Modulation of ATPase Activities of Human Erythrocyte Membranes by Free Fatty Acids or Phospholipase A$_2$

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Summary. The artificial insertion of increasing amounts of unsaturated fatty acids into human erythrocyte membranes modulated ATPase activities in a biphasic manner, depending on the number and position of double bonds, their configuration, and the chain length. Uncharged long-chain fatty acid derivatives with double bonds and short-chain fatty acids were ineffective. Stearic acid stimulated Na$^+$K$^+$-ATPase only. Anionic and non-ionic detergents and α-lysophosphatidylcholine failed to stimulate ATPase activities at low, and inhibited them at high concentrations.

Mg^{2+}-ATPase activity was maximally enhanced by a factor of 2 in the presence of monoenoic fatty acids; half-maximal stimulation was achieved at a molar ratio of *cis*(*trans*)-configurated C18 acids/membrane phopholipid of 0.16 (0.26).

Na$^+$K$^+$-ATPase activity was maximally augmented by 20% in the presence of monoenoic C18 fatty acids at 37 °C. Half-maximal effects were attained at a molar ratio oleic (elaidic) acid/phospholipid of 0.032 (0.075). Concentrations of free fatty acids which inhibited ATPase activities at 37 °C were most stimulatory at reduced temperatures. At 10 °C, oleic acid increased Na$^+$K$^+$-ATPase activity fivefold (molar ratio 0.22).

Unsaturated fatty acids simulated the effect of calmodulin on Ca^{2+}-ATPase of native erythrocyte membranes (i.e., increase of V_{max} from 1.6 to 5 μmol PO$_4^{3-}$ · phospholipid^{-1} · hr^{-1}, decrease of K'_{Ca} from 6 μM to 1.4–1.8 μM). Stearic acid decreased K'_{Ca} (2 μM) only, probably due to an increase of negative surface charges.

A stimulation of Mg^{2+}-ATPase, Na$^+$K$^+$-ATPase, and Ca^{2+}-ATPase could be achieved by incubation of the membranes with phospholipase A$_2$.

An electrostatic segregation of free fatty acids by ATPases with ensuing alterations of surface charge densities and disordering of the hydrophobic environment of the enzymes provides an explanation of the results.

Key words ATPase activity · human erythrocyte membranes · free fatty acids · phospholipase A$_2$ · membrane fluidity · surface charges

Introduction

Alterations of the activity of membrane-embedded enzymes result from changes in the fatty acid composition of membrane phospholipids [for a review, see Ref. 19]. However, in recent years there is increasing evidence that even free fatty acids may serve as modulators of membrane functions: free fatty acids were found to stimulate guanylate cyclase [35], cyclic nucleotide phosphodiesterase [14, 37], brain phosphatidylinositol phosphodiesterase [16], and either to stimulate [13, 23] or to inhibit adenylate cyclase [2, 9]. For example, Orly and Schramm [23] found that the insertion of free fatty acids enhanced hormone-activated adenylate cyclase from turkey erythrocytes up to 25-fold. Fluorescence polarization measurements [13] confirmed their suggestion that unsaturated fatty acids which were most potent in stimulation of adenylate cyclase may fluidize plasma membranes. Additionally, free fatty acids increase the density of negative surface charges of cell membranes which *per se* may act as regulators of membrane-bound enzymes [1, 36]. The work of Fiehn and Hasselbach [12] and The and Hasselbach [33] has shown that fatty acids are sufficient to reconstitute the calcium-dependent ATPase of delipidated sarcoplasmic membranes.

Since fluidizing and electrostatic effects should also affect other lipid-dependent reactions, the influence of free fatty acids on ATPases of human erythrocyte membranes was investigated. In the present study it could be shown that the incorporation of free fatty acids into native erythrocyte membranes or their liberation by phospholipase A$_2$ enhances Mg^{2+}-ATPase, Na$^+$K$^+$-ATPase, and Ca^{2+}-ATPase activities. Unsaturated fatty acids mimick the effect of calmodulin by increasing V_{max} and Ca^{2+} affinity, whereas stearic acid increases Ca^{2+} affinity only.

Materials and Methods

Chemicals

Fatty acids and their derivatives, α-lysophosphatidylcholine, phospholipase A$_2$ (from *Naja naja* venom), fatty acid-free albumin, Triton X-100, and Triton WR-1339 were obtained through Sigma (München, FRG), aprotinin through Serva (Heidelberg, FRG), and ethylphenylpolyethyleneglycol (on the average nonamer, Nonidet®) through Fluka (Neu-Ulm, FRG). Detergents were

chromatographically purified by a weak cation exchange resin before use. $(1-^{14}C)$oleic acid with a specific activity of 57 mCi/mmol was from Amersham Buchler GmbH (Braunschweig, FRG) and Protosol® (a 0.5 M quarternary ammonium solution) from NEN Chemicals GmbH (Dreieichenhain, FRG). All other substances were purchased in analytical grade from Merck (Darmstadt, FRG).

Preparation of Human Erythrocyte Membranes

Membranes were prepared from freshly drawn and washed red cells in analogy to the procedure of Dodge et al. [8]. The use of 10 mM Tris/HCl buffer of pH 7.6 with 0.1 mM EGTA yielded calmodulin-deficient membranes [11]. Calmodulin-containing membranes were prepared according to the method of Farrance and Vincenzi [10]: erythrocytes were hemolyzed in isosmotic (310 mosM) imidazole/HCl buffer of pH 7.4, followed by washes with 10 mM Tris/HCl buffer, pH 7.6, without EGTA.

An aliquot of the hemoglobin-free membrane suspension was dissolved in NaOH (final concentration 0.5 M) to determine the protein concentration by the Coomassie method of Sedmak and Grossberg [28]; bovine serum albumin served as standard. The membrane suspension was adjusted to 1 mg protein per ml with buffer and dithioerythritol (final concentration 2 mM). In order to measure the phospholipid-P of the cell membranes, 0.1 ml of the membrane suspension was digested with 0.5 ml of perchloric acid (70%, wt/vol) at 180 °C. The colorless and cooled digest was diluted with 2 ml of aqua bidest. Phosphate standards were handled in the same way and phosphate was determined using malachite green as described below. Membranes were stored at 4 °C and used only within 2 days after preparation; during this time ATPase activities remained stable.

ATPase Assay[1]

Cleavage of ATP was measured in a total volume of 1 ml in the presence of (final concentrations) 2 mM $MgCl_2$, 2 mM Na_2ATP, 100 mM NaCl, 100 mosM Tris/HCl of pH 7.4; to this basal medium the following agents were added: for Mg^{2+}-ATPase activity 0.1 mM ouabain + 2 mM EGTA; for Mg^{2+}, Na^+K^+-ATPase 20 mM KCl + 2 mM EGTA; for Mg^{2+}, Ca^{2+}-ATPase 0.1 mM ouabain +

i) 0–150 μM $CaCl_2$ or (ii) 5 mM EGTA + 1.5–4.5 mM $CaCl_2$, respectively. The pH values of the media were adjusted to 7.4 at the temperatures at which the ATPase assays were carried out. The incubation temperatures are given in the legends to the figures.

After temperature adjustment of the ATPase media, the ATPase reactions were initiated by addition of 0.2 ml (Mg^{2+}-ATPase and Mg^{2+}, Ca^{2+}-ATPase) or 0.5 ml (Mg^{2+}-ATPase and Mg^{2+}, Na^+K^+-ATPase), respectively, of the membrane suspension. Unless otherwise noted, after 5 and 20 min of incubation 0.2 ml were withdrawn from the reaction mixture and thoroughly mixed with an equal volume of $CHCl_3$ (this procedure requires less than 10 sec to stop the reaction and avoids an acid hydrolysis of ATP). After short centrifugation in a Beckman microfuge, the precipitated protein was found at the water/$CHCl_3$ interface. The samples were stored on ice until inorganic phosphate was measured spectrophotometrically by the Penney method [24] with a slight modification, since it was found that the addition of Nonidet® (600 mg malachite green per liter of aqua bidest. with 0.1 % Nonidet®) increased the accuracy of the assay [for details,

see 27]. ATPase activities were calculated in terms of inorganic phosphate liberated between 5 and (usually) 20 min.

Calculation of free Ca^{2+} Concentrations

The apparent dissociation constant K' of EGTA for Ca in dependence of pH and temperature and the corresponding free Ca^{2+} concentrations of the Ca/EGTA buffers were calculated according to equations given by Scharff [26]. K' was found to be 0.1 μM for pH 7.4 and 0.57 μM for pH 7.0. With molar ratios of Ca/EGTA > 0.9, the calculated free Ca^{2+} concentrations deviate considerably from values measured with the calcium electrode [26]. Therefore, in order to obtain free Ca^{2+} concentrations in the range of 1–5 μM at a molar ratio of Ca/EGTA < 0.9, Ca^{2+}-ATPase was also analyzed at pH 7.0.

For even higher concentrations of free Ca^{2+}, Ca^{2+}-ATPase activities were measured in EGTA-free media. In the absence of added calcium, the calcium content of these media and of the red cell membranes was estimated by atomic absorption spectrophotometry (Philips SP 9 atomic absorption spectrophotometer) according to the method of McDonald et al. [21]. The calculation of free Ca^{2+} was based on total calcium concentrations (endogenous Ca + added Ca) and the logarithmic stability constants of ATP for Mg and Ca quoted by Scharff [26].

Insertion of Fatty Acids into Erythrocyte Membranes

Fatty acids (and derivatives) were dissolved in absolute ethanol; 5-μl aliquots were added from adequate stock solutions to the sonicated ATPase medium without red cell membranes. Controls without fatty acids received an equal volume of ethanol. The sonication of the medium with added fatty acids was terminated after about 1 min; immediately thereafter, the ATPase reaction was started by addition of the membrane suspension as described above.

The completeness of the incorporation of fatty acids into erythrocyte membranes was confirmed with ^{14}C-oleic acid. Red cell membranes corresponding to 0.45 μmol of membrane phospholipid were added to 0.5–200 nmol of ultrasonically dispersed ^{14}C-oleic acid and incubated with magnetic stirring at 37 °C for 15 min. After three ensuing washes by centrifugation and resuspension in 10 ml of 10 mM Tris/HCl buffer at 4 °C, the pellet was dissolved in 0.5 ml of Protosol® (a tissue solubilizer compatible with toluene-based scintillators). The radioactivity was estimated in 10 ml of scintillator (5 g PPO and 0.2 g POPOP in 1 liter of toluene) by liquid scintillation spectrometry in a Packard Tricarb® 2660. At a molar ratio of ^{14}C-oleic acid/membrane phospholipid below one, 95% (±4%, SD) of the added radioactivity was recovered; the incorporation decreased to about 90%, when the molar ratio exceeded one.

Insertion of α-lysophosphatidylcholine (oleoyl) into Erythrocyte membranes

α-Lysophosphatidylcholine was added to the sonicated ATPase media without membranes as described for fatty acids; however, the volume of the ethanolic solution was increased to 10 μl since this led to a higher incorporation into cell membranes. In order to ascertain the completeness of incorporation, membranes were incubated with the lysolipids at 37 °C with magnetic stirring as described above for fatty acids in the absence of ATP. After 15 min of incubation, an aliquot was diluted with a 10-fold volume of Tris/EGTA buffer. Membranes were spun down at 34,000 × g for 20 min in a Sorvall centrifuge. The pellet was resuspended in a small volume of buffer; protein and phospholipid-P were measured as described above. The lipid-P/protein ratio increased

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[1] The terms Mg^{2+}, Ca^{2+}-ATPase and Mg^{2+}, Na^+K^+-ATPase characterize the corresponding enzyme activities including Mg^{2+}-ATPase activity. The terms Ca^{2+}-ATPase and Na^+K^+-ATPase mean that Mg^{2+}-ATPase activity has been determined in parallel and has been subtracted from total enzyme activity.

linearly when increasing amounts of α-lysophosphatidylcholine were added to erythrocyte membranes. The recovery of α-lysophosphatidylcholine-P was found to be $72\pm9\%$ in the range of 32–760 nmol per 0.45 μmol of membrane phospholipid. Higher concentrations of α-lysophosphatidylcholine led to a reduction of recovery of cell membranes by centrifugation. This was probably due to the disintegration of red cell membranes by α-lysophosphatidylcholine micelles.

Treatment with Phospholipase A_2

For measuring the influence of phopholipase A_2 on Mg^{2+}, Ca^{2+}-ATPase, 20 μl of phospholipase A_2 (corresponding to 2 μg) and 0.4 ml of membrane suspension were simultaneously added to the EGTA- free Ca^{2+}-ATPase medium (100 μM $CaCl_2$, total volume of 2 ml). After 5, 10, 15, 20, 25, 30 and 35 min of incubation at 37 °C, 0.2 ml samples were withdrawn from this reaction mixture and assayed for PO_4^{3-} as described above. In parallel control experiments, Mg^{2+}, Ca^{2+}-ATPase was measured either in the presence of fatty acid-free albumin (final concentration 1 mg/ml) with and without phospholipase A_2 or in the absence of both albumin and phospholipase A_2. Additional controls contained 1.4–14 units/ml (final concentration) of the trypsin inhibitor aprotinin.

In similar experiments, after 15 and 25 min of incubation in the presence of phospholipase A_2, the cleavage of ATP by the Ca^{2+}-ATPase was terminated by complexation of free Ca^{2+} with 50 μl of a concentrated EGTA solution (final concentration 2 mM). The residual Mg^{2+}-ATPase activity was determined by following the liberation of PO_4^{3-} until 20 min after addition of EGTA.

The effect of phospholipase A_2 on Mg^{2+}-ATPase and Mg^{2+}, Na^+K^+-ATPase activities was examined by preincubating the original membrane suspension (1 mg protein/ml) at 100 μM $CaCl_2$ in the absence or presence of 0.1 μg (1 μg) of phospholipase A_2 per mg of membrane protein at 37 °C (30 °C). After different lengths of preincubation, 0.5-ml samples of this reaction mixture were withdrawn and subject to the assay for Mg^{2+}-ATPase and Mg^{2+}, Na^+K^+-ATPase activities in the presence of 2 mM EGTA, sufficient for an effective complexation of Ca. The membranes were incubated at the same temperatures at which they had been pretreated. Na^+K^+-ATPase activity was calculated as ouabain inhibitable activity.

Results

1. Preparation of Red Cell Membranes

Different procedures for the preparation of red cell membranes were reinvestigated. An increase of the pH value of the hypotonic lysis and washing buffer from pH 6.4 to 8.4 led to a decrease of protein content (including hemoglobin) relative to phospholipid-P of red cell ghosts. In parallel, all ATPase activities increased. Red cell ghosts prepared with 10 mM buffer of pH 7.6 were hemoglobin-free and exhibited maximal ATPase activities. Independent of the nature of the buffer substance (Tris, Hepes, imidazole), pH 7.6 membranes of red cells from one donor yielded nearly identical Mg^{2+}-ATPase and Na^+K^+-ATPase activities. The results were not influenced by the mode of hemolysis, which was car-

ried out isosmotically in 310 mosm imidazole/HCl buffer or hypotonically in 10 mM Tris/HCl or Hepes/ NaOH or imidazole/HCl buffer of pH 7.6, respectively. Highest Ca^{2+}-ATPase activities of 4.8– 5.0 μmol PO_4^{3-} liberated per μmol membrane phospholipid per hour were measured after isosmotic lysis and subsequent washing with an EGTA-free hypotonic buffer. If 0.1 mM EGTA was included into the hypotonic washing solution, Ca^{2+}-ATPase amounted to 1.5–1.8 μmol PO_4^{3-} per μmol phospholipid per hour only, irrespective of whether the initial lysis was carried out isosmotically or hypotonically. Intermediate Ca^{2+}-ATPase activities were measured after hypotonic lysis and washing of the membranes in an EGTA-free buffer of pH 7.6.

The differences of Ca^{2+}-ATPase activities are apparently due to the binding of calmodulin [4, 10]. Therefore, for defined states of the Ca^{2+}-ATPase, erythrocyte membranes were prepared by two different procedures: 10 mM buffer of pH 7.6 with 0.1 mM EGTA was used to prepare calmodulin-free membranes. For calmodulin containing membranes, red cells were isosmotically lysed in imidazole/HCl buffer of pH 7.6; for the subsequent hypotonic washing, EGTA was omitted.

2. Inhibition of ATPase Activities of pH 7.6 Membranes by Detergents

ATPase activities of pH 7.6 membranes remained unchanged at low concentrations and were inhibited at higher concentrations of detergents like Nonidet® (Fig. 1), Triton X-100, Triton WR-1339, Na-desoxycholate, or saponin, respectively. Similarly, freezing and thawing and ultrasonication of pH 7.6 membranes led to a decrease of Mg^{2+}-ATPase, Na^+K^+- ATPase and Ca^{2+}-ATPase activities. On the other hand, the submaximal ATPase activities of membranes prepared at a pH below 7.2 were found to increase upon addition of detergents and freeze-thawing (Table 1). However, even in comparison with these detergent-treated or freeze-thawed membranes, ATPase activities of untreated pH 7.6 membranes were considerably higher (Table 1).

3. Modulation of ATPase Activities by Free Fatty Acids

The insertion of increasing amounts of unsaturated fatty acids into native erythrocyte membranes modulated Mg^{2+}-ATPase, Na^+K^+-ATPase, and Ca^{2+}- ATPase activities in a biphasic manner. The number and the position of double bonds, their configuration, and the chain length of the fatty acids proved to be critical for the modulation.
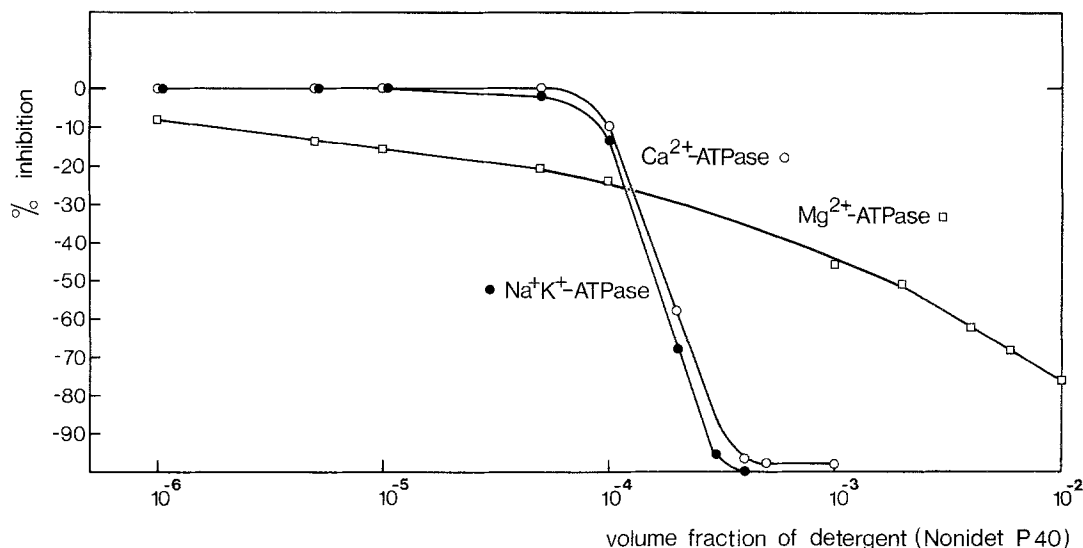
Fig. 1. Inhibition of ATPase activities of pH 7.6 membranes by a non-ionic detergent (Nonidet®). Red cell membranes were incubated at 37 °C in the presence of varying amounts of Nonidet®. The volume fraction of Nonidet® in the reaction mixture is indicated on the abscissa. Ca^{2+}-ATPase activity was measured in an EGTA-free medium at a total calcium concentration of 150 µM. ATPase activities are given in percent of basal enzyme activities without detergent. Each point represents the mean of three experiments. Assuming a molecular weight of about 600 for Nonidet®, concentrations of 160 µM (Na^+K^+-ATPase), 180 µM (Ca^{2+}-ATPase) and 1.6 mM (Mg^{2+}-ATPase) inhibited half-maximally

Table 1. Effect of freeze-thawing and detergent on ATPase activities of pH 6.8 and pH 7.6 membranes

Treatment		Mg^{2+}-ATPase membranes prepared at		Na^+K^+-ATPase membranes prepared at	
		pH 6.8	pH 7.6	pH 6.8	pH 7.6
None		0.21	0.43	0.16	0.77
Freeze-thawing		0.24	0.43	0.24	0.75
Nonidet® 0.01	µl/ml	0.26	0.37	0.16	0.77
Nonidet® 0.1	µl/ml	0.30	0.33	0.38	0.68
Nonidet® 1.0	µl/ml	0.22	0.22	0.00	0.00

ATPase activities are given in µmol PO_4^{3-} liberated per µmol membrane phospholipid per hour. All incubations were carried out at pH 7.4 and 37 °C.

3.1. Modulation of Mg^{2+}-ATPase

Figure 2 shows the concentration-dependent effect of long-chain fatty acids on Mg^{2+}-ATPase activity. The largest increase, about 90%, was produced by monoenoic acids with the double bond in the position 9 or 11 (myristoleic acid, oleic acid, elaidic acid, cis- and trans-vaccenic acid). The activation exhibited a maximum at a molar ratio of cis-configurated fatty acids/membrane phospholipid of about 0.9 (petroselinic acid, oleic acid, cis-vaccenic acid, nervonic acid) or 0.4 (myristoleic acid), respectively. The trans-configurated elaidic acid and trans-vaccenic acid stimulated the enzyme maximally at a

molar ratio of 1.8. The ED_{50} was about the same for the cis-configurated C-18-acids (molar ratio 0.15–0.17), whereas the trans-configurated isomers activated half-maximally at a molar ratio of 0.26. Stearic acid was ineffective in stimulating Mg^{2+}-ATPase, but inhibited the enzyme at a molar ratio of free fatty acid/phospholipid above 3. Cis-vaccenyl alcohol, cis-vaccenic acid methyl ester, and cis-vaccenyl acetate were found to be without an effect, just as short-chain fatty acids (valeric acid, caproic acid, crotonic acid) in the concentration range investigated.

Polyunsaturated fatty acids stimulated Mg^{2+}-ATPase maximally at a molar ratio of fatty acid/phospholipid of 0.3 (Fig. 2B). Fatty acids with 2–3 double bonds already attained their half-maximal effects at a molar ratio of 0.046–0.056. Concerning the maximal effect, they were inferior to the monoenoic analogues. The maximal stimulation was also smaller when fatty acids contained a double bond in position 6; this results from a comparison of petroselinic acid with oleic acid and cis-vaccenic acid (Fig. 2A), and of γ-linolenic acid with linolenic acid (Fig. 2B). Arachidonic acid and ricinoleic acid which activated half-maximally at molar ratios of 0.03 or 0.002, respectively, were found to be particularly effective to stimulate Mg^{2+}-ATPase at low concentrations; however, the maximal increase amounted to only 40% in the case of ricinoleic acid.

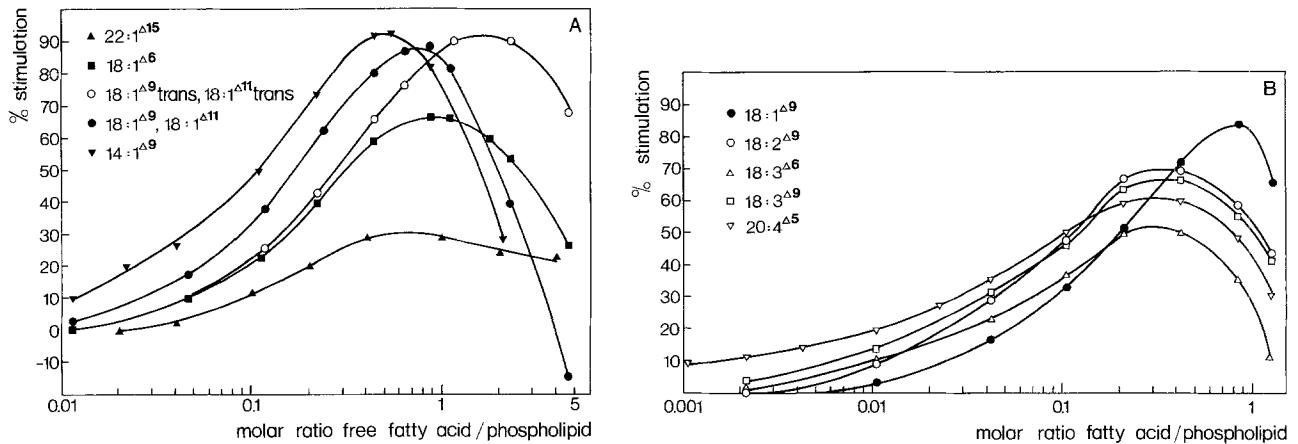Figure 3 demonstrates alterations in the temperature dependency of the Mg^{2+}-ATPase of eryth-

Fig. 2. Effect of various monoenoic (*A*) or polyunsaturated (*B*) fatty acids, respectively, on Mg^{2+}-ATPase activity of erythrocyte membranes. Human erythrocyte membranes (0.45 μmol phospholipid per ml of reaction mixture) were incubated with ultrasonically dispersed fatty acids in a magnetically stirred medium at 37 °C (final volume 1 ml). Mg^{2+}-ATPase activity in the presence of fatty acids is given in percent of the basal enzyme activity without free fatty acids (0.42 μmol PO_4^{3-} liberated per μmol phospholipid per hour). Each point represents the mean of 3–5 experiments. (*A*): Myristoleic acid (14:1$^{\Delta 9}$, ▼), petroselinic acid (18:1$^{\Delta 6}$, ■), oleic acid (18:1$^{\Delta 9}$, ●), elaidic acid (18:1$^{\Delta 9}$, *trans*, ○), *cis*-vaccenic acid (18:1$^{\Delta 11}$, ●), *trans*-vaccenic acid (18:1$^{\Delta 11}$ *trans*, ○), nervonic acid (22:1$^{\Delta 15}$, ▲). (*B*): Linoleic acid (18:2$^{\Delta 9}$, ○), γ-linolenic acid (18:3$^{\Delta 6}$, △), linolenic acid (18:3$^{\Delta 9}$, □), arachidonic acid (20:4$^{\Delta 5}$, ▽). The dose-response curve of oleic acid (●) is shown to facilitate the comparison with monoenoic acids (*A*)
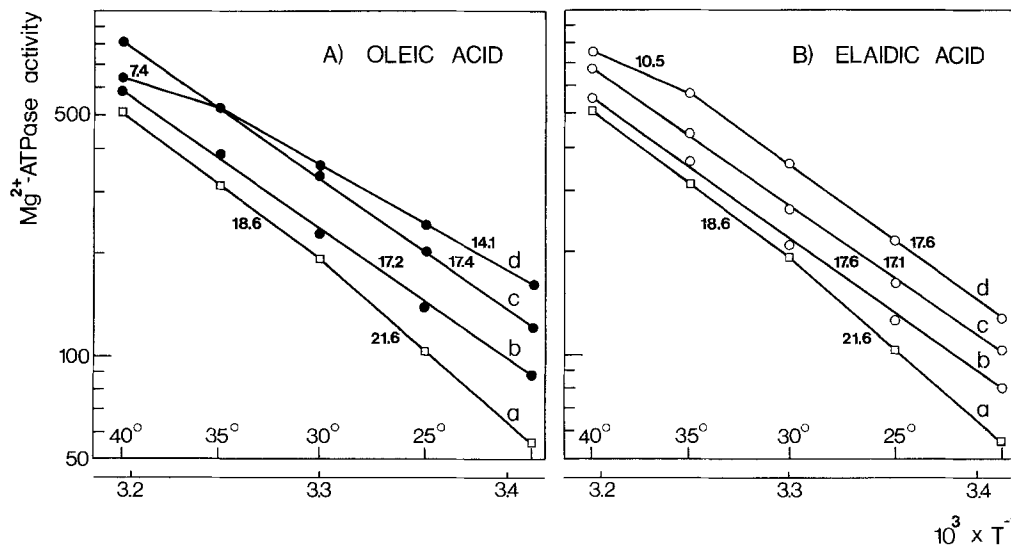

Fig. 3. Effect of oleic and elaidic acid on the temperature dependency of Mg^{2+}-ATPase. Mg^{2+}-ATPase activities were assayed at the following molar ratios of fatty acids/membrane phopholipid: (*a*) 0; (*b*) 0.022; (*c*) 0.22; (*d*) 2.2. The reaction mixture contained 0.45 μmol phospholipid-P in a total volume of 1 ml. The incubations were carried out as described in Materials and Methods; however, the incubation time was 30 min for the temperature range 20–25 °C. Control experiments confirmed that the media employed to measure ATPase activities in the absence of free fatty acids always gave maximal velocities of the enzyme reaction at the different temperatures required for Arrhenius plots. Mg^{2+}-ATPase activity is expressed as nmol PO_4^{3-} liberated per μmol membrane phospholipid per hour. Numbers shown in the figure give the energy of activation in kcal/mol

rocyte membranes, into which different amounts of oleic acid (Fig. 3*A*) or elaidic acid (Fig. 3*B*) have been inserted. Without free fatty acids, the Arrhenius plot shows a weak break at 30 °C (*a* in Fig. 3), but it is practically linear in the presence of low concentrations of free fatty acids (*b*, *c*). Below 30°C oleic and elaidic acid decreased the energy of activation (E_a) from 22 to 17 kcal/mol; in the temperature range 30-40 °C the slopes are similar.

At a molar ratio of fatty acid/membrane phospholipid of 2.2, fatty acids altered the thermal behavior of the enzyme more markedly. Inflection points occurred at 35 °C with distinct decreases of activation energies. In the temperature range above 35 °C, this decrease of E_a led to a reduction of the enzyme stimulation. Hence, this behavior is the reason for the biphasic effect of fatty acids: when the amount of oleic acid/phospholipid was raised above

the molar ratio of 1, the enzyme stimulation decreased at 37 °C, even though these high concentrations were most effective at reduced temperatures. These alterations were more obvious with oleic acid (Fig. 3A) than with the trans-configurated isomer (Fig. 3B). Similar results as presented in Fig. 3 were obtained with cis- and trans-configurated vaccenic acid (data not shown).

3.2. Modulation of Na^+K^+-ATPase Activity

The stimulating effect of unsaturated fatty acids on Na^+K^+-ATPase at 37 °C was much less pronounced than that on Mg^{2+}-ATPase. Na^+K^+-ATPase activity was maximally augmented by 20% (±4%, SD) at a molar ratio of fatty acid/phospholipid of 0.1 (oleic acid and petroselinic acid), 0.2 (cis-vaccenic acid), 0.3 (elaidic acid) or 1 (trans-vaccenic acid), respectively. Oleic acid and petroselinic acid activated half-maximally at a molar ratio of 0.032, cis-vaccenic acid at a molar ratio of 0.05, elaidic acid at a molar ratio of 0.075, and trans-vaccenic acid at a molar ratio of 0.12. Stearic acid produced a variable, but significant increase of Na^+K^+-ATPase activity of maximally 15% at a molar ratio of 0.42–2.0. A steep decline of Na^+K^+-ATPase activity was observed when the fatty acid/phospholipid ratio was raised above 0.3 (oleic acid and petroselinic acid), 0.5 (cis-vaccenic acid), 1 (elaidic acid) or 2 (trans-vaccenic acid), respectively. Cis-vaccenic acid methyl ester and cis-vaccenyl acetate were found to be ineffective in modulating Na^+K^+-ATPase activity.

The analysis of the temperature dependency of Na^+K^+-ATPase in the presence of different amounts of artificially inserted oleic acid (Fig. 4) revealed that the stimulating effect was much more distinct at reduced temperatures: for example, at a molar ratio oleic acid/membrane phospholipid of 0.22, Na^+K^+-ATPase activity was increased fivefold at 10 °C. At the higher molar ratios employed, the thermal behavior of the enzyme became markedly altered. The transition temperature was shifted by 5 °C from 30 to 25 °C, and activation energies were reduced below and above the transition temperature. As in the case of the Mg^{2+}-ATPase, the reduction of activation energies at high concentrations of free fatty acids led to a decrease of the stimulating effect or even an inhibition of enzyme activity at higher temperatures.

3.3. Modulation of V_{max} and Ca^{2+} Affinity of the Ca^{2+}-ATPase

The modulating effect of free fatty acids on Ca^{2+}-ATPase depended on both the amount of free fatty acid inserted into membranes and the free Ca^{2+}
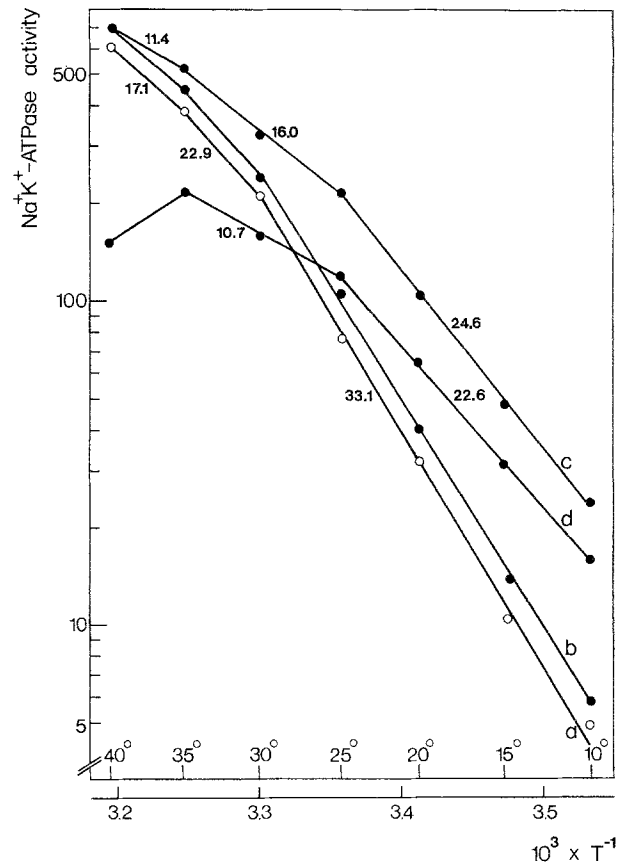

Fig. 4. Effect of oleic acid on the temperature dependency of the Na^+K^+-ATPase of erythrocyte membranes. Mg^{2+}-ATPase and Mg^{2+}, Na^+K^+-ATPase activities were analyzed in parallel at the following molar ratios of oleic acid/membrane phospholipid: (a) 0; (b) 0.022; (c) 0.22; (d) 2.2. Each assay system contained 0.45 μmol membrane phospholipid in a total volume of 1 ml. The incubation time was 30 min for the temperature range 10–25 °C. Control experiments showed that the ATPase media in the absence of free fatty acids always gave V_{max} of the enzyme reaction. Na^+K^+-ATPase activity was obtained by subtracting Mg^{2+}-ATPase activity and is expressed as nmol PO_4^{3-} liberated per μmol phospholipid per hour. Numbers shown in the figure give the energy of activation in kcal/mol

concentration. Therefore, the studies were carried out either at a constant concentration of free Ca^{2+} or at a constant molar ratio of free fatty acid/membrane phopholipid.

3.3.1 Constant concentration of free Ca^{2+}. The concentration-dependent effect of free fatty acids on Ca^{2+}-ATPase activities of calmodulin-deficient and calmodulin-containing membranes were investigated at a free Ca^{2+} concentration of 0.2 μM in a Ca/EGTA-buffered medium. In the absence of free fatty acids, Ca^{2+}-ATPase activities were below detectability at this Ca^{2+} concentration even in the presence of calmodulin. With both membrane preparations, unsaturated fatty acids produced about the
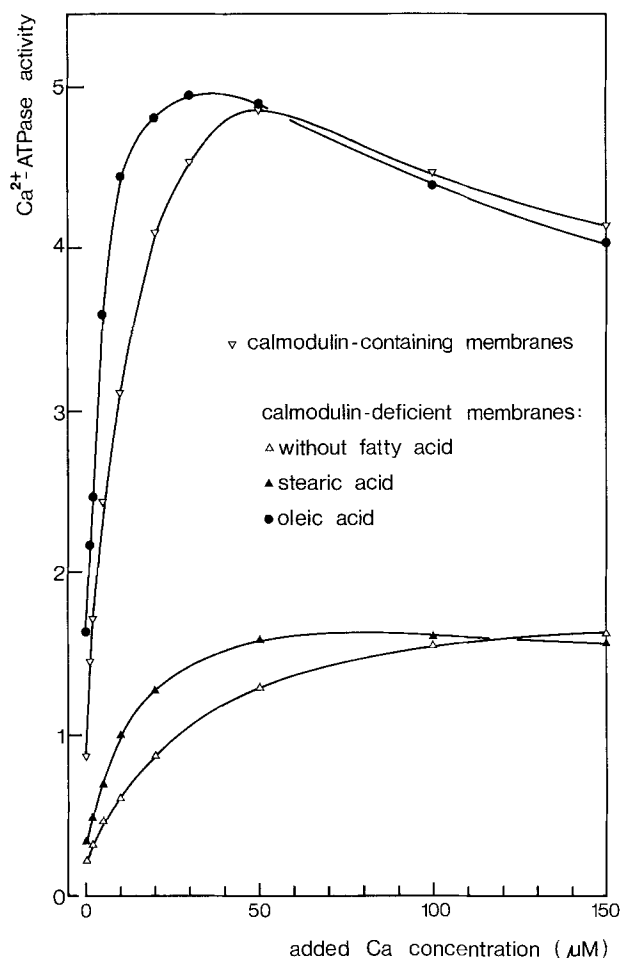
Fig. 5. Ca^{2+}-ATPase activities of calmodulin-deficient membranes in the absence and presence of free fatty acids compared with Ca^{2+}-ATPase activities of calmodulin-containing membranes. Calmodulin-deficient membranes were additionally washed twice with 10 mM Tris/HCl buffer of pH 7.6 without EGTA by centrifugation and resuspension and were adjusted to the original protein concentration (1 mg/ml). 0.2 ml of membranes equivalent to 0.2 mg protein were incubated in the absence (△) and presence of stearic acid (▲) or oleic acid (●), respectively (molar ratio fatty acid/phospholipid = 0.56) in an EGTA-free Ca^{2+}-ATPase medium (total volume 1 ml) at 37 °C with magnetic stirring. Calmodulin-containing membranes prepared by isosmotic lysis of red cells from the same subject were incubated without free fatty acids only (▽). Ca^{2+}-ATPase activities in terms of μmol PO_4^{3-} liberated per μmol membrane phospholipid per hour were calculated by subtracting Mg^{2+}-ATPase from Mg^{2+}, Ca^{2+}-ATPase activities; both activities were measured in parallel for each type of membrane preparation. Atomic absorption measurements demonstrated a calcium content of ATPase medium and membranes of 2 μM at zero calcium added

same maximal stimulation of Ca^{2+}-ATPase to 0.7–0.8 μmol PO_4^{3-} liberated per μmol phospholipid per hour. Half-maximal (maximal) stimulation was achieved at a molar ratio of 0.33 (0.56) with oleic acid, linoleic acid, γ-linolenic acid, and linolenic acid; of 0.42 (0.67) with cis-vaccenic acid and arachidonic acid; of 0.58 (1.1) with petroselinic acid.

3.3.2 Constant molar ratio of free fatty acid/phospholipid of 0.56.

With Ca/EGTA buffers the free Ca^{2+} concentration amounts to only 5 μM at a molar ratio Ca/EGTA of 0.9. For examining the Ca^{2+}-ATPase at higher free Ca^{2+} concentrations, activities were measured in an EGTA-free medium with total calcium concentrations between 2 and 150 μM. In Fig. 5 the effects of free fatty acids on Ca^{2+}-ATPase activities are compared with those of calmodulin. In the absence of free fatty acids, $V_{max}/2$ of the Ca^{2+}-ATPase of calmodulin-deficient membranes was achieved at 6 μM free Ca^{2+}. Oleic acid increased both Ca^{2+} affinity (K'_{Ca} 1.3 μM) and V_{max} of calmodulin-deficient membranes to an extent similar to that of calmodulin (K'_{Ca} 2 μM) without any effect on V_{max}.

In order to compare the effects of different unsaturated fatty acids, the stimulation of Ca^{2+}-ATPase activities at a molar ratio of 0.56 was examined in a range of free Ca^{2+} concentrations between 0.04 and 5 μM, adjusted by a Ca/EGTA buffer (Fig. 6). Fatty acids with one or the first of several double bonds in position 9 (oleic acid, cis-vaccenic acid, linoleic acid, linolenic acid) attained their half-maximal effects at a free Ca^{2+} concentration of 1.4 μM (K'_{Ca}). Petroselinic acid and γ-linolenic acid with the double bonds in position 6 were slightly less effective (K'_{Ca} 1.8 μM). Trans-configurated monoenoic acids activated half-maximally at 3 μM free Ca^{2+}. All these fatty acids evoked about the same increase of Ca^{2+}-ATPase activity of calmodulin-deficient membranes to 4.95 μmol (±0.1 μmol, SD, for 9 different fatty acids) PO_4^{3-} liberated per μmol of membrane phospholipid per hour and mimicked the effect of calmodulin ($V_{max} = 4.85$ μmol PO_4^{3-} liberated per μmol of membrane phospholipid per hour; $K'_{Ca} = 1.9$ μM).

The inset in Fig. 6 shows Ca^{2+}-ATPase activities of calmodulin-containing membranes with and without oleic acid. Even in the presence of calmodulin, oleic acid caused a distinct increase of enzyme activity; however, only at low Ca^{2+} concentrations (0.1–1.0 μM). At 8 μM Ca^{2+}, V_{max} of the calmodulin-stimulated enzyme was the same in the presence and in the absence of oleic acid. Therefore, in the presence of calmodulin, oleic acid increased Ca^{2+} affinity only, reducing K'_{Ca} from 1.9 to 1.4 μM.

4. Modulation of ATPase Activities by Phospholipase A_2

Since phospholipase A_2 requires Ca^{2+} for activity, only Mg^{2+}, Ca^{2+}-ATPase could be modulated directly (Fig. 7). The addition of 1 μg of phospholipase A_2 per 0.18 μmol of membrane phospholipid increased Mg^{2+}, Ca^{2+}-ATPase activity at 100 μM $CaCl_2$ immediately. The complexation of Ca by a
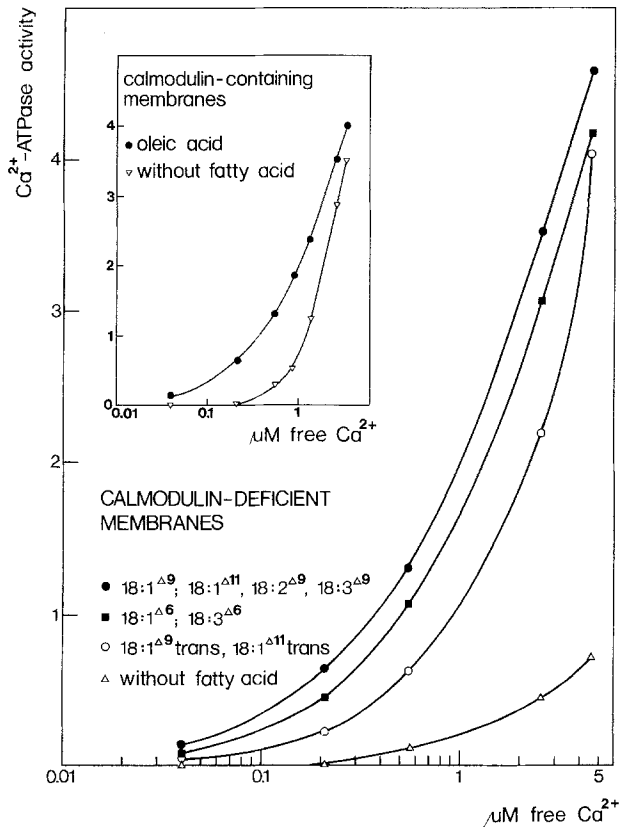
Fig. 6. Effect of unsaturated fatty acids on Ca^{2+}-ATPase activity of erythrocyte membranes in Ca/EGTA buffers. Calmodulin-deficient membranes (0.18 μmol phospholipid/ml reaction mixture) were added to 100 nmol of ultrasonically dispersed fatty acids (corresponding to a molar ratio of fatty acid-membrane phospholipid of 0.56) and incubated at 37 °C with magnetic stirring. The different concentrations of free Ca^{2+} were achieved with Ca/EGTA ratios <0.9 (5 mM EGTA) at pH 7.4 (0.04–1 μM Ca^{2+}) and pH 7.0 (1–5 μM Ca^{2+}). Ca^{2+}-ATPase activity was calculated by subtracting Mg^{2+}-ATPase from Mg^{2+}, Ca^{2+}-ATPase activity; both activities were measured in parallel in the presence of equal amounts of free fatty acids and membrane phospholipid. In analogous incubations, Ca^{2+}-ATPase activity was assayed in the absence of free fatty acids (Δ) yielding 1.6 μmol PO_4^{3-} liberated per μmol of phospholipid per hour, measured in an EGTA-free medium at 150 μM $CaCl_2$; $V_{max}/2$ was reached at 6 μM free Ca^{2+}. *Inset:* Calmodulin-containing membranes were treated with oleic acid (●) as described for the calmodulin-deficient membranes. In the absence of fatty acids V_{max} of the calmodulin-stimulated Ca^{2+}-ATPase (▽) was 4.85 μmol PO_4^{3-} liberated per μmol of phospholipid per hour at 8 μM free Ca^{2+}

Fig. 7. Effect of phospholipase A_2 on Mg^{2+}, Ca^{2+}-ATPase activity of erythrocyte membranes. Erythrocyte membranes (0.36 μmol of membrane phospholipid in a total volume of 2 ml) were incubated in the medium for Mg^{2+}, Ca^{2+}-ATPase (without EGTA at 100 μM $CaCl_2$ with 1 μg/ml phospholipase A_2 (■). In parallel experiments, the enzyme activity was measured in the presence of 1 μg/ml phospholipase A_2 + 1 mg/ml fatty acid-free albumin (●). Control experiments were carried out with and without 1 mg/ml fatty acid-free albumin (○). The PO_4^{3-} concentration in the reaction mixture is plotted as a function of time

20-fold excess of EGTA demonstrated that the rise of PO_4^{3-} liberation is mainly due to the enhancement of Ca^{2+}-ATPase activity, since only 13 % of the PO_4^{3-} was released by the stimulated Mg^{2+}-ATPase. When 1 μg of phospholipase A_2 and 1 mg of delipidated albumin were simultaneously added per ml of membrane suspension, the Mg^{2+}, Ca^{2+}-ATPase activity was even slightly lower than in control incubations without phospholipase A_2 in the presence or absence of albumin. Addition of 1.4–14
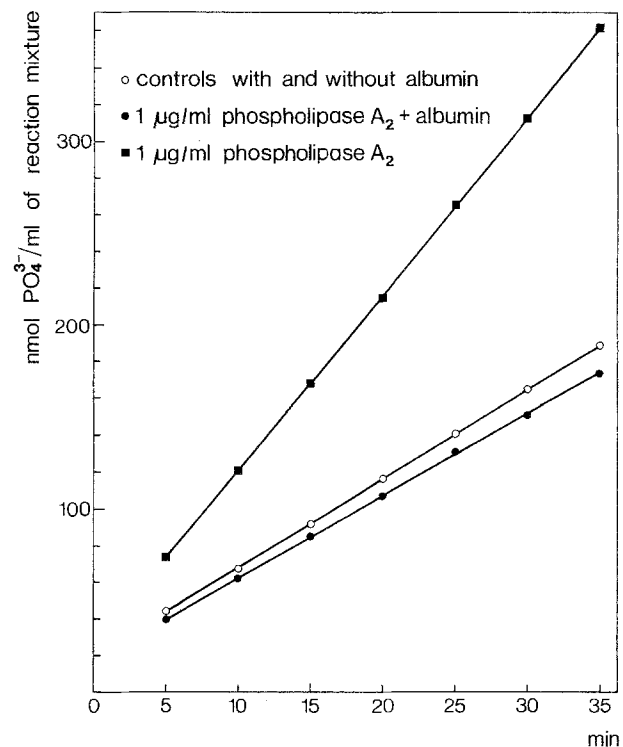
units/ml of the trypsin inhibitor aprotinin left the stimulating effect of phospholipase A_2 unaffected.

In order to investigate the action of phospholipase A_2 on Mg^{2+}-ATPase and Na^+K^+-ATPase, freshly prepared erythrocyte membranes were preincubated at 100 μM $CaCl_2$ in the absence and in the presence of phospholipase A_2 (Fig. 8) at 30 and 37 °C. The complexation of Ca with 2 mM EGTA in the ATPase media allowed the immediate analysis of Mg^{2+}- and Mg^{2+}, Na^+K^+-ATPase. Both activities were augmented in the time range 0.5–4 min by action of phospholipase A_2; however, Na^+K^+-ATPase activity also increased slightly in control experiments without exogenous phospholipase A_2 by preincubation at 100 μM $CaCl_2$. Prolonged preincubation of erythrocyte membranes in the presence of phospholipase A_2 led to a steep decline of Na^+K^+-ATPase activity after about 6 min and of Mg^{2+}-ATPase activity after about 15 min.
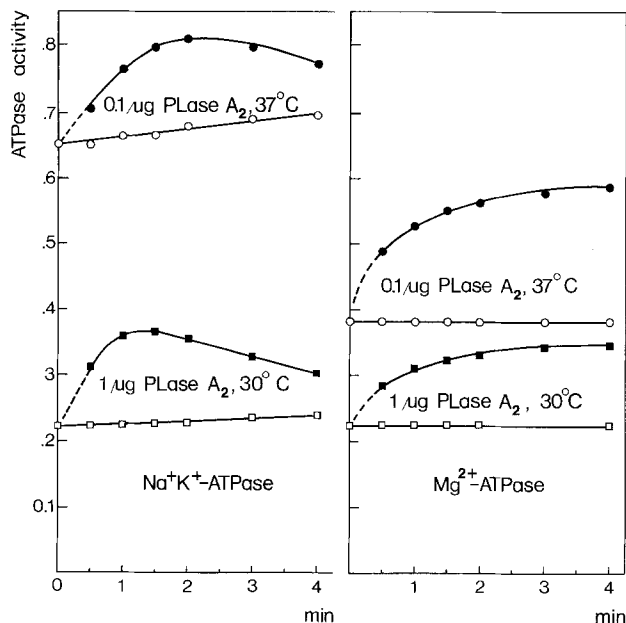
Fig. 8. Effect of phospholipase A_2 on Mg^{2+}-ATPase and Na^+K^+-ATPase activities of erythrocyte membranes. The original membrane suspension (1 mg of protein per ml) was preincubated at 30 °C with 1 µg (□) or at 37 °C with 0.1 µg (○), respectively, of phospholipase A_2 per ml of membranes at 100 µM $CaCl_2$. Control experiments without phospholipase A_2 at 30 °C (■) and 37 °C (●) were carried out in parallel. At the time indicated, samples were withdrawn for Mg^{2+}-ATPase and Mg^{2+}, Na^+K^+-ATPase assays. ATPase activities are expressed as µmol PO_4^{3-} liberated per µmol of membrane phospholipid per hour

Fig. 9. Inhibition of ATPase activities by α-lysophosphatidylcholine. Human erythrocyte membranes were incubated with ultrasonically dispersed α-lysophosphatidylcholine (oleoyl) in magnetically stirred ATPase media at 37 °C. Ca^{2+}-ATPase activity was measured in an EGTA-free medium at 150 µM $CaCl_2$. ATPase activities in the presence of α-lysophosphatidylcholine are given in percent of the basal enzyme activity without the lysolipid. Each point represents the mean of three experiments

5. Inhibition of ATPase Activities by α-Lysophosphatidylcholine

α-Lysophosphatidylcholine was found to leave ATPase activities unaffected at lower and to inhibit them at higher concentrations (Fig. 9). Half-maximal inhibition was observed at molar ratios of lysolipid/membrane phospholipid of 0.6 (Na^+K^+-ATPase) and 1 (Mg^{2+}-ATPase and Ca^{2+}-ATPase).

Discussion

When the activity of an integral membrane enzyme is found to be modulated by free fatty acids, the first question arising is whether fatty acids unscreen latent ATPase activity or in fact increase the catalytic activity of the enzymes. An unscreening of latent ATPase activity by free fatty acids can be expected when the membrane preparation yields ATP-impermeable ghosts. Therefore, different procedures for the preparation of red cell membranes were reinvestigated. Membranes prepared at pH 7.6 which exhibited maximal ATPase activities were subjected to procedures which are thought to increase permeability, i.e., freeze-thawing, ultrasonication, and addition of detergents. The failure of all of these
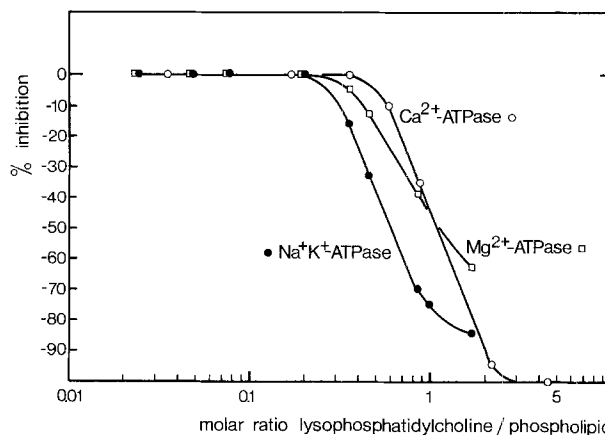
procedures to increase ATPase activities proves that the effect of fatty acids cannot be explained by a detergent-like action. The results are in accordance with those of Bramely et al. [5] who reported that ghosts prepared at low ionic strength at pH 7.4 with accordingly low hemoglobin content exhibited maximal ATPase activities, which were not increased by sonication or detergents. In membranes prepared at a pH below 7.2, the low ATPase activities are principally due to the impermeability of the membranes to ATP.

The modulation of ATPase activities of erythrocyte membranes by free fatty acids reported here confirms the claim of Orly and Schramm [23] who suggested that free fatty acids are useful tools to probe membrane-linked reactions. Microviscosity measurements indicated that the insertion of cis-vaccenic acid progressively increased the fluidity of turkey erythrocyte membranes [13]. As in the case of catecholamine-stimulated adenylate cyclase [13, 23], the effects of exogenous fatty acids on ATPases were probably caused by alterations of the hydrophobic environment of the enzymes. The following data of the present work support this hypothesis: only unsaturated fatty acids stimulated ATPases of erythrocyte membranes (except for the effect of stearic acid on Na^+K^+-ATPase); differences in the efficacies depended on the degree of unsaturation, the position of double bonds, and their configuration (Figs. 2 and 6); unsaturated fatty acids altered the thermal behavior of the ATPases (data for Ca^{2+}-ATPase not shown) with decreases of activation energies (Figs. 3 and 4) and a shift of the break to a lower temperature (Fig. 4). The greater potency of

cis-configurated monoenoic acids can be attributed to the permanent kink in the fatty acyl chain that perturbates the packing of the acyl chains of membrane phospholipids at lower concentrations than are required with trans-configurated isomers. These effects are all attributable to the hydrophobic part of the fatty acids. If this is true, why are the uncharged derivatives of cis-vaccenic acid ineffective as modulators of ATPase activities?

A differential partitioning of fatty acids by electrostatic interactions may give an answer to this question. Studies of Birrel et al. [3] in chromatophores with fatty acid spin-labels indicated that most of the negatively charged fatty acids were immobilized on the hydrophobic surfaces of membrane proteins in contrast to analogous quarternary amines. The authors suggested that this charge selectivity may be due to electrostatic interactions with basic amino acid side-chains at the interface where polar groups of proteins and lipids adjoin. Brotherus et al. [6] provided evidence that the Na^+K^+-ATPase influences its lipid environment by segregating negatively charged lipid species and claimed that the phenomenon may have general significance.

The electrostatic accumulation of fatty acids not only alters the surrounding lipids and consequently hydrophobic interactions between the lipid bilayer and the floating enzyme, but also modulates surface charges in the environment of the enzyme. Since stearic acid was found to be stimulatory for Na^+K^+-ATPase only, this effect may be caused by the occurrence of additional negative charges. The requirement of the Na^+K^+-ATPase for acidic lipids [15] and the lower activity in the absence of negative charges [7] support this consideration.

The increase in the density of negative surface charges may also account for the decrease of K'_{Ca} of the Ca^{2+}-ATPase in the presence of free fatty acids. Interestingly, the Ca^{2+}-ATPase stimulator calmodulin is a highly acidic protein [20]. One of the functions of the negative charges may be the binding of calmodulin to the ATPase, similar to the electrostatic segregation of free fatty acids. As calmodulin, the fatty acids mask positive charges on the enzyme and increase the density of negative charges in its environment. There is evidence from the present study that this increase is sufficient to explain the increase of Ca^{2+} affinity: stearic acid evoked an increase of Ca^{2+} affinity similar to that of unsaturated fatty acids and calmodulin, leaving V_{max} unaffected. Only the additional perturbation of hydrophobic interactions between the Ca^{2+}-ATPase and the surrounding lipids led to the marked increase of V_{max}. This results from a comparison between the effects of stearic acid and unsaturated fatty acids on

V_{max} (Fig. 5). In accordance with this, hydrophobic properties of the Ca^{2+}-calmodulin complex were shown to be important for the activation of calmodulin-dependent enzymes [31]. It should also be mentioned that several enzymes activated by calmodulin [20] can also be stimulated by free fatty acids [13, 14, 23, 35, 37].

Taken together, these results give evidence that the Ca^{2+}-ATPase of native erythrocyte membranes can exist in three defined states:

I. basal activity and low Ca^{2+} affinity

II. basal activity and high Ca^{2+} affinity (requirement: increase in the density of negative surface charges as caused by stearic acid)

III. stimulated activity and high Ca^{2+} affinity (increase of negative surface charges and additional perturbation of hydrophobic interactions as caused by calmodulin or unsaturated fatty acids).

Niggli et al. [22] recently reported that acidic lipids including unsaturated fatty acids mimick the effect of calmodulin on purified Ca^{2+}-ATPase of human erythrocyte membranes. According to their experiments, the Ca^{2+}-ATPase exists either in a low affinity state (low V_{max}, low Ca^{2+} affinity) or in the high affinity state (high V_{max}, high Ca^{2+} affinity). The present results demonstrate that also in native erythrocyte membranes the Ca^{2+}-ATPase exists in a high affinity state (III) in the presence of unsaturated fatty acids, and they provide evidence for the additional occurrence of state II.

The artificial insertion of increasing amounts of free fatty acids into erythrocyte membranes eventually inactivated ATPases. From Fig. 4 this temperature dependency of this effect is apparent: the decrease of the stimulating effect at a high molar ratio of fatty acid/membrane phospholipid occurs at higher temperatures as a result of a further decrease of activation energies. These findings indicate that a limited extent of membrane perturbation facilitates the conformational changes that the ATPases have to undergo during their action; a further increase of the disordering of membrane structure probably led to a loss of the lateral constraints of lipids on membrane proteins, which apparently are necessary to maintain the active enzyme configurations or to stabilize the enzyme in a more reactive conformation [29].

Naturally occurring phospholipids usually carry one saturated and one unsaturated fatty acyl chain, predominantly with the unsaturated chain in the 2 position. This fatty acid becomes liberated by the action of phospholipase A_2. The enhancement of Mg^{2+}-ATPase, Na^+K^+-ATPase (Fig. 8) and Ca^{2+}-ATPase (Fig. 7) by the action of purified phospho-

lipase A_2 from *Naja naja* venom agrees well with the stimulation caused by exogenously added unsaturated fatty acids (Figs. 2, 4 and 6). Experiments of Roelofsen et al. [25] demonstrated the purity and high activity of this commercially available enzyme preparation with only one isoenzyme present. The action of phospholipase A_2 additionally leads to the production of lysophospholipids. The failure of α-lysophosphatidylcholine to stimulate ATPase activities (Fig. 9) indicates that the initial enhancement of the cleavage of ATP resulted from the liberation of fatty acids by phospholipase A_2. The prevention of the increase of Ca^{2+}-ATPase activity by phospholipase A_2 in the presence of albumin (Fig. 7) supports this assumption, since albumin was found to extract especially unsaturated fatty acids from the membrane [30]. The production of lysolipids may contribute to the final inactivation of Mg^{2+}-ATPase and Na^+K^+-ATPase activities after prolonged incubation with phospholipase A_2. Previously, a similar rise of Mg^{2+}, Ca^{2+}-ATPase activity was observed with phospholipase A_2-treated preparations of sarcoplasmic reticulum [30] and human erythrocyte membranes [32].

In view of the multiple membrane-bound enzymes which are affected by the occurrence of free fatty acids as reported in literature [2, 9, 13, 14, 16, 23, 35, 37] and found here, the question arises whether or not free fatty acids may physiologically act as modulators of enzyme activity. On the one hand, the amounts of unesterified fatty acids in mammalian cell membranes are low in comparison with the relatively high molar ratio of free fatty acid/membrane phospholipid required for a marked stimulation of ATPase activities. On the other hand, membrane-bound phospholipase A_2 activities are present in almost every cell [*see* 34 for references] and show an absolute requirement for Ca^{2+}. The activation of an endogenous phospholipase A_2 by a rise in cytoplasmic free Ca^{2+} secondary to a hormone- or transmitter-dependent Ca^{2+} influx may cause a small and transient increase in the concentration of free fatty acids. In the presence of equimolar proportions of fatty acids and lysophospholipids the bilayer structure of membranes remains preserved [17]. The limitation of hydrolysis to phospholipase A_2-susceptible domains [18] together with an electrostatic segregation of fatty acids may restrict the effect to specific membrane-bound enzymes. In the work of Taverna and Hanahan [32], the liberation of less than 1% of total membrane fatty acids resulted in a marked stimulation of Mg^{2+}, Ca^{2+}-ATPase activity. The rise of free fatty acids in the environment of the Ca^{2+}-ATPase may even contribute to terminate the Ca^{2+} signal by

increasing the Ca^{2+} efflux. The ensuing fall of intracellular free Ca^{2+} would reduce phospholipase A_2 activity, allowing the reacylation of previously liberated lysolipids with restoration of the initial state.

The stimulating effect — especially on Na^+K^+-ATPase — was more pronounced at reduced temperatures (Figs. 4 and 8). Therefore, it is conceivable that this alteration of the temperature dependency of the enzymes may have a functional role in cold acclimatation, since poikilotherms and hibernators are known to alter enzyme activities by increasing the fluidity of membrane lipids at lower ambient temperatures [*see* 19, for a review]. An increase of free fatty acids in the plasma membranes of poikilotherms as the result of stimulation of endogenous phospholipase A_2 activity would permit an immediate adaptation of certain transport functions to a sudden drop in environmental temperature, followed by a slower increase in the degree of unsaturation of membrane phospholipids. The rapid onset of the distinct enhancement of ATPase activities by phospholipase A_2 may be taken as an indication of a physiological role of the modulating effect of free fatty acids. If this assumption is correct, phospholipids may be conceived as donors of unsaturated fatty acids, providing an instrument to the cell for concomitant alteration of surface charge densities and the hydrophobic interior of membranes with subsequent modulation of enzyme activity.

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