mg/mg of ATPase). For re-activation, PC (●) or a mixture of 80% PC and 20% PtdIns4P (○) was used. The specific activity of the PC-re-activated enzyme was $0.6 \mu\text{mol/min per mg}$ of protein, whereas that of the PtdIns4P-re-activated enzyme was $1.8 \mu\text{mol/min per mg}$ of protein. The re-activated ATPase was preincubated at 25°C for the time intervals indicated in a medium containing 5 mM-phenylglyoxal, 0.1 M-N-ethylmorpholine, 40 mM-KCl, 6 mM-Hepes, 0.7 mM- MgCl_2 and 1.2 mM-EGTA at pH 7.7. The $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity remaining after this preincubation was measured at $6 \mu\text{M}$ - Ca^{2+} , without calmodulin, and is expressed as a percentage of the control, which was preincubated in the same medium without phenylglyoxal. The results are means $\pm$ S.E.M. for three determinations.

concentrations of 0.1 μM , 0.3 μM , 1.0 μM , 3.0 μM , 10 μM and 100 μM . The Mg^{2+} concn. was kept constant at 1 mM.

The linearity of the ATPase activity with time indicated that after diluting the reaction mixture in the ATPase assay medium, the phenylglyoxal-induced inhibition of the ATPase remained unchanged.

Protein concentrations were determined by the method of Lowry *et al.* (1951), using serum albumin as a standard.

RESULTS AND DISCUSSION

The purification of the plasma-membrane Ca^{2+} -transporting ATPase in the absence of lipids yields an inactive enzyme that can be re-activated by adding phospholipids (Vrolix *et al.*, 1988; Missiaen *et al.* 1989a). The ATPase was re-activated by adding PC, or PC plus acidic phospholipid, and then the mixture was preincubated at 25°C for various times in the presence or in the absence of 5 mM-phenylglyoxal. The $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity was subsequently determined. During preincubation in the absence of phenylglyoxal, the ATPase activity decreased by up to 15% and this decline was identical regardless of the presence or absence of negatively charged phospholipids (results not shown). The

Fig. 2. Effect of phenylglyoxal on the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity measured at different concentrations of free Ca^{2+}

The $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity (in $\mu\text{mol/min per mg}$ of protein) is expressed as a function of the free Ca^{2+} concentration (in μM) either without (○) or after preincubation for 2.5 min with 5 mM-phenylglyoxal (●) at 25°C . The enzyme was re-activated by 100% PC. The results are expressed as means $\pm$ S.E.M. for three determinations.

values obtained in the presence of phenylglyoxal were normalized to those obtained in its absence.

As shown in Fig. 1, the inactivating effect of 5 mM-phenylglyoxal on ATPase preparations previously re-activated by 100% PC was greater than that on the ATPase re-activated by 80% PC and 20% PtdIns4P. Although this inactivating effect of phenylglyoxal was consistently observed, its magnitude differed amongst the different batches of enzyme. The reason for this variability was not clear. The effect of 5 mM-phenylglyoxal on the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase re-activated by PC was measured at different concentrations of free Ca^{2+} (Fig. 2). Preincubation of the enzyme for 2.5 min with 5 mM-phenylglyoxal significantly reduced the V_{max} , whereas the affinity of the enzyme for Ca^{2+} was not affected.

In the experiment in Fig. 1, both the preincubation and the subsequent measurement of the ATPase were done either in the presence of PC alone, or in the presence of PC plus PtdIns4P. In the experiment represented in Table 1, the protocol was altered such that the preincubation was done either in PC alone, or in PC plus PtdIns4P, and the ATPase activity of both of these enzymes was subsequently measured in the presence of both PC and PtdIns4P. The PC-re-activated ATPase was first incubated in a medium containing 0.1 M-N-ethylmorpholine, 40 mM-KCl, 6 mM-Hepes, 0.7 mM- MgCl_2 and 1.2 mM-EGTA (in the absence of phenylglyoxal), and after 150 s PtdIns4P was added (condition 1, Table 1). The enzyme activity subsequently measured was identical with that obtained by immediately re-activating the enzyme with PC plus PtdIns4P and then preincubating the enzyme in the above medium (condition 3, Table 1). If, however, PtdIns4P had been added before or after the preincubation in the presence of 5 mM-phenylglyoxal, the ATPase activity was significantly altered ($P < 0.05$). The

Table 1. Inactivation of the plasma-membrane Ca²⁺ pump by phenylglyoxal

In conditions 1 and 2, the ATPase was re-activated with 1 mg of PC/mg of ATPase and thereupon the enzyme was further preincubated for 150 s at 25 °C in a medium containing 0.1 M-N-ethylmorpholine, 40 mM-KCl, 6 mM-Hepes, 0.7 mM-MgCl₂ and 1.2 mM-EGTA at pH 7.7 (condition 1) or in the same medium supplemented with 5 mM-phenylglyoxal (condition 2). The ATPase activity was finally determined after addition of another 1 mg of lipid mixture, containing 80 % PC plus 20 % PtdIns4P, to 1 mg of ATPase. In conditions 3 and 4, the re-activation of the ATPase was obtained by addition of 1 mg of pure PC and 1 mg of an 80 % PC and 20 % PtdIns4P mixture to 1 mg of ATPase. Subsequently the enzyme was preincubated in the medium described above without (condition 3) or with (condition 4) 5 mM-phenylglyoxal. The (Ca²⁺ + Mg²⁺)-ATPase activities after this preincubation were measured at 6 μM-Ca²⁺, without calmodulin, and expressed as μmol/min per mg of protein (means ± S.E.M. for four determinations on four different batches of enzyme).

	Preincubation		(Ca ²⁺ + Mg ²⁺)-ATPase activity (μmol/min per mg of protein)
	PtdIns4P	Phenylglyoxal	
1.	—	—	1.62 ± 0.04
2.	—	+	0.69 ± 0.02
3.	+	—	1.78 ± 0.08
4.	+	+	1.29 ± 0.06

inhibition of the ATPase activity was more pronounced if PtdIns4P was absent from the preincubation medium than if it was present (conditions 2 and 4 in Table 1). This finding again indicates that PtdIns4P partly protects the enzyme against arginine modification.

We also have compared the protecting effect of several negatively charged lipids, each at a relative concentration of 20 % balanced by 80 % PC, on the inactivation of the ATPase by phenylglyoxal. As compared with PC, a significant protection was observed after re-activation of the enzyme by PtdIns4P, PS and, to a lesser extent, PtdIns. Whereas 5 mM-phenylglyoxal inactivated the PC-stimulated enzyme by 51.0 ± 1.6 % (*n* = 4), re-activation by 80 % PC and 20 % PtdIns4P, PS or PtdIns was only inhibited by 34.4 ± 2.7 % (*n* = 4), 37.8 ± 1.6 % (*n* = 4) or 41.8 ± 1.7 % (*n* = 4) respectively. No protection was however observed after re-activating the enzyme with 80 % PC plus 20 % PA, or, surprisingly, with 80 % PC plus 20 % PtdIns(4,5)P₂ [inhibitions of 51.8 ± 1.5 % (*n* = 4) and 51.1 ± 1.5 % (*n* = 4) respectively]. We previously observed that the stimulative effect of the latter two lipids on the activity of the purified plasma-membrane Ca²⁺ pump only occurred at low concentrations of the lipid, whereas at higher concentrations [over 10 % PtdIns(4,5)P₂ and 20 % PA] the enzyme was inhibited (Missiaen *et al.* 1989a). Although the reason for this biphasic action is not well understood, it was of interest to investigate whether lower concentrations of PtdIns(4,5)P₂ would protect the enzyme against inactivation by phenylglyoxal. This is illustrated in Table 2. Whereas addition of 20 % PtdIns(4,5)P₂ failed to protect

Table 2. Inactivation of the (Ca²⁺ + Mg²⁺)-ATPase by phenylglyoxal at different concentrations of PtdIns(4,5)P₂ and PtdIns4P

The ATPase was re-activated by adding different concentrations of PtdIns(4,5)P₂ or PtdIns4P, balanced with PC. The percentage inactivation of the (Ca²⁺ + Mg²⁺)-ATPase at 6 μM-Ca²⁺ by 5 mM-phenylglyoxal was determined as described in the legend of Fig. 1. The results are expressed as means ± S.E.M. for four determinations.

Lipid	Concentration (%)	Inactivation (%)
PtdIns(4,5)P ₂	0	59.3 ± 1.7
	2	48.1 ± 2.5
	5	53.1 ± 2.5
	20	62.7 ± 3.5
PtdIns4P	0	60.5 ± 3.4
	5	46.0 ± 4.4
	10	44.7 ± 3.7
	20	46.9 ± 1.4
	50	55.2 ± 2.1

the enzyme, 2 or 5 % of this lipid actually did so. A similar effect as that seen for PtdIns(4,5)P₂ could be observed for low concentrations of PA (results not shown). Also, the protective effect of PtdIns4P against arginine modification was dependent on the concentration of the lipid. Whereas PtdIns4P at 20 % effectively prevented the inactivation of the enzyme, as shown above, this effect became less pronounced at higher concentrations (Table 2): the protection at 50 % PtdIns4P was less than that at 20 %. The explanation of this phenomenon is not straightforward. However, the inhibition of the ATPase activity above a certain lipid concentration (Missiaen *et al.*, 1989a) might be linked to the decline of the protective effect of acidic lipids at higher concentrations.

The kinetics of this inhibition of the smooth-muscle enzyme did not depend on the presence of 1 mM-ATP during the incubation with phenylglyoxal (results not shown), and ATP was therefore omitted in the present experiments. These results on the smooth-muscle enzyme thereby differ from those obtained on the membrane-bound human erythrocyte (Ca²⁺ + Mg²⁺)-ATPase. In the latter enzyme, the binding of ATP to its low-affinity binding site decreased the effect of arginine modification (Raess *et al.*, 1985). A possible explanation for this discrepancy could be that we have studied the purified enzyme in the presence of detergent, whereas this was not the case in the study of Raess *et al.* (1985). Detergents have been reported to occupy the low-affinity ATP-regulatory site on the (Na⁺ + K⁺)-ATPase (Huang *et al.* 1985). If the same phenomenon applied to the plasma-membrane (Ca²⁺ + Mg²⁺)-ATPase, the low-affinity ATP-binding site would be occupied by the detergent and therefore no effect at ATP on the arginine modification would be observed.

The stimulation of the (Ca²⁺ + Mg²⁺)-ATPase by negatively charged phospholipids is not restricted to the smooth-muscle enzyme, but it is also observed for the corresponding ATPases from human (Enyedi *et al.*, 1987) and pig (Missiaen *et al.*, 1989a) erythrocytes. It may therefore be a general characteristic for different isoforms

of this transport enzyme. The ATPase from pig stomach smooth muscle used in this study has not yet been sequenced but it is probably very similar to sequenced isoforms from rat brain (Shull & Greeb, 1988) and from a human teratoma cell line (Verma *et al.*, 1988). At present, one can only speculate about the location of the arginine residue(s) involved in the activation of the plasmalemmal ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase by negatively charged phospholipids. It is likely that several arginines, and possibly also other amino acids, contribute to the ionic interaction with the negatively charged phospholipids. However, according to studies on the erythrocyte enzyme, the phospholipid-binding domain would be restricted to a 5 kDa tryptic fragment (Enyedi *et al.*, 1987). It is not known at present whether this segment is located at the *N*-terminal or at the *C*-terminal end of a 81 kDa tryptic fragment (Enyedi *et al.*, 1987). It may be worth mentioning that an arginine residue is present at a strategic position in a region of the sequence that is very probably located within the 5 kDa *C*-terminal region of the 81 kDa-fragment [Arg-1015 in the class 1 and Arg-993 in the class 2 sequence from rat brain (Shull & Greeb, 1988) and Arg-1065 in the sequence from a human teratoma cell line (Verma *et al.*, 1988)]. This location is between the putative last *C*-terminal transmembrane segment and a regulatory domain known to modulate the activity of the ATPase by phosphorylation and also by interaction with calmodulin (Verma *et al.*, 1988). The location of this arginine residue close to a hydrophobic stretch of amino acids is a prerequisite for interaction with the head group of a phospholipid of the membrane bilayer.

In conclusion, the arginine-modifying reagent phenylglyoxal inhibited the ATPase activity in a time-dependent fashion. This indicates that one or more arginine residues might be crucial for optimal function of the enzyme. Very low concentrations of negatively charged lipids partially prevented this inactivation, indicating that these lipids interact at least partially with the protein via

electrostatic interaction with arginine residues. The loss of protective effect at higher concentrations of these lipids remains unexplained, but could be related to the inhibition of the enzyme if it is surrounded by too high a concentration of these negatively charged phospholipids.

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