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EFFECTS OF FATTY ACIDS ON ACTIVITY AND CALMODULIN BINDING OF Ca^{2+}-ATPase OF HUMAN ERYTHROCYTE MEMBRANES

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Activation and inhibition of Ca^{2+}-ATPase of calmodulin-depleted human erythrocyte membranes by oleic acid and a variety of other fatty acids have been measured. Low concentrations of oleic acid stimulate the enzyme activity, both in the presence and in the absence of calmodulin. Concomitantly, the affinity of the membrane bound enzyme to calmodulin progressively decreases due to competitive interactions of calmodulin and oleic acid with the enzyme. Removal of oleic acid from the membrane by serum albumin extinguishes the activating effect of oleic acid and restores the ability of the enzyme to bind calmodulin with high affinity. High concentrations of oleic acid induce an almost complete and irreversible loss of enzyme activity which cannot be abolished by removal of oleic acid. Despite a complete loss of enzyme activity, binding of calmodulin to membranes is approximately normal after removal of oleic acid. Activities of (Na^+ + K^+)-ATPase, Mg^{2+}-ATPase and acetylcholine esterase, as well as the total protein content, show no gross changes upon treatment of membranes with increasing amounts of oleic acid, which seems to exclude that membrane solubilisation by oleic acid causes an inactivation of the enzyme.

Introduction

Previous studies have shown that calmodulin is not unique as an activator of the Ca^{2+}-transport ATPase of erythrocyte membranes. The activity of the enzyme can be considerably increased, in the absence of calmodulin, by unsaturated fatty acids, acidic phospholipids, phospholipase A_2, trypsin and even by Ca^{2+} itself [1–5]. Considering the effects of fatty acids and acidic phospholipids on the activity of the purified Ca^{2+}-ATPase, interactions with a regulatory portion of the enzyme of the basis of the net negative charges and/or on the basis of hydrophobic interactions have been suggested to be common mechanisms [3].

In the present paper, the effects of oleic acid and other fatty acids on the membrane bound Ca^{2+}-ATPase have been studied, especially with respect to changes of calmodulin binding to ghosts. Analysis indicates that oleic acid and calmodulin competitively interact with the calmodulin binding part of the membrane-bound Ca^{2+}-ATPase. Low concentrations of fatty acids activate the enzyme even in its calmodulin-activated form whilst high concentrations cause an irreversible inactivation.

Materials and Methods

Materials. All chemicals were of reagent grade. Fatty acids were the products of Serva (Heidelberg), and fatty-acid free human serum albumin was from VEB Institut für Impfstoffe (Dessau).

General. The techniques for collection of blood, preparation of calmodulin, assay of Mg^{2+}-ATPase and Ca^{2+}-ATPase by P_i liberation, assay of calmodulin binding to ghosts and calculation of free Ca^{2+} concentration have been described in recent publications [5–7]. A slight modification

was introduced in the assay of calmodulin binding to ghosts [6] consisting of 60 min incubation instead of 15 min, in order to establish equilibrium between free and bound calmodulin which takes about 45 min in the presence of oleic acid and only 5-10 min in its absence. The activities of acetylcholine esterase and ouabain sensitive ($Na^+ + K^+$)-ATPase of ghosts were determined according to Weber [8] and Post et al. [9], respectively. The Ca^{2+}-ATPase reported is the difference between the activity in the presence of 2.6 μM free Ca^{2+} and 1 mM free Mg^{2+} and the activity in the presence of 10^{-8} M free Ca^{2+} and 1 mM free Mg^{2+}. The latter activity is referred to as Mg^{2+}-ATPase. Membrane sidedness assays showed that our preparations did not contain resealed vesicles.

Preincubation of ghosts with fatty acids. Ghosts were preincubated with shaking at 37°C in preincubation medium containing about 3 mg ghost protein/ml, 50 mM Tris-HCl (pH 7.2), 50 mM KCl, 0.05 mM EGTA and appropriate additions of potassium salts of oleic acid and a few other fatty acids from 50 mM stock solutions. The preincubation was stopped after 10 min by the addition of 10 vols. of ice-cold preincubation medium without fatty acids. The ghosts were pelleted by centrifugation for 10 min at 23000 × *g*. Aliquots of the pelleted ghosts were mixed with assay medium (0.1 mg of ghost protein/ml, 50 mM Tris-HCl (pH 7.2), 50 mM KCl, 3 mM $MgCl_2$, 2 mM ATP, 2 mM EGTA and calculated amounts of $CaCl_2$ in order to obtain free Ca^{2+} concentrations as indicated or with 'calmodulin loading medium' (50 mM Tris-HCl (pH 7.2), 50 mM KCl, 0.05 mM $CaCl_2$, 1–100 μg of purified calmodulin from human erythrocytes and about 2 mg of ghost protein/ml).

Stock solutions of potassium salts of fatty acids were prepared by appropriate additions of free fatty acids to 50 mM KOH, boiling for 2 min and cooling the clear solution to about 40°C. A single batch of ghosts was used for the assay reported in a single figure of this paper.

Removal of fatty acids from ghosts. After preincubation of ghosts with fatty acids, the incubates were mixed with 6 vols. of a medium containing 50 mM Tris-HCl (pH 7.2), 50 mM KCl, 0.1 mM EGTA and 2% (w/v) fatty acid-free serum albumin. After 10 min incubation at 37°C, the ghosts were pelleted by centrifugation, washed twice with 50 vols. of wash solution containing 50 mM Tris-HCl (pH 7.2), 50 mM KCl and 0.05 mM EGTA. Determinations of free fatty acid content of membranes revealed that more than 90% of fatty acids were removed from the membranes by this procedure. Aliquots of the pelleted ghosts were resuspended in assay medium or in calmodulin loading medium and used for determinations of enzyme activity and calmodulin binding to ghosts.

Determinations of free fatty acids. Loading of membranes with fatty acids was generally determined by measuring the disappearance of fatty acids from the preincubation medium according to Ducombe [10]. Up to 16 μmol fatty acid per mg membrane protein, more than 95% of the fatty acids disappeared from the medium during preincubation. The amount of fatty acids remaining in the medium progressively increased at higher fatty acid concentrations. In order to check the reliability of this method, and also the efficacy of fatty acid removal by serum albumin, in a series of experiments, lipids of the pelleted membranes were extracted and separated according to Taverna and Hanahan [2]. Free fatty acids were then removed from the corresponding spot of the thin layer chromatogram and subjected to a quantitative determination according to Novak [11]. Within the range of experimental error, the results obtained with both methods were in agreement.

Results

Effects of oleic acid on Ca^{2+}-ATPase activity

In the absence of calmodulin, increasing concentrations of oleic acid progressively stimulated Ca^{2+}-ATPase activity severalfold up to a maximum at 0.75 μmol oleic acid/mg membrane protein (Fig. 1A). High concentrations of oleic acid progressively inhibited enzyme activity, which approached zero above 2 μmol oleic acid/mg protein.

In the presence of calmodulin, small concentrations of oleic acid induced a significant stimulation of enzyme activity, which also proceeded to a progressive inhibition with further increasing concentrations of oleic acid. The stimulation by oleic acid is of interest as fatty acids bring about an additional activation of the enzyme, which is maxi-

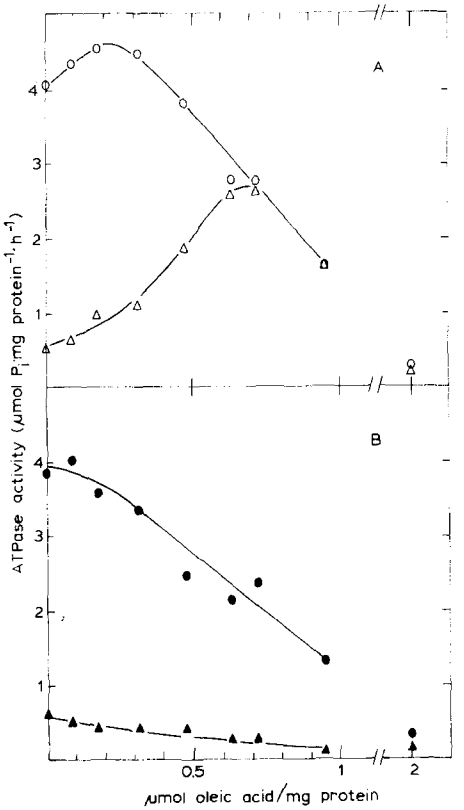

Fig. 1. Concentration dependence of the effect of oleic acid on Ca^{2+}-ATPase activity at 2.6 μM free Ca^{2+}. The results are typical of three similar experiments. (A) In the presence of oleic acid. △, in the absence of calmodulin; ○, in the presence of 0.18 μM calmodulin. (B) After removal of oleic acid. ▲, in the absence of calmodulin; ●, in the presence of 0.18 μM calmodulin.

mally activated by calmodulin.

In order to prove the reversibility of these effects on ATPase activity we removed oleic acid by serum albumin. As shown in Fig. 1B, the stimulation of enzyme activity in the absence or presence of calmodulin was completely abolished. Instead, a progressive inactivation was observed in parallel to the pretreatment with increasing oleic acid concentrations. This holds good for the low activity enzyme in the absence of calmodulin, as well as for the enzyme which is maximally stimulated by addition of calmodulin. Enzyme activity which was near zero in the presence of high concentrations of oleic acid (Fig. 1A), was likewise near zero after removal of oleic acid (Fig. 1B). It could not be restimulated by a second addition of oleic acid (data not shown).

The inactivation of the Ca^{2+}-ATPase by high oleic acid concentrations might be due to a non-specific membrane solubilization. This possibility could be excluded since the activities of other membrane-bound enzymes (Mg^{2+}-ATPase, ($Na^+ + K^+$)ATPase and acetylcholin esterase) showed only a negligible decrease at 2 μmol oleic acid which completely inactivated Ca^{2+}-ATPase (Mg^{2+}-ATPase, 80%; ($Na^+ + K^+$)-ATPase, 96%; acetylcholinesterase, 88%; Ca^{2+}-ATPase, 6% of activity without oleic acid). Moreover, a complete recovery of membrane protein was obtained after pretreatment with oleic acid and pelleting the membranes under standard conditions. No Ca^{2+}-ATPase activity could be detected in the supernatant.

As could be expected, activating and inhibitory effects of oleic acid were not specific and could be mimicked by other saturated and unsaturated fatty acids, and even by sodium dodecyl sulfate in a similar concentration range (Table I). There was no obvious dependence of the activating effect of different fatty acids on their structure.

Effects of oleic acid on calmodulin binding to ghosts

Previous work indicated that the number of calmodulin binding sites in erythrocyte membranes corresponds to the number of Ca^{2+}-ATPase molecules [12,13]. Thus, the effects of oleic acid on calmodulin binding to ghosts were examined. Increasing concentrations of oleic acid progressively

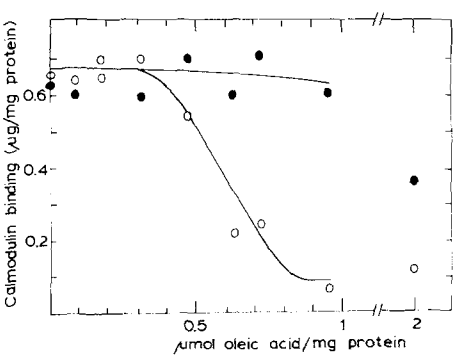

Fig. 2. Concentration dependence of the effect of oleic acid on calmodulin binding to ghosts at 0.6 μM calmodulin and 50 μM free Ca^{2+}. ○, in the presence of oleic acid; ●, after removal of oleic acid.

TABLE I

MAXIMUM ACTIVATION OF Ca^{2+}-ATPase BY DIFFERENT FATTY ACIDS AND SDS

The enzyme activities found in untreated ghosts at 2.6 μM free Ca^{2+}, in the presence and in the absence of 0.18 μM calmodulin, were taken as 100%. The other data are relative values obtained under identical conditions and related to the corresponding controls.

Fatty acid species	Relative activity		Fatty acid concentration at maximum activation (μmol per mg protein)	
	without calmodulin	with calmodulin	without calmodulin	with calmodulin
Control	100	100	-	-
12:0	157	110	1.1	0.7
14:0	329	118	1.1	0.7
16:0	239	111	0.7	0.7
18:0	184	114	0.4	0.4
20:0	141	109	0.4	0.4
18:1	400	117	0.7	0.4
20:4	232	110	0.5	0.4
SDS	380	110	0.9 [a]	0.3 [a]

[a] Final concentration in the assay medium.

diminished calmodulin binding to membranes when measured in the presence of 0.6 μM calmodulin under standard conditions [6]. After removal of oleic acid, however, calmodulin binding was almost completely restored (Fig. 2). This suggests that Ca^{2+}-ATPase was present within the membrane and its ability to bind calmodulin was unaffected.

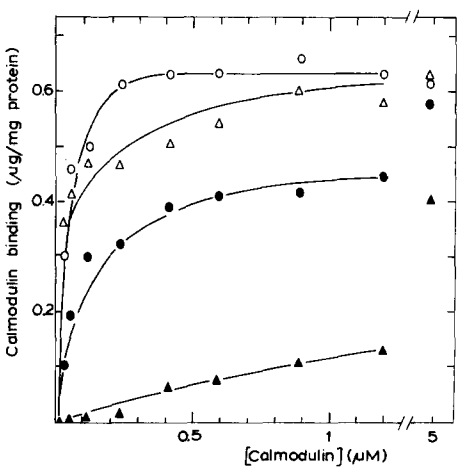

Fig. 3. Effect of increasing calmodulin concentrations on calmodulin binding to ghosts. ○, 0 μmol oleic acid/mg protein; △ 0.4 μmol; ●, 0.8 μmol; ▲1.2 μmol oleic acid/mg protein.

The reversibility of the effect of oleic acid on calmodulin binding suggests a competitive interaction of fatty acids and calmodulin for the Ca^{2+}-ATPase molecule. Thus, we measured calmodulin binding to ghosts in the presence of varying concentrations of calmodulin and oleic acid. The results are shown in Fig. 3. In the presence of different constant concentrations of oleic acid, calmodulin binding to ghosts progressively increased at increasing calmodulin concentrations. Calmodulin concentrations which were necessary to obtain the maximum amount of bound calmodulin increased with increasing concentrations of oleic acid within the membrane. From the binding data presented it can be concluded that oleic acid decreases the affinity of the membrane bound enzyme to calmodulin but does not alter the number of calmodulin binding sites. Binding curves obtained after removal of oleic acid (not shown) coincided exactly with that of untreated ghosts.

Discussion

Activating effects of fatty acids and/or acidic phospholipids seem to be a common phenomenon of calmodulin-dependent enzymes, as shown for

cyclic nucleotide phosphodiesterase [14,15], myosin light chain kinase [16] and purified Ca^{2+}-ATPase from erythrocyte membranes [3]. The results presented in this paper indicate that fatty acids exert different effects on the membrane-bound Ca^{2+}-ATPase. At low to moderate oleic acid concentrations an activation of the enzyme predominates in the absence of calmodulin. In addition, our results show for the first time that the activation of Ca^{2+}-ATPase by oleic acid is accompanied by a decrease of calmodulin affinity to the enzyme. Both effects can be abolished by removal of oleic acid. These data clearly indicate that there is a competitive interaction of oleic acid and calmodulin for the calmodulin binding site of the enzyme. It seems possible that acidic phospholipids, besides their ability to activate the Ca^{2+}-ATPase [12], also decrease the calmodulin affinity to the enzyme.

When the enzyme is fully activated by calmodulin very low concentrations of oleic acid cause an additional activation. Possibly, this effect is the result of an increase of membrane fluidity induced by fatty acids [17]. It is probable that a limited degree of membrane structure perturbation facilitates the conformational change of the ATPase necessary for optimal activity [18].

At high oleic acid concentrations only a progressive and irreversible loss of enzyme activity could be observed in the presence and in the absence of calmodulin. Removal of oleic acid, which abolishes its activating effect, reveals that enzyme inactivation begins even at very low oleic acid concentrations and proceeds in parallel to the amount of oleic acid applied during pretreatment. Despite a complete inactivation of Ca^{2+}-ATPase by high oleic acid concentrations, the activities of three other membrane-bound enzymes as well as the binding of calmodulin to Ca^{2+}-ATPase show only minor changes. Thus, the loss of Ca^{2+}-ATPase activity is not a consequence of membrane solubilization. Probably, minor membrane components required for Ca^{2+}-ATPase activity are specifically removed during pretreatment with oleic acid. Experiments in this direction are being made.

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