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# Binding of unsaturated fatty acids to $Na^+,K^+$-ATPase leading to inhibition and inactivation

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**The effects of free fatty acids on the mechanism of action of $Na^+,K^+$-ATPase were studied. Unsaturated free fatty acids (palmitoleic acid, oleic acid, linoleic acid and arachidonic acid) inhibited the $Na^+,K^+$-ATPase activity within a narrow range, while saturated and methylated fatty acids had little or no effect. The following effects of oleic acid were found: (1) The affinity for $K^+$ on the overall ATPase and the *p*-nitrophenylphosphatase reaction as well as the maximal activities were decreased. (2) The $Na^+$-ATPase activity was also inhibited but the '0'-ATPase activity was hardly changed. (3) The steady-state ATP phosphorylation level in the presence of $Na^+$ was not influenced. (4) The dephosphorylation rate constant of the phosphointermediate was slightly decreased, resulting in elevated phosphorylation levels in the absence of $Na^+$. (5) The inhibitory effect of ATP on the dephosphorylation rate was not affected. (6) The $K^+$ sensitivity of the phosphoenzyme in the presence as well as in the absence of $Na^+$ was decreased. (7) Ouabain binding was inhibited. Both the affinity and the number of binding sites were lowered. In addition it was found that $Na^+,K^+$-ATPase binds oleic acid linearly with the fatty acid concentration up to more than 100 mol oleic acid per mol $\alpha\beta$ oligomer of $Na^+,K^+$-ATPase. Prolonged incubation with oleic acid led to irreversible inactivation of the enzyme. This inactivation was dependent on the reaction conditions: ligands, temperature, enzyme concentration, time and fatty acid concentration. The combined presence of inactivation (long term effects) and the effects on the ($K^+$-activated) dephosphorylation (short term effects) explain the mixed type inhibition of free fatty acids as observed in assays for $K^+$-activated ATPase, $K^+$-activated *p*-nitrophenylphosphatase and ouabain binding. It also explains the sharp inhibition curve in the $Na^+,K^+$-ATPase activity test.**

## Introduction

Free fatty acids, present in highly purified $Na^+,K^+$-ATPase preparations [1], have been isolated as an 'ouabain-like' substance from the electric organ of *Electrophorus electricus* [2] and from plasma [3–6]. These free fatty acids may act as physiological regulators of the $Na^+,K^+$-ATPase activity and so have their clinical implications. Recently, we observed increased levels of free fatty acids in plasma of patients with essential hypertension [7].

In the past several studies have been carried out showing that fatty acids inhibit the overall $Na^+,K^+$-ATPase activity [3–4,8–10]. In some of these studies inhibitory effects on $K^+$-activated *p*-nitrophenylphosphatase activity [9,10] and ouabain-binding [2–4,11] have also been reported. From these studies preliminary conclusions have been reached that fatty acids (in particular unsaturated fatty acids) inhibit the enzyme activity by decreasing the affinity for $K^+$ [8,9] and by promoting the $E_1$-state of the enzyme [10,12]. A comprehensive study with the purified enzyme towards the mechanism of inhibition is still lacking.

In this study we investigated short and long term effects of free fatty acids on the activities of $Na^+$-ATPase, $Na^+$ and $K^+$ free ATPase ('0'-ATPase), on the phosphorylation and dephosphorylation reaction and determined the fatty acid binding capacity, in an attempt to gain more information on the mechanism of action of these free fatty acids on $Na^+,K^+$-ATPase.

Abbreviations: $Na^+,K^+$-ATPase, sodium and potassium activated adenosine triphosphatase; $Na^+$-ATPase, ouabain-sensitive adenosine triphosphatase activity in the presence of sodium; '0'-ATPase, ouabain-sensitive adenosine triphosphatase activity in the absence of sodium and potassium.

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## Materials and Methods

*Enzyme preparation.* $Na^+,K^+$-ATPase was purified from rabbit kidney outer medulla according to Jørgensen [13]. After removal of contaminating ATP [14] the preparation was stored at −20 °C in 0.25 M sucrose and 50 mM imidazole-acetate pH 7.0. Protein was determined by the Lowry method [15] using bovine serum albumin as standard. The specific $Na^+,K^+$-ATPase activity of the preparations ranged from 900 to 1400 μmol $P_i$/mg protein per h.

*Free fatty acids.* Concentrated solutions of free fatty acid (Supelco, Inc., Leusden, The Netherlands) were prepared by dissolving the free fatty acids in methanol. In the experiments they were diluted to their final concentrations (1–300 μM) in 3% (v/v) methanol. This concentration of methanol did not influence the $Na^+,K^+$-ATPase activity and was always used as a control.

*$Na^+,K^+$-ATPase assay.* To 0.4 ml medium, which contained either 110 mM $Na^+$, 10 mM $K^+$, 5 mM $Mg^{2+}$, 5 mM ATP, 0.1 mM EDTA and 30 mM imidazole-HCl (pH 7.4) (medium A) or 110 mM $Na^+$, 5 mM $Mg^{2+}$, 5 mM ATP, 0.1 mM EDTA, 0.1 mM ouabain and 30 mM imidazole-HCl (pH 7.4) (medium B), 0.25–2 μg $Na^+,K^+$-ATPase was added. After the incubation at 37 °C or at 22 °C, 1.5 ml 8.6% (w/v) trichloroacetic acid and 1.5 ml ammonium heptamolybdate (1.15%, w/v) in 0.66 M sulfuric acid with 9.2% (w/v) $FeSO_4 \cdot 7H_2O$ was added. The blue phosphomolybdate complex was analysed after 30 min at 700 nm on a Philips PU8620 spectrophotometer and $Na^+,K^+$-ATPase activity calculated as the ouabain-sensitive ATPase activity (difference in medium A and B). For determination of the $K_{0.5}$ value for ATP, a similar radioactive procedure as described in the $Na^+$-ATPase assay (see below) was used.

*$K^+$-p-nitrophenylphosphatase assay.* To 0.4 ml medium (A) containing: 7.5 mM $Mg^{2+}$, 10 mM $K^+$, 5 mM *p*-nitrophenyl phosphate, 1 mM CDTA and 50 mM imidazole-HCl (pH 7.4) and to medium (B) of the same composition but without $K^+$ and with 0.1 mM ouabain, 2.5 μg $Na^+,K^+$-ATPase per ml was added and incubated for 30 min at 37 °C. The *p*-nitrophenol concentration was determined by measuring the 410 nm absorbance after stopping the reaction by adding 2 ml 0.5 M NaOH to 0.4 ml incubation mixture. The $K^+$ *p*-nitrophenylphosphatase activity was calculated as the difference in medium A and medium B.

*$Na^+$-ATPase and '0'-ATPase assay.* At 22 °C $Na^+$, $K^+$-ATPase (10 μg/ml) was incubated for 15 s in 0.1 ml medium containing: 2 μM [γ-$^{32}$P]ATP, specific activity 0.3 Ci/mmol (Radiochemical Center Amersham, Amersham, U.K.), 0.1 mM $Mg^{2+}$, 50 mM imidazole-acetate (pH 7.0), with 100 mM $Na^+$ ($Na^+$-ATPase activity) or without $Na^+$ ('0'-ATPase activity). The reaction was stopped by adding 0.2 ml 8.6% (w/v) trichloroacetic acid and the temperature was decreased to 0 °C. One part (2/3rd) was used for determination of the steady-state phosphorylation level (see next section) and 1/3rd was used for determination of the amount of $^{32}P_i$ formed by ATP hydrolysis. For this purpose 0.1 ml was mixed with 0.5 ml charcoal suspension (20% (w/v) in 8.6% (w/v) trichloroacetic acid) and after 15 min at 0 °C it was centrifugated at 1250 × *g*. To 0.2 ml of the clear supernatant 4 ml Aqua-luma Plus (Lumac, Landgraaf, The Netherlands) was added and the mixture was analysed by liquid scintillation analysis. Blanks were prepared by preincubating the enzyme (1 mg/ml) with 5 mM $Mg^{2+}$, 2 mM ouabain and 50 mM imidazole-acetate (pH 7.0), for 60 min whereafter they were diluted with imidazole buffer and handled in the same way.

*Steady-state ATP phosphorylation.* Two-third of reaction volume, obtained in the $Na^+$-ATPase or '0'-ATPase assay (see previous section), was diluted with 3 ml 5% (w/v) trichloroacetic acid in 0.1 M phosphoric acid and filtered over a Schleicher & Schüll (Dassel, F.R.G.) filter (type AE95, 1.2 μm). After washing two times with 5 ml trichloroacetic acid, the filters were analysed for their $^{32}$P-protein content. Blanks were prepared by denaturing the enzyme before the incubation with the stopping solution [16].

*Dephosphorylation studies.* After phosphorylation of $Na^+,K^+$-ATPase for 10 s at 22 °C (see previous section), the reaction was quenched by adding 5 volumes non-radioactive ATP (0.01–10 mM) in 50 mM imidazole-acetate (pH 7.0) and the ligands to be tested. The dephosphorylation reaction was stopped after 3–10 s with 3 ml 5% (w/v) trichloroacetic acid in 0.1 M phosphoric acid and the phospho-level was determined as described in the previous section.

*Ouabain binding studies.* $Na^+,K^+$-ATPase (5 μg/ml) was incubated at 22 °C with 0.1–3 μM [$^3$H]ouabain (NEN, 's Hertogenbosch, The Netherlands; spec. act. 26.9 Ci/mmol) in the presence of 5 mM $Mg^{2+}$, 100 mM $Na^+$, 5 mM ATP and 50 mM imidazole-acetate (pH 7.0). After reaching equilibrium (60 min), the samples were diluted with 3 ml ice-cold wash solution (2 mM ATP, 100 mM $Na^+$, 5 mM $Mg^{2+}$ and 50 mM imidazole-acetate (pH 7.0)) and immediately filtered over a Schleicher & Schüll (Dassel, F.R.G.) membrane filter (type BA85, 0.45 μm) and rinsed two times with 3 ml wash-solution. Thereafter the filters were incubated with 0.5 ml 10% (w/v) sodium dodecylsulfate for 60 min at room temperature, to dissolve the protein. The solution was mixed with 10 ml Instagel (Packard, Tilburg, The Netherlands) and analysed by liquid scintillation analysis. From these data the amount of ouabain binding was calculated, which was corrected for aspecific binding ([$^3$H]ouabain binding in the presence of 1 mM non-radioactive ouabain).

*Oleic acid binding.* 1250 nmol [$^{14}$C]oleic acid, specific activity 2 mCi/mmol (Radiochemical Center Amersham, Amersham, U.K.) was evaporated to dryness, dissolved in 300 μl methanol and thoroughly mixed with 9.7 ml 50 mM imidazole-acetate (pH 7.0). An 0.5-ml aliquot of this solution was diluted to the appropriate concentration and 0–50 μg $Na^+,K^+$-ATPase or bovine serum albumin was added. After incubation for 30 min at 37°C the solution was cooled in ice, whereafter 0.5 ml ice-cold Lipidex-1000 (Packard, Tilburg, The Netherlands) suspension (20% (w/v) in 50 mM imidazole-acetate (pH 7.0)) was added under stirring to remove the unbound fatty acids [17]. The samples were incubated for 10 min at 0°C and centrifuged for 2 min at 2000 × *g* at 4°C. An aliquot of the supernatant, containing the fatty acids bound to the protein, was analysed for radioactivity by liquid scintillation analysis.

*Calculations.* The results are expressed as average values with standard error of the mean. Linear regression analysis was used for determination of the $K_d$ and maximal binding capacity in the ouabain-binding studies (Scatchard-plots). $K_{0.5}$ is defined as the concentration of effector giving the half-maximal stimulation and $I_{50}$ as the value giving 50% inhibition of the activity.

Other methods, like determination of $Na^+$ and $K^+$ and conversion of ATP to its imidazole salt, were carried out as described previously [18].

## Results

*Effects of free fatty acids on the $Na^+,K^+$-ATPase activity*

Free fatty acids were first tested on the overall $Na^+,K^+$-ATPase activity. Fig. 1 shows that oleic acid (18 : 1) inhibits at 37°C as well as at 22°C the activity of purified $Na^+,K^+$-ATPase from rabbit kidney. The degree of inhibition of ATP hydrolysis depended on the assay conditions. If the $Na^+,K^+$-ATPase concentration, at 37°C, was decreased from 25 to 0.25 μg/ml and at the same time the incubation time was increased from 2 to 120 minutes, the $I_{50}$ value of oleic acid for $Na^+,K^+$-ATPase decreased from 71 to 9 μM (Fig. 1A). At 22°C the same effect of oleic acid was observed (Fig. 1B) but the $I_{50}$ values were about twice as high as at 37°C. At low oleic acid concentrations a small increase in the rate of ATP hydrolysis was noticed.

TABLE I

*Effect of free fatty acids on the $Na^+,K^+$-ATPase activity at 37°C*

Mean values with standard error are given for $n = 3$.

| | $I_{50}$ (μM) | |
|---|---|---|
| Incubation time (min) | 15 | 120 |
| $Na^+,K^+$-ATPase concn. (μg/ml) | 2.75 | 0.35 |
| Myristic acid (14 : 0) | 86 ± 3 | 42 ± 15 |
| Palmitic acid (16 : 0) | > 100 | > 100 |
| Palmitoleic acid (16 : 1) | 26 ± 1 | 17 ± 1 |
| Stearic acid (18 : 0) | > 100 | > 100 |
| Oleic acid (18 : 1) | 16 ± 2 | 10 ± 2 |
| Linoleic acid (18 : 2) | 20 ± 3 | 14 ± 2 |
| Arachidonic acid (20 : 4) | 19 ± 3 | 13 ± 2 |

The data of Table I show that the unsaturated free fatty acids, palmitoleic acid (16 : 1), oleic acid (18 : 1), linoleic acid (18 : 2) and arachidonic acid (20 : 4), effectively inhibited the $Na^+,K^+$-ATPase activity ($I_{50} \leq 30$ μM), while saturated fatty acids, myristic acid (14 : 0), palmitic acid (16 : 0) and stearic acid (18 : 0), at concentrations below 0.1 mM had little or no effect.

The inhibitory effect of oleic acid on $Na^+,K^+$-ATPase activity was abolished upon methylation leading to removal of its negative charge. Changing the pH between pH 6 and pH 9 had only a minor effect on the $I_{50}$ value (data not shown).

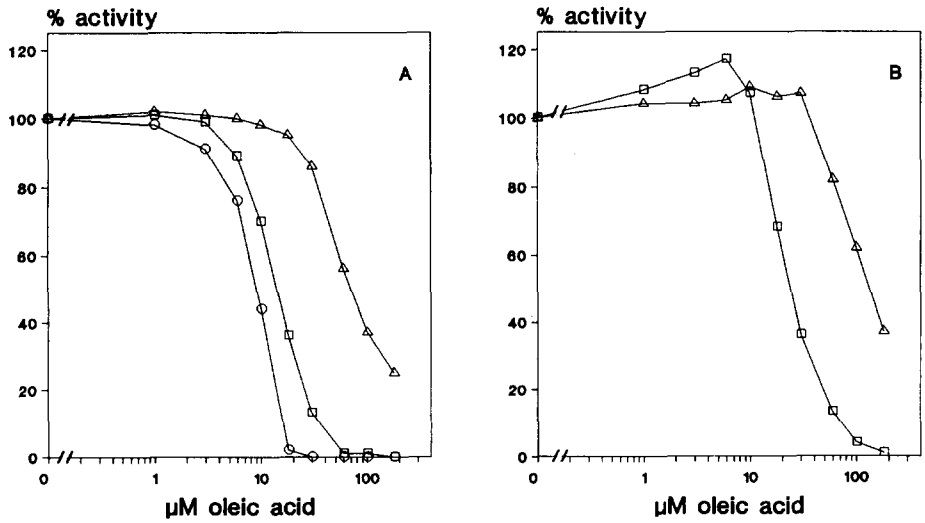


Fig. 1. The effect of oleic acids on the $Na^+,K^+$-ATPase activity. $Na^+,K^+$-ATPase, 0.25 (○), 2.5 (□) or 25 (△) μg/ml was incubated for 120 (○), 20 (□) or 2 (△) min at 37°C (A) or incubated at 22°C (B) for 60 (□) or 6 (△) min. The 100% value in (A) equals 1227 μmol $P_i$/mg protein per h and in B 319 μmol $P_i$/mg protein per h. The $I_{50}$ values of oleic acid are 9, 15 and 71 μM in (A) and in (B) 24 and 141 μM, respectively.

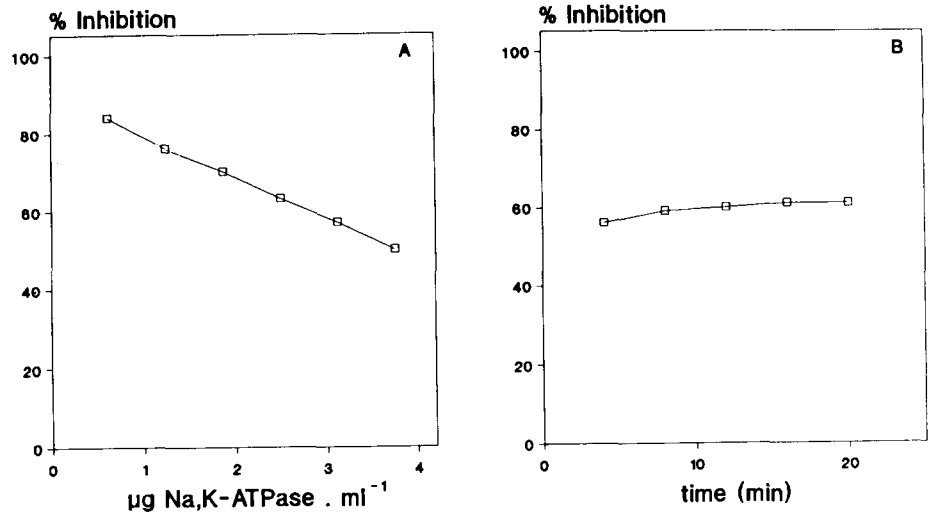


Fig. 2. The effect of the $Na^+,K^+$-ATPase concentration (A) and incubation time (B) on the inhibition by 15 μM oleic acid of the $Na^+,K^+$-ATPase activity at 37°C. (A) Constant incubation time (15 min) and varying $Na^+,K^+$-ATPase concentrations (0.625–3.75 μg/ml). (B) Constant protein concentration (2.5 μg Na,K-ATPase per ml) at variable incubation times (4–20 min). The degree of inhibition as percent of the control is plotted.

Table I also shows that the degree of inhibition of the $Na^+,K^+$-ATPase activity depends on the assay conditions. The higher the protein concentration (hence the lower the incubation time) the higher the $I_{50}$ value of the unsaturated free fatty acids for $Na^+,K^+$-ATPase activity. This phenomenon was further investigated in an experiment in which the incubation time was kept constant and the $Na^+,K^+$-ATPase concentration varied (Fig. 2A). The amount of inhibition by 15 μM oleic acid of the $Na^+,K^+$-ATPase activity decreased from 84% at a $Na^+,K^+$-ATPase concentration of 0.625 μg/ml to 50% in the presence of 3.75 μg/ml. When a constant enzyme concentration was used and the incubation time varied no effect on the percentage inhibition by 15 μM oleic acid was observed (Fig. 2B).

To get more information on the kind of inhibition, the properties of the overall $Na^+,K^+$-ATPase activity in the presence and absence of oleic acid, as most potent inhibitor, were analysed. The affinity for $Na^+$ was hardly affected. The $K_{0.5}$ was $5.5 \pm 0.4$ mM in the absence and $6.8 \pm 0.6$ mM in the presence of 15 μM oleic acid ($n = 3$). The $K_{0.5}$ for $K^+$ increased from 1.6 mM in the control to 2.8 mM at 13 μM of oleic acid. There was no change in the Hill coefficient or cooperativity index ($n_H = 1.25$). The decreased $V_{max}$, together with the increase in $K_{0.5}$, indicates mixed-type inhibi-

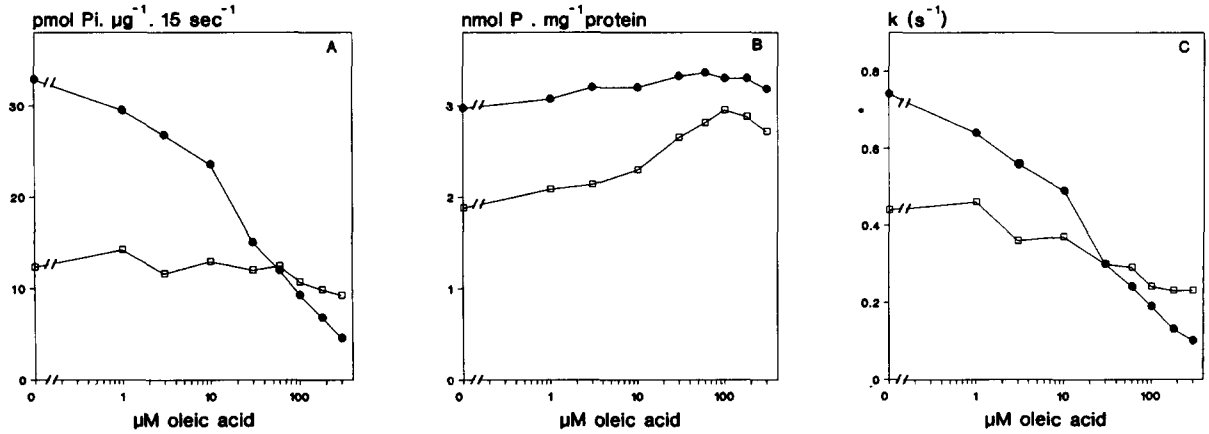


Fig. 3. Effect of oleic acid (18:1) on the ATP hydrolysis (A), on the steady-state ATP phosphorylation (B) and the dephosphorylation rate constant (C). $Na^+,K^+$-ATPase (10 μg/ml) was incubated with 2 μM [$\gamma$-$^{32}$P]ATP, 0.1 mM $Mg^{2+}$, 50 mM imidazole-acetate (pH 7.0) in the presence (100 mM, ●) or absence of $Na^+$ (□) and the oleic acid concentrations as indicated, for 15 s at 22°C and further processed as indicated in Materials and Methods. From the data of ATP hydrolysis ($v$) and the phosphorylation level (E-P) the dephosphorylation rate constant ($k$) was calculated using the equation $v = k$[E-P] (C). Mean values of two experiments are presented.

tion for $K^+$. Oleic acid (15 μM) stimulated, similarly as observed before [4,19], at suboptimal (30–100 μM) ATP concentration the hydrolysis of ATP (data not shown).

*The effect of oleic acid on the $K^+$-p-nitrophenylphosphatase activity*

The $K^+$-*p*-nitrophenylphosphatase reaction was also inhibited by oleic acid with an $I_{50}$ of 5–10 μM. Like in the $Na^+$,$K^+$-ATPase activity assay an antagonism between oleic acid and $K^+$ was found. In the absence of $Na^+$, where a cooperativity index of 1.4–1.5 for $K^+$ is found, oleic acid inhibited the activity by mixed-type kinetics, decreasing both the affinity for $K^+$ and the $V_{max}$ (data not shown). In the presence of 10 mM $Na^+$, however, when the $n_H$ value for $K^+$ decreased to 1.0 (see also Ref. 20), oleic acid decreased only the affinity for $K^+$ (the $K_{0.5}$ rising from 3.3 mM in the absence of oleic acid to 25 mM at 15 μM inhibitor) without affecting the $V_{max}$ and thus inhibited only competitively.

*Effects of oleic acid on $Na^+$-ATPase and "0"-ATPase*

Oleic acid (18 : 1) inhibited at room temperature the $Na^+$-ATPase activity with an $I_{50}$ of 25 μM (Fig. 3A). In the absence of $Na^+$ ('0'-ATPase) the ATP hydrolysis was less influenced by oleic acid (Fig. 3A). A maximal inhibition of 30% at 0.3 mM oleic acid was found.

*Effects of oleic acid on the steady-state phosphorylation*

Under the $Na^+$-ATPase assay conditions oleic acid did not affect the steady-state ATP phosphorylation level (Fig. 3B). Under '0'-ATPase conditions (absence of $Na^+$ and $K^+$), where a high steady-state phosphorylation level is measured due to the inhibitory effect of imidazole on the dephosphorylation reaction [21], oleic acid (30–100 μM) further increased the phosphorylation level. Theoretically this could result from activation of phosphorylation or from a decreased dephosphorylation rate (see also next session). Only at very high oleic acid concentrations a small reduction of the phosphoenzyme level was observed.

Directly measuring the phosphorylation rate constant at 0 °C [22] in the absence of $Na^+$, did not reveal any changes brought about by oleic acid. At this temperature there was also an increase in the steady state phosphorylation level.

*Effects of oleic acid on the dephosphorylation rate*

From the data, obtained in the ATP hydrolysis (Fig. 3A) and in the steady-state phosphorylation experiments (Fig. 3B) the dephosphorylation rate constant can be calculated using the equation $v = k \cdot [E\text{-}P]$. The results are plotted in Fig. 3C and show that both in the absence and in the presence of $Na^+$ the dephosphorylation rate constant decreased upon increasing the oleic acid concentration. The $I_{50}$ value for oleic acid was > 100 μM in the absence and 28 μM in the presence of $Na^+$. The results were confirmed by directly measuring the rate of hydrolysis of the phosphointermediate. Oleic acid (0.3 mM) reduced the dephosphorylation rate constant in the presence of 100 mM $Na^+$ by 70% (from $0.23 \pm 0.01$ $s^{-1}$ to $0.07 \pm 0.01$ $s^{-1}$, $n = 8$ and Fig. 4A). In the absence of $Na^+$ the basal dephosphorylation rate constant was less, as $Na^+$ is an activator of the dephosphorylation step [21]. Under these conditions oleic acid decreased the rate constant only from $0.09 \pm 0.04$ $s^{-1}$ to $0.06 \pm 0.02$ $s^{-1}$ ($n = 4$), see also Fig. 4B.

The dephosphorylation studies were carried out in the presence of 0.5 mM ATP, which also inhibits the dephosphorylation rate [22]. To cancel out this inhibitory effect, the inhibition by oleic acid was studied at varying concentrations of ATP in the presence of $Na^+$. Although the basal dephosphorylation rate constant

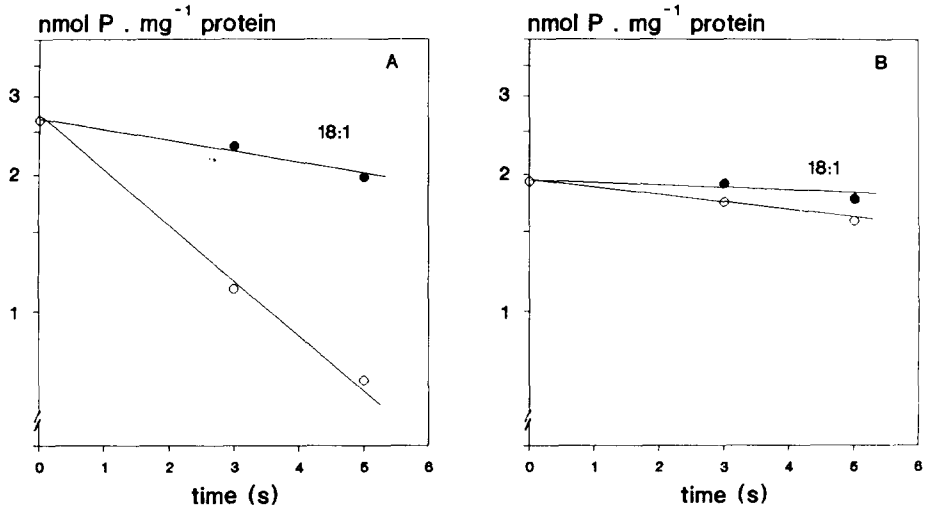


Fig. 4. Effect of oleic acid (18 : 1) on the rate of the dephosphorylation reaction in the absence or presence of $Na^+$. After phosphorylation for 10 s at 22 °C in the presence (A) or absence (B) of 100 mM $Na^+$, the phosphoenzyme (1.67 μg·$ml^{-1}$) was incubated in a medium containing 100 (A) or 0 (B) mM $Na^+$, 0.1 mM $Mg^{2+}$, 0.5 mM ATP, 50 mM imidazole-acetate (pH 7.0) with (●) or without (○) 0.3 mM oleic acid. After the indicated incubation time at room temperature the reaction was terminated and processed as described in Materials and Methods.

was double at low ATP (10 μM), oleic acid (0.3 mM) also reduced the dephosphorylation rate constant by 70% (from 0.45 $s^{-1}$ to 0.15 $s^{-1}$) at this low ATP concentration. The $I_{50}$ for ATP in the dephosphorylation remained almost the same whether oleic acid was absent ($I_{50} = 0.25$ mM) or present ($I_{50} = 0.20$ mM). This indicated that oleic acid had no influence on the inhibition of the dephosphorylation by ATP and that inhibition by both ATP and oleic acid must be additive.

The $K^+$ sensitivity of the phosphointermediate decreased upon addition of 0.3 mM oleic acid both in the presence and in the absence of $Na^+$ (Fig. 5). With the dephosphorylation technique used in this study we were unable to determine the maximal dephosphorylation rate constant and so the observed $K_{0.5}$ values for $K^+$ in the absence and presence of free fatty acids are apparent ones.

*The effects of oleic acid on the ouabain-binding*

Ouabain has a $K_d$ of 0.19 ± 0.01 μM for $Na^+,K^+$-ATPase (Table II). In the presence of oleic acid both the maximal ouabain-binding level and the affinity were affected. The maximal ouabain-binding level decreased by 27% and the affinity of ouabain for $Na^+,K^+$-ATPase decreased 2.4-times to 0.45 μM, again indicating a mixed-type inactivation process, in which the ouabain binding capacity has to be attributed to residual active enzyme molecules [23].

*Oleic acid binding to $Na^+,K^+$-ATPase*

Oleic acid binding experiments were performed by the Lipidex-1000 method [17]. After incubation with $Na^+,K^+$-ATPase the fatty acids, which were not bound, were removed by Lipidex-1000. Fig. 6A shows that the amount of oleic acid bound to $Na^+,K^+$-ATPase increases linearly with the oleic acid concentration, reaching a level of 700 nmol/mg protein (about 100 mol oleic acid per mol $\alpha\beta$ oligomer $Na^+,K^+$-ATPase [24,25]) at 0.1 mM oleic acid. The binding capacity of free fatty acids to bovine serum albumin, a well known fatty acid binding protein, is much lower (40–100 nmol/mg protein (about 5 mol/mol) with a $K_d$ of 1 μM [17]).

If, at a fixed oleic acid concentration, the $Na^+,K^+$-ATPase concentration was varied, we observed (Fig. 6B) that the amount of oleic acid bound was not linear with the enzyme concentration. So at high protein concentrations the amount of oleic acid per mg protein decreases.

The amount of bound oleic acid in the presence of 50 mM imidazole-acetate (pH 7.0) at a medium concentration of 10.5 μM oleic acid was 88 nmol/mg protein. Upon addition of 100 mM $Na^+$, 100 mM $K^+$, 10 mM $Mg^{2+}$, 5 mM ATP or 1 mM ouabain + 10 mM $Mg^{2+}$ the binding level was 86, 93, 79, 85, 76 nmol/mg protein, respectively. Hence, the binding of the fatty acid seems to be independent of the binding of effectors involved in $Na^+,K^+$-ATPase activity and of conformations ($E_1$, $E_2$) induced by them.

TABLE II

*Effect of oleic acid (0.1 mM) on the ouabain binding to $Na^+,K^+$-ATPase*

Mean values with S.E. for $n = 4$. * Significant different from controls ($P < 0.05$).

| | $K_d$ (μM) | nmol ouabain/ mg protein | % |
|---|---|---|---|
| Control | 0.19 ± 0.01 | 2.50 ± 0.26 | ≡100 |
| + oleic acid 0.1 mM | 0.45 ± 0.05 * | 1.86 ± 0.36 | 73 ± 7 * |

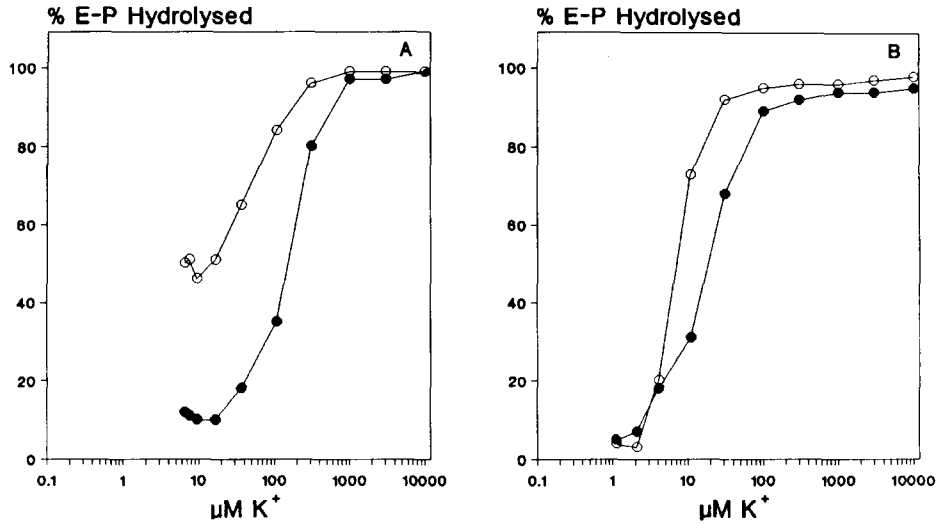


Fig. 5. Effect of oleic acid on the $K^+$ sensitivity of the phosphointermediate. Phosphorylation occurred in the presence (A) or absence (B) of 100 mM $Na^+$, 0.1 mM $Mg^{2+}$, 5 μM [$\gamma$-$^{32}$P]ATP, 50 mM imidazole-acetate (pH 7.0) and $Na^+,K^+$-ATPase (10 μg/ml) for 10 s at 22°C. Dephosphorylation was studied by adding 0.5 mM ATP, plus (A) or minus (B) 100 mM $Na^+$, 50 mM imidazole-acetate (pH 7.0), the $K^+$ concentrations as indicated and either (●) or not (○) 0.3 mM oleic acid. The percent E-P, hydrolysed in 3 s, is plotted.

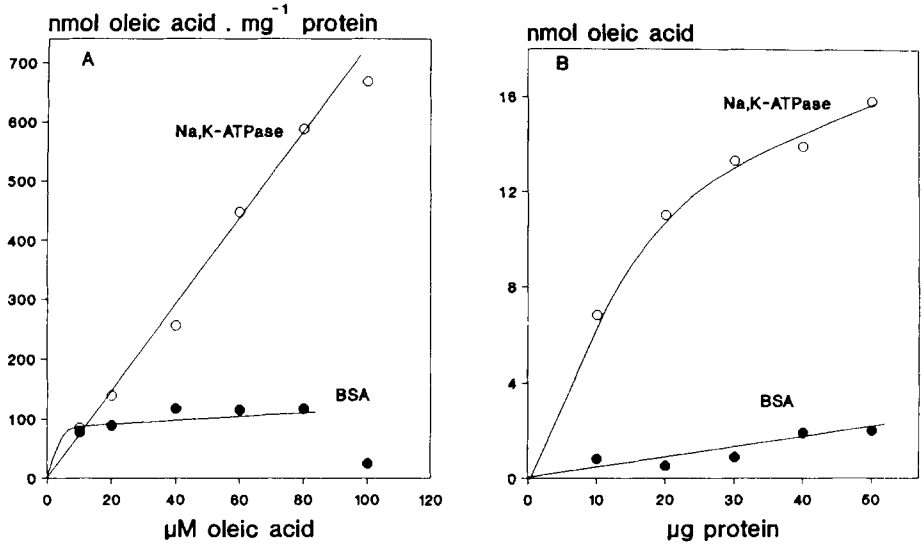


Fig. 6. Oleic acid binding. (A) $Na^+,K^+$-ATPase or bovine serum albumin (BSA), 20 μg in 0.5 ml 50 mM imidazole acetate (pH 7.0) was incubated with [$^{14}$C]oleic acid at the indicated concentrations. The amount of bound [$^{14}$C]oleic acid was determined as described in Materials and Methods. (B) Effect of $Na^+,K^+$-ATPase or BSA concentration (0–50 μg per 0.5 ml) on the amount of bound oleic acid at 0.1 mM oleic acid.

*Effect of preincubation with oleic acid on enzyme activity*

Since fatty acids did not influence the steady-state ATP phosphorylation level in the presence of $Na^+$ (see Fig. 3B) we used this assay as a test for the activity of the enzyme after prolonged oleic acid treatment.

Fig. 7 shows the effect of preincubating $Na^+,K^+$-ATPase with 0.1 mM oleic acid in the absence and presence of 100 mM $Na^+$. There was a very fast decrease in the steady-state phosphorylation level in the absence of $Na^+$. The presence of $Na^+$, however led to reduction of the inactivation rate. This inactivation of $Na^+,K^+$-ATPase depended on the fatty acid concentration, enzyme concentration (higher protein concentrations protected) and also on the incubation temperature (cf. Fig. 8B with Fig. 8A). $Mg^{2+}$ partly protected the enzyme against inactivation by 0.1 mM oleic acid, while dithioerythritol, EDTA or sucrose had no effect (Table III). In the presence of choline chloride an enhancement of the inactivation rate was even observed. Exclusion of oxygen, by flushing with nitrogen, did not alter the inactivation process. There was no or little inactivation

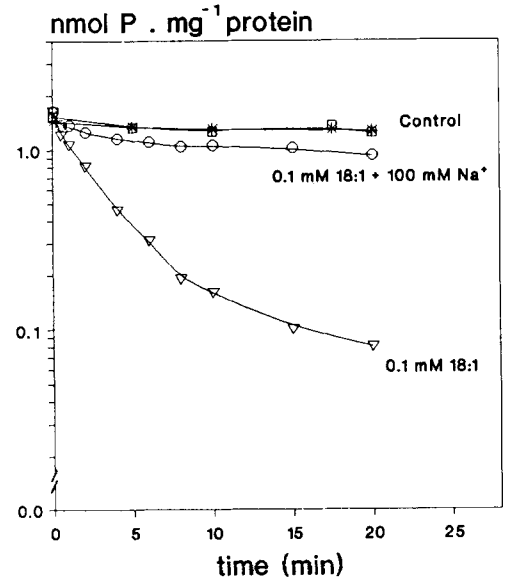


Fig. 7. Time dependence of inactivation of $Na^+,K^+$-ATPase by oleic acid. $Na^+,K^+$-ATPase (10 μg·ml$^{-1}$) was incubated with (○, ▽) or without (*, □) 0.1 mM oleic acid (18:1) in the absence (▽, *) and presence (○, □) of 100 mM NaCl for various times at 22°C in 50 mM imidazole-acetate (pH 7.0). The phosphorylation capacity was determined as 3 s level in the presence of 50 mM imidazole-acetate (pH 7.0), 2 μM [γ-$^{32}$P]ATP, 0.1 mM $Mg^{2+}$ and 40 mM $Na^+$.

TABLE III

*Effect of fatty acids on the steady state phosphorylation level during different preincubation conditions*

$Na^+,K^+$-ATPase (10 μg/ml) was preincubated at 22°C in the presence of 50 mM imidazole-acetate (pH 7.0) and the ligands as indicated. After 30 min 0.2 nmol [γ-$^{32}$P]ATP, 2 μmol $Na^+$ and 0.2 μmol $Mg^{2+}$ was added and after 3 s the phospho-level was determined as described in Materials and Methods and expressed as percentage of the level in which no fatty acid was present during the preincubation period.

| Fatty acid (0.1 mM) | Additions | Residual activity (%) | *n* |
|---|---|---|---|
| 18:1 | none | 13.9±4.2 | 6 |
| | sucrose (100 mM) | 9.6±4.4 | 4 |
| | dithioerythritol (1 mM) | 13.5±3.6 | 4 |
| | EDTA (1 mM) | 6.4±2.8 | 4 |
| | $Mg^{2+}$ (5 mM) | 34.8±4.5 | 4 |
| | $Na^+$ (100 mM) | 68.6±4.6 | 5 |
| | choline$^+$ (100 mM) | 1.2±0.5 | 5 |
| 18:0 | none | 76.3±1.8 | 3 |
| 18:1 Methyl | none | 86.2±3.2 | 3 |

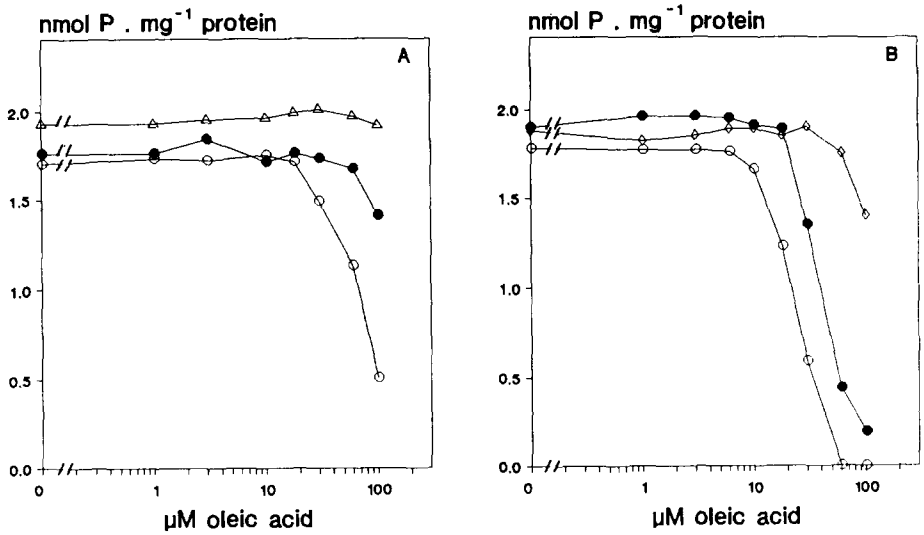


Fig. 8. Effect of oleic acid on $Na^+,K^+$-ATPase inactivation. $Na^+,K^+$-ATPase (○ and ●, 10 μg/ml; △, 50 μg/ml; or ◇, 100 μg/ml) was incubated for 30 min at 22°C (A) or 37°C (B) in 50 mM imidazole-acetate (pH 7.0), the oleic acid concentrations as indicated, in the absence (○, △, ◇) or presence of 100 mM $Na^+$ (●). The phosphorylation capacity was determined as 3-s level at the experimental temperature in the presence of 50 mM imidazole-acetate (pH 7.0), 2 μM [$\gamma$-$^{32}$P]ATP, 0.1 mM $Mg^{2+}$ and 100 mM $Na^+$.

either by saturated fatty acids (stearic acid, 18 : 0) or by methylated unsaturated fatty acids (methylated 18 : 1).

After treating $Na^+,K^+$-ATPase with oleic acid (0.1 mM) in the absence of $Na^+$, the latter ion was unable to restore the enzyme activity even when phosphorylation was prolonged until 3 min (data not shown). Also incubation with either Lipidex-1000 or bovine serum albumin (5 mg/ml) was ineffective.

Since $Na^+,K^+$-ATPase is a particulate enzyme it is possible to concentrate the enzyme nearly completely by centrifugation (60 min, 100 000 × $g$). However, after oleic acid treatment the recovery of the protein decreased with increasing oleic acid concentrations, with and without $Na^+$ present. Apparently part of the enzyme is solubilized by this treatment. No activity in the supernatant was found, suggesting that the solubilized enzyme was rapidly inactivated. In the pellet fraction both the ATPase activity (assayed at high protein concentrations) and the ATP phosphorylation capacity decreased to the same extent (data not shown).

## Discussion

Unsaturated fatty acids are known for a long time to be inhibitors of $Na^+,K^+$-ATPase [3–4,8–10,18]. Despite these studies the mechanism by which these fatty acids act is not elucidated yet.

Incubation of $Na^+,K^+$-ATPase with oleic acid had at least two effects. There is an irreversible inhibition of the enzyme activity which depends on incubation temperature and concentration of fatty acids, $Na^+,K^+$-ATPase and ligands. This inactivation can already be seen from the very steep slopes of the curves in Fig. 1, which cannot be interpreted as simple inhibition curves acting through a single site [26]. High $Na^+$ and low temperature counteract this inactivation. This inactivation process is relatively slow, taking 10–30 min for completion. Restoration of the enzyme activity by increasing $Na^+$ or addition of bovine serum albumin or Lipidex-1000, which can remove fatty acids from the protein [17], is not possible. This suggest that under these conditions the lipid–protein interaction is modified in such a way that the $Na^+,K^+$-ATPase protein cannot be restored to its original conformation. This modified lipid–protein interaction would also lead to the observed partial solubilization of the enzyme.

As inactivation of the enzyme activity only occurred in the presence of unsaturated fatty acids and not in the presence of methylated or saturated fatty acids this indicates that both the carboxyl group (charge) and the double bound (fluidity) are important for this phenomenon.

In addition, fatty acids have short-term effects on $Na^+,K^+$-ATPase which are revealed in the steady-state phosphorylation level, in the dephosphorylation step and in the $Na^+$-ATPase activity. These activities can be measured within 15 s at 22°C. An inhibition of the basal and $K^+$-activated dephosphorylation reaction was found. The inhibition of the basal dephosphorylation rate explains the increase in the steady-state level in the absence of $Na^+$. Since the steady-state phosphorylation level in the presence of $Na^+$ is not affected by oleic acid and the $Na^+$-ATPase activity under the same condition is decreased, the $Na^+$-activated dephosphorylation rate must be decreased too, which is confirmed by direct measurement of the dephosphorylation rate. $Na^+$, in high concentrations, probably stimulates dephosphorylation by a $K^+$-like effect [21]. Little fatty acid inhibi-

tion is seen on the '0'-ATPase activity (absence of $Na^+$ and $K^+$), which is in agreement with the finding that also the basal dephosphorylation rate constant is only slightly affected.

The shift of the $K^+$-stimulated dephosphorylation process towards higher $K^+$ concentrations suggests a lowering of the affinity of the enzyme for $K^+$ by oleic acid. Such a shift is also seen in the measurements of the $Na^+,K^+$-ATPase activity and the *p*-nitrophenylphosphatase activity. However, the accompanying inactivation of the enzyme makes a kinetic analysis difficult, but explains the noncompetitive effects on enzyme activity and binding of ouabain as revealed in the mixed-type inhibition. Binding of $Na^+$, which prevents the inactivation, also abolished the non-competitive effect in the *p*-nitrophenylphosphatase activity, but not in the overall $Na^+,K^+$-ATPase activity. The difference may be explained by $Na^+$ being temporarily off the enzyme in the $Na^+,K^+$-ATPase cycle, but not in the *p*-nitrophenylphosphatase activity assay.

In this study we have shown that purified $Na^+,K^+$-ATPase from rabbit kidney medulla has the capacity to bind oleic acid to very high amounts. The binding was shown to be linear with the oleic acid concentration leading to an incorporation of about 100 mol oleic acid per $\alpha\beta$ oligomer of the enzyme at 0.1 mM oleic acid. The amount of binding is hardly affected by ligands which induce specific conformations of $Na^+,K^+$-ATPase, such as the $E_1,E_1$-ATP or the E-ouabain complex. It is not known what the physical status of these fatty acids in the membrane is. They can be either incorporated in the lipid bilayer or adsorbed to the protein.

This study does not give an answer to the question as to whether fatty acids present in serum are physiological regulators of $Na^+,K^+$-ATPase present in smooth muscle cell membranes and whether these are involved in the pathogenesis of hypertension. Although fatty acids present in serum are increased in plasma of hypertensive patients [7] and inhibit $Na^+,K^+$-ATPase in vitro, it has to be established whether they also inhibit the in-vivo situation, i.e., in the presence of plasma proteins and at the extracellular $K^+$-activating side.

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