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Effect of free fatty acids and detergents on H,K-ATPase. The steady-state ATP phosphorylation level and the orientation of the enzyme in membrane preparations

Herman G.P. Swarts [1], Tom J.F. Van Uem [1], Sjouke Hoving [1], Jack A.M. Fransen [2] and Jan Joep H.H.M. De Pont [1]

[1] *Department of Biochemistry, University of Nijmegen, Nijmegen (The Netherlands)* and [2] *Laboratory for Electron Microscopy, University of Leiden, Leiden (The Netherlands)*

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The effects of detergents and free fatty acids on the K^+-activated ATPase activity and on the steady-state phosphorylation level of pig gastric H,K-ATPase were studied. Unsaturated free fatty acids inhibited the K^+-activated ATPase activity, due to inactivation of the enzyme (long-term effects) and to a decrease in the K^+-sensitive dephosphorylation rate (short-term effects). The degree of inhibition depended on the reaction conditions: the protein concentration, the temperature and the ligands used. No effect was observed when saturated- or methylated unsaturated fatty acids were tested. Free fatty acids and the detergent $C_{12}E_8$ increased the steady-state ATP phosphorylation level, indicating the presence of vesicular structures in the H,K-ATPase preparations. At higher concentrations these compounds inactivated H,K-ATPase, which was measured as a decrease in phosphorylation capacity. By combining the data from the ATP phosphorylation level in the absence and presence of $C_{12}E_8$ (without inactivation) and the data from the K^+-activated ATPase activity with and without ionophore the tightness of vesicular preparations and the orientation of H,K-ATPase was determined. A rather simple method for the isolation of H,K-ATPase is reported, which yields highly purified H,K-ATPase preparations with a ATP phosphorylation capacity of 3.9 nmol P per mg protein or 0.57 mol P per mol $\alpha\beta$ protomer. This number suggests that each α-subunit H,K-ATPase can be phosphorylated at the same time.

Introduction

H,K-ATPase is an intrinsic membrane protein complex which is responsible for acid secretion [1]. It is a member of the class of P-type membrane bound ion-transporting ATPases. There are numerous structural and kinetic similarities between H,K-ATPase and Na,K-ATPase. Both enzymes contain a catalytic α-subunit of 114 kDa [2,3] and 112 kDa [4], respectively, as well as a glycosylated β-subunit with a protein moiety of 33 kDa [5–7] and 35 kDa [8], respectively. The catalytic α-subunit contains the ion- and ATP-binding site(s) as well as the ATP phosphorylation site. There is one apparent difference. Whereas with Na,K-ATPase it has been established that each α-subunit can be phosphorylated at the same time [9], the maximal phosphorylation level found until now with H,K-ATPase has been 1.5–2.3 nmole per mg protein, which is less then 0.35 mol/mol catalytic subunit [10–13].

One of the reasons for this difference might be the presence of inaccessible substrate sites in the enzyme preparations. With Na,K-ATPase most studies have been carried out with a detergent treated kidney preparation prepared according to Jørgensen [14]. In these preparations the enzyme is present in membrane sheets, so that both sides of the membrane are available for substrate and ions. Several types of H,K-ATPase preparations have been described [15,16], which are characterized, with regard to leakiness and

Abbreviations: $C_{12}E_8$, octaethyleneglycoldodecyl monoether; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

Correspondence: J.J.H.H.M. de Pont, Department of Biochemistry, University of Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands.

orientation, by using ionophores and detergents in the K^+-activated ATPase assay.

We studied the effect of detergents and free fatty acids on the K^+-activated ATPase activity as well as on the ATP phosphorylation level in various H,K-ATPase preparations. Free fatty acids are interesting since they were reported to be inhibitors of K^+-activated ATPase activity [17]. Moreover, in a recent study with Na,K-ATPase we found short-term inhibitory effects as well as long term irreversible inactivation effects of unsaturated free fatty acids [18]. In view of the structural and functional similarities between the two enzymes it is interesting to compare the effects of fatty acids on both ATPases.

In the present paper we show that in all H,K-ATPase preparations used by us vesicular structures are present. Detergents and free fatty acids open these structures leading to increased phosphorylation levels whereas at higher concentrations these compounds inactivate the enzyme. By using limited concentrations of detergents and ionophores and by measuring the phosphorylation level and the K^+-stimulated ATPase activity, we could determine the tightness and orientation of H,K-ATPase preparations. We show moreover that in membrane vesicles, treated by osmotic shock, it is possible to obtain ATP phosphorylation levels above 0.5 mol per mole of α-subunit, suggesting that in H,K-ATPase preparations each α-subunit can be phosphorylated at the same time.

Materials and Methods

H,K-ATPase preparations

H,K-ATPase, from gastric mucosa, was prepared as reported previously [19] with some modifications. Stomachs of freshly slaughtered pigs were rinsed with tap water, transported on ice and further processed at 0–4 °C. The pink fundic region was placed in 0.25 M sucrose in 20 mM Tris-HCl (pH 7.0) (homogenization buffer) for 30 min. The tissue was wiped with paper towels to remove the mucus and the mucosa was scraped from the underlying muscular layer with a surgical blade. After 10-times dilution with homogenization buffer, the tissue was homogenized by 3–5 up and down strokes at 300 rev./min with a Braun teflon-glass homogenizer. The homogenate was centrifuged for 30 min at 20000 × *g*. The resulting supernatant was centrifuged for 30 min at 100000 × *g*. The pellet was collected and resuspended in the homogenization buffer. This microsomal preparation (fraction M) was layered on top of a gradient of 7% Ficoll in 0.25 M sucrose and 37% sucrose in 20 mM Tris-HCl (pH 7.0) and centrifuged for 60 min at 100000 × *g*. The membrane fraction at the 7% (w/v) Ficoll and the 37% (w/v) sucrose interface (fraction A) was 5-times diluted with 20 mM Tris-HCl (pH 7.0), spun down, homogenized in homogenization buffer and stored at −20 °C. The fraction at the 0.25 M sucrose and 7% (w/v) Ficoll in 0.25 M sucrose interface (fraction B) was collected and stored at 4 °C. Sucrose was removed from fraction B by dilution with 20 mM Tris-HCl (pH 7.0) and centrifugation, whereafter the membranes were freeze-dried overnight, resuspended in homogenization buffer and stored at 4 °C (fraction C). Alternatively sucrose was removed from fraction B, whereupon the resuspended fraction was incubated overnight in 20 mM Tris-HCl (pH 7.0) at 4 °C and layered on a 7% (w/v) Ficoll in 0.25 M sucrose cushion, followed by centrifugation for 60 min at 100000 × *g*. The pellet was resuspended in homogenization buffer and stored at 4 °C (fraction D).

Protein determination

Protein was determined according to the Lowry et al. [20] method following trichloroacetic acid precipitation [21], or the Bio-Rad protein assay [22]. In either case bovine serum albumin was used as a standard. The ratio between the measurements with the Lowry-trichloroacetic acid method and Bio-Rad protein assay was 1.50 ± 0.03 (mean ± S.E., $n = 20$). All data are expressed in Lowry protein values.

Gel electrophoresis

The H,K-ATPase preparations (10 μg) were electrophoresed on 10% (w/v) SDS-polyacrylamide slab gel according to Laemmli [23]. The protein bands were stained with Coomassie blue.

Steady-state ATP phosphorylation level

H,K-ATPase (10–50 μg/ml) was incubated at 22 °C for 10 s in 0.1 ml medium containing: 1–20 μM [γ-^{32}P]ATP, spec. act. 0.03–0.3 Ci/mmol (Amersham International, U.K.), 0.1–1.0 mM $MgCl_2$ and either 50 mM imidazole-acetate (pH 7.0) or 0.25 M sucrose in 20 mM Tris-HCl (pH 7.0). The reaction was stopped by adding 5 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 stopping solution, the filters were analyzed for their ^{32}P-protein content. Blanks were prepared by denaturing the enzyme prior to incubation with stopping solution [24].

Dephosphorylation studies

After 10 s phosphorylation (see above) 5 volumes of 0.6 mM nonradioactive ATP with the ligand to test were added and incubated for another 10 s at room temperature. The reaction was stopped by adding 5 ml stopping solution. The ^{32}P-phosphoenzyme was determined as described above.

Hydroxylamine assay

The sensitivity of the phosphointermediates formed by ATP, in the presence or absence of detergent (as described above), to hydroxylamine was performed as described by Schuurmans Stekhoven et al. [25].

K^+-activated ATPase assay

To 0.4 ml medium, containing either (medium A) 20 mM choline chloride, 5 mM $MgCl_2$, 5 mM Na_2ATP, 0.1 mM EDTA, 0.1 mM ouabain and 30 mM imidazole-HCl (pH 7.0) or (medium B) same as A but 20 mM K^+ replacing choline chloride, 20 μg H,K-ATPase was added. Osmolarity of the assay medium was kept constant at 0.25 osM with sucrose. After incubation at 37 °C or at 0 °C for 10 min 1.5 ml 8.6% (w/v) trichloroacetic acid and 1.5 ml ammonium heptamolybdate (1.15%, w/v) in 0.66 M sulphuric acid with 9.2% (w/v) $FeSO_4 \cdot 7H_2O$ was added. The blue phosphomolybdate complex was analyzed after 30 min at 700 nm. The aspecific Mg^{2+}-ATPase activity was calculated as the difference between activity at 37 °C and 0 °C in medium A and the K^+-activated ATPase activity as the difference in activity at 37 °C in medium A and B [26].

p-Nitrophenylphosphatase assay

K^+-activated *p*-nitrophenylphosphatase activity was determined under the same conditions as the K^+-activated ATPase activity with *p*-nitrophenyl phosphate as substrate, according to Schrijen et al. [26].

Electron microscopy

The H,K-ATPase preparations were pelleted by centrifugation for 60 min at 100000 × *g* and resuspended, at 4 °C, in 1.5% (w/v) glutaraldehyde in phosphate-buffered saline. After 30 min the fixed material was centrifuged again for 30 min at 150000 × *g*. The pellets were rinsed with phosphate-buffered saline and fragments of the pellets were stained with 1% (w/v) osmium tetroxide, dehydrated with ethanol and embedded in Epon LX112. Thin sections were studied in a Philips EM410 electron microscope.

Calculation of relative H,K-ATPase orientation in the membrane preparation

In the preparations different structures of membrane bound H,K-ATPase can be distinguished: leaky or ruptured vesicles (open structures, S1), vesicles with the ATP-site at the outside (S2) and vesicles in which the ATP-site is not accessible (S3) (see Fig. 1). These can either be unilamellar vesicles with the ATP-site at the inside (S3a) or multilamellar vesicles (S3b). In leaky or ruptured vesicles both ATPase activity and phosphorylation level are maximal and detergents (at low concentrations) as well as ionophores have no effect. In the S2 vesicles phosphorylation can occur, but ATP hydrolysis is not possible since K^+ is needed at the inside of the vesicles. With ionophores or detergents the ATPase activity can increase up to its maximal level. In the S3 vesicles no phosphorylation or ATP hydrolysis takes place. Ionophores have no effect but detergents increase both the activity and the phosphorylation level to its maximal value.

In this study we calculated, from the ratio of the K^+-activated ATPase activity in the presence ($ATPase_{S1+S2}$) and absence ($ATPase_{S1}$) of nigericin and from the ratio of the phosphorylation level in the absence ($E\text{-}P_{S1+S2}$) and presence ($E\text{-}P_{S1+S2+S3}$) of the non-ionic detergent $C_{12}E_8$, the fractional occurrence (f) of S1, S2 and S3 in the H,K-ATPase preparations as follows:

$$f_{S3} = (E\text{-}P_{S1+S2+S3} - E\text{-}P_{S1+S2})/(E\text{-}P_{S1+S2+S3})$$

$$f_{S2} = (ATPase_{S1+S2} - ATPase_{S1}) \cdot (1 - f_{S3})/(ATPase_{S1+S2})$$

$$f_{S1} = (ATPase_{S1}) \cdot (1 - f_{S3})/(ATPase_{S1+S2})$$

Chemicals

Free fatty acids (Supelco, Inc. Leusden, The Netherlands) and nigericin (Sigma, St. Louis, MO, U.S.A.) were dissolved in methanol or ethanol (nigericin) and diluted to their final concentrations in 3% (w/v) methanol or ethanol. $C_{12}E_8$, octaethyleneglycoldodecyl

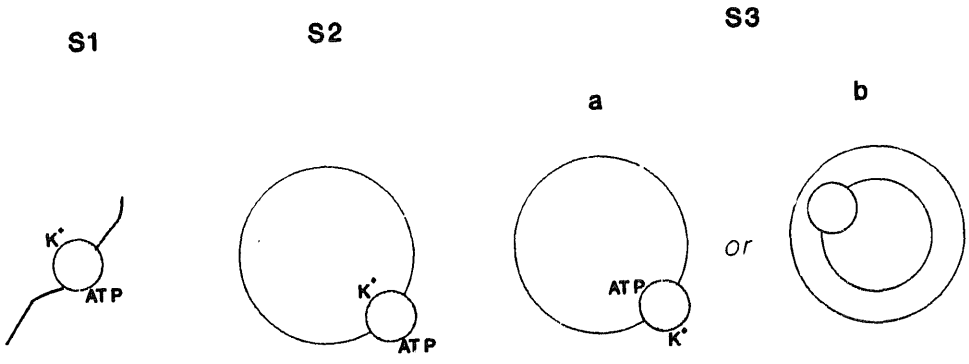

Fig. 1. Possible orientation of H,K-ATPase with its ATP- and K^+-site(s) in the different lipid bilayer membrane structures. S1, open structures; S2, closed vesicles with the ATP phosphorylation site at the outside; S3, closed vesicles with the ATP-site at in the inside: unilamellar (S3a) or multilamellar vesicles (S3b).

monoether, was obtained from Nikko Chemicals, Tokyo, Japan. Cholate, from Matheson Coleman & Bell, Norwood, OH, U.S.A., was recrystallised in 50% ethanol, following destaining with charcoal and dissolved as a Tris salt. All other chemicals were of analytical grade.

Results

Effect of free fatty acids

In this study we compared the effect of free fatty acids on H,K-ATPase with the results obtained by us with Na,K-ATPase [18]. In general the findings were rather similar. (i) Both the K^+-activated ATPase and the K^+-activated *p*-nitrophenylphosphatase activity, assayed with 10 μg H,K-ATPase per ml during 60 min incubation at 37 °C, were inhibited by oleic acid with an I_{50} value of 10 μM. (ii) Both higher enzyme concentrations (or shorter incubation times needed in order to avoid a too high substrate conversion) and the lowering of the temperature decrease the inhibitory effect of free fatty acids. (iii) Saturated- or methylated fatty acids had little or no effect on the enzyme, indicating that both the carboxyl group (charge) and the double bound (fluidity) are important for this inhibition process.

To get more information about the nature of this inhibition we studied the effect of free fatty acids on the partial reactions of H,K-ATPase. Oleic acid increased the steady-state ATP phosphorylation level (Fig. 2A), while there was a decrease in the K^+-sensitivity of the phosphoenzyme from 0.16 mM to 0.40 mM in the presence of 0.18 mM oleic acid (Fig. 2B). If H,K-ATPase was preincubated for 60 min with oleic acid prior to the phosphorylation reaction an enhancement of the phosphorylation level was only observed at low concentrations of oleic acid (10–20 μM). At higher concentrations there was a complete inhibition. The $t_{1/2}$ for this inactivation process, also observed with Na,K-ATPase [18], was about 5 min with 0.1 mM oleic acid and could be reduced to 30 min by including 100 mM NaCl in the incubation medium.

The enhancement of the ATP phosphorylation level can be explained by assuming the presence of inaccessible ATP-sites, which are set free in the presence of fatty acids. Fatty acids apparently act as detergents [27]. Therefore we carried out comparative experiments with the detergents $C_{12}E_8$, cholate and SDS.

Effect of detergent on the ATP phosphorylation level

In Fig. 3A the effect of the detergents $C_{12}E_8$, cholate and SDS is shown. They influence the ATP phosphorylation level of a freeze dried and resuspended H,K-ATPase preparation, similarly to free fatty acids. Increasing the concentration of detergent in the phosphorylation medium resulted, at a H,K-ATPase concentration of 50 μg per ml, first in an enhancement of the phosphorylation level. Maximal activation occurred at the following concentrations: SDS at 0.1 mg/ml, cholate at 6 mg/ml and $C_{12}E_8$ at 0.1 mg/ml. At higher detergent concentrations a decrease in the ATP phosphorylation level was observed. The

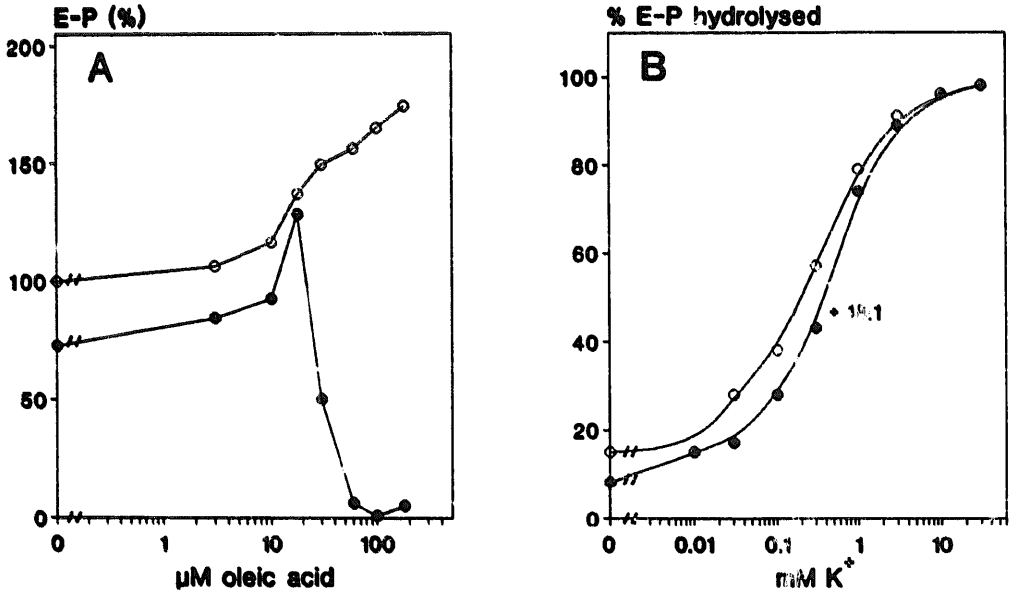

Fig. 2. Effect of oleic acid on ATP phosphorylation and dephosphorylation. (A) H,K-ATPase (10 μg/ml) was preincubated for 0 min (○———○) or 60 min (●———●) at 22 °C in 50 mM imidazole acetic acid (pH 7.0) with the indicated oleic acid concentrations, whereafter it was phosphorylated for 10 s at 22 °C with 2 μM [γ-^{32}P]ATP, 0.1 mM $MgCl_2$, 50 mM imidazole-acetate (pH 7.0). Further processing as described in Materials and Methods. Data are presented as % of the control (no preincubation, no oleic acid). (B) After phosphorylation 5 volumes of 0.6 mM non-radioactive ATP with (●———●) and without (○———○) 0.18 mM oleic acid (18:1) in the presence of the indicated K^+ concentrations was added and incubated for another 10 s. The amount (% of original) of E-P hydrolysed in 10 s is plotted.

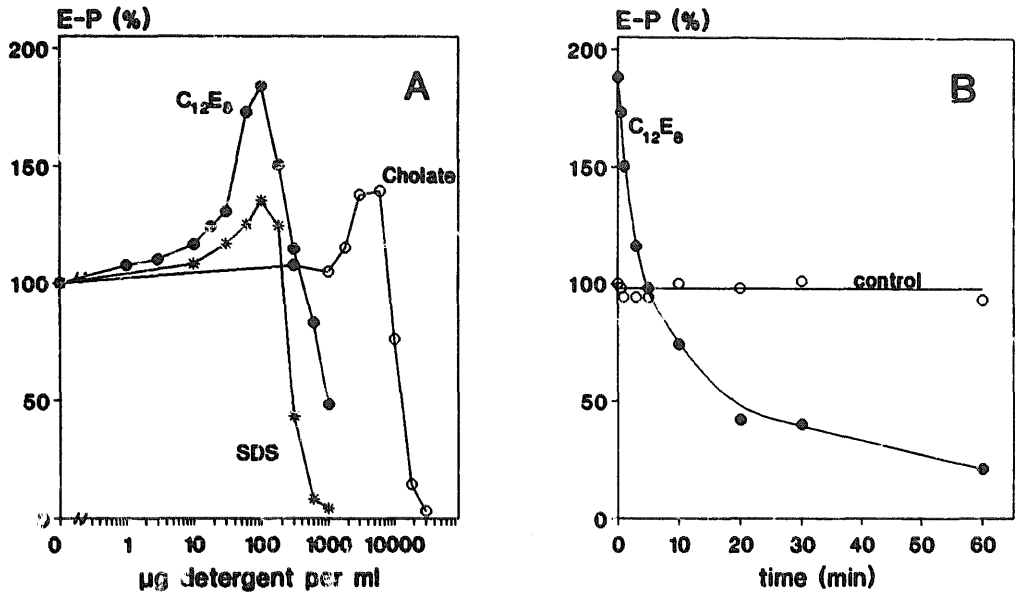

Fig. 3. Effect of $C_{12}E_8$, cholate and SDS on the ATP phosphorylation level. (A) H,K-ATPase (50 µg/ml) was incubated for 10 s at 22 °C with 2 µM [γ-^{32}P]ATP, 0.1 mM $MgCl_2$, 50 mM imidazole-acetate (pH 7.0) in the presence of the indicated detergent concentrations (* ——— *, SDS; ● ——— ●, $C_{12}E_8$; ○ ——— ○, cholate). Further processing as described in Materials and Methods. (B) H,K-ATPase was incubated at 22 °C with (●) and without (○) 0.1 mg $C_{12}E_8$ per ml, 0.1 mM $MgCl_2$ and 50 mM imidazole acetate (pH 7.0). After the indicated preincubation time the phosphorylation capacity was determined by the addition of 5 µl [γ-^{32}P]ATP (40 µM) to the reaction medium (95 µl). After 10 s the reaction was terminated and the samples were processed as described in Materials and Methods. Data are presented as percentage of the control: (E-P)%.

phosphorylation experiments were carried out under steady-state conditions as the ATP phosphorylation level was not influenced by increasing the incubation time (up to 1 min), the temperature (0–37 °C) or the concentration of either Mg^{2+} (0.1–1 mM) or ATP (2–10 µM). An increase of the incubation time of the enzyme with $C_{12}E_8$ prior to the phosphorylation experiment resulted, at a fixed $C_{12}E_8$ concentration (0.1 mg/ml), in a rapid decrease in the phosphorylation level of the enzyme, with a $t_{1/2}$ of about 5 min (Fig. 3B). This inactivation process is much slower than the enhancement in phosphorylation level which takes place in less than 10 s (Fig. 3A).

The increase of the steady-state ATP phosphorylation level by $C_{12}E_8$ was not due to a decrease in the turnover of the phosphointermediate, since the rate of

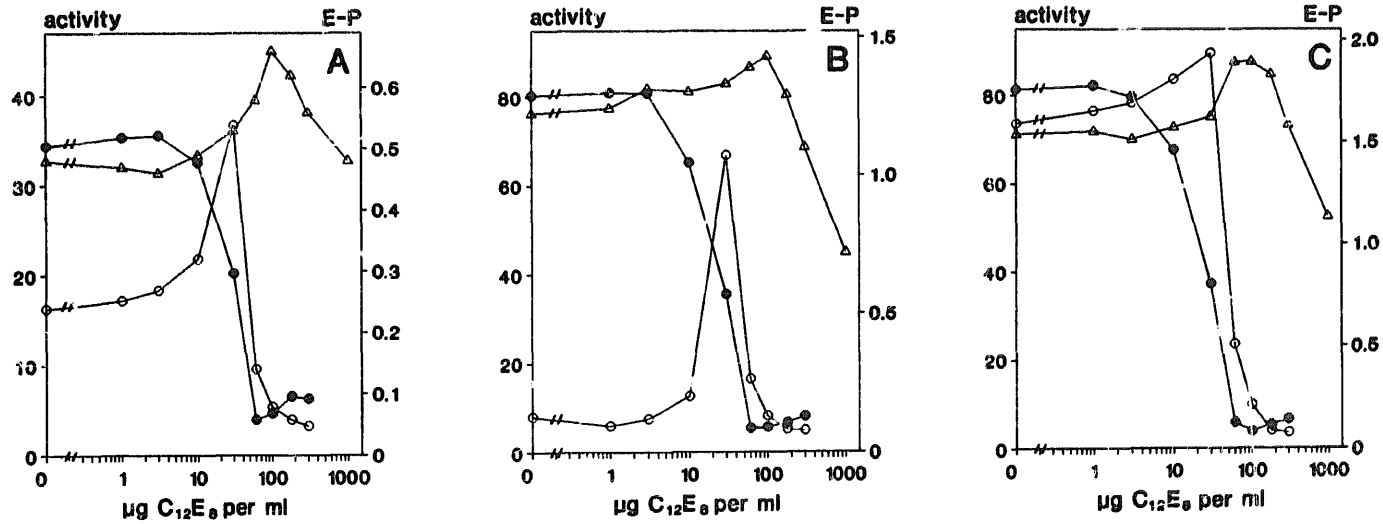

Fig. 4. Effect of $C_{12}E_8$ on K^+-activated ATPase activity and ATP phosphorylation capacity of preparation A, B, and C. Phosphorylation capacity (△ ——— △): H,K-ATPase (50 µg/ml) was incubated with the $C_{12}E_8$ concentrations as indicated in the presence of 1 mM $MgCl_2$, 20 µM [γ-^{32}P]ATP and 0.25 M sucrose/20 mM Tris-HCl (pH 7.0), for 10 s at 22 °C. E-P is expressed as nmol P per mg protein. K^+-activated ATPase activity: H,K-ATPase (50 µg/ml) was incubated with (● ——— ●) and without (○ ——— ○) 10 µM nigericin for 10 min at 37 °C in an iso-osmotic medium (250 mM) as indicated in Materials and Methods. The activity is expressed as µmol P_i per mg protein per h.

ATP hydrolysis measured under phoshorylation conditions (low ATP, no K^+, 30 s) was also increased by $C_{12}E_8$ (data not shown).

The phosphoenzymes formed in the presence and absence of $C_{12}E_8$ showed identical behavior towards hydroxylamine (data not shown). This indicates that under both conditions a carboxyl residue was phosphorylated.

The above findings indicate that freeze-dried and resupended H,K-ATPase preparations still have closed structures. As the increase in the steady-state ATP phosphorylation level was most pronounced in the presence of $C_{12}E_8$ we reinvestigated with the aid of $C_{12}E_8$ the properties of the different kinds of H,K-ATPase preparations that can be isolated from gastric mucosa (fractions A, B, C or D, see Materials and Methods).

Effect of $C_{12}E_8$ on the K^+-activated ATPase activity

Under iso-osmotic conditions the K^+-activated ATPase activity of these different preparations were also influenced by $C_{12}E_8$, (Figs. 4A, 4B and 4C). Enhancement of the activity occurred at concentrations up to 0.03 mg $C_{12}E_8$ per ml. Higher concentrations of this detergent decreased the enzyme activity. The K^+-activated ATPase activity was inhibited more strongly by $C_{12}E_8$ than the steady-state ATP phosphorylation level. This was due to the longer incubation period and higher temperature involved in the ATPase assay, giving rise to inactivation, as shown above.

In the presence of the ionophore nigericin (absence of detergent), an activation (being maximal at 10 μM nigericin) of the K^+-activated ATPase activity took place, indicating the presence of vesicular structures with the ATP-site at the outside (K^+ activation at the inside). The increase in K^+-activated ATPase activity by $C_{12}E_8$ was most pronounced in preparation B (Fig. 4B) and can be interpreted as the conversion of closed to open structures. However, the K^+-activated ATPase activity in the presence of $C_{12}E_8$ alone did not always reach the same level as in the presence of nigericin (Fig. 4B), indicating that these limited concentrations of detergent already caused some enzyme inactivation. In the presence of nigericin the inhibitory effect of $C_{12}E_8$ was enhanced and no activation was observed, the activation of H,K-ATPase probably being overruled by the inactivation process. Comparison of Figs. 4A and 4C with 4B indicate that in the preparations A and C the ATPase activity in the absence of ionophore is much higher than in preparation B and that the relative stimulation by ionophore is much less. This suggests that fractions A and C contain more open vesicles or sheet structures than fraction B. Fraction A was obtained from the Ficoll/37% sucrose interface, whereas fraction B was obtained from the 0.25 M sucrose/Ficoll interface, after centrifugation of the microsomal fraction. Fraction C was obtained after freeze drying of fraction B. This procedure apparently opens closed vesicles.

The orientation of H,K-ATPase in membrane preparations

Three different orientations of the H,K-ATPase with respect to the lipid bilayer can be distinguished: open structures (S1), closed structures with the ATP-site at the outside (S2) and closed structures with the ATP-site at the inside either unilamellar (S3a) or multilamellar (S3b), see Fig. 1. The experiments described in Fig. 4 make it possible to calculate the contribution of each of the three different orientations for the various ATPase preparations. In the absence of $C_{12}E_8$ the ATP-phosphorylation level of S1 plus S2 could be measured. In the presence of the detergent probably all vesicular structures disappeared, the ATP-site at the inside (S3) became available too and the ATP phosphorylation level of S1 + S2 + S3 could be measured. In the K^+-activated ATPase assay, under standard conditions, only the activity of S1 could be measured, since the ATP- as well as the K^+-site (opposite to the ATP-site) must be accessible for this activity. In the presence of the K^+-ionophore nigericin the inner K^+ sites became accessible and the enzyme molecules present in S2 vesicles start to contribute to the enzyme activity too (S1 + S2). As in the presence of low concentrations of $C_{12}E_8$ in combination with 10 μM nigericin inactivation of the enzyme activity occurred (see previous section) it was not possible to measure under these conditions the K^+-activated ATPase activity (S1 + S2 + S3).

From the ratio of the K^+-activated ATPase activity in the presence (S1 + S2) and absence (S1) of nigericin and from the ratio of the phosphorylation level in the absence (S1 + S2) and presence (S1 + S2 + S3) of $C_{12}E_8$, we calculated the relative amounts of S1, S2 and S3 in the H,K-ATPase preparations (see Materials and Methods). The data in Table I show that there is a large variety in the orientation of H,K-ATPase in membrane preparations. Fraction A contained, when assayed under iso-osmotic conditions considerable amounts of vesicular structures (36% S2 and 27% S3). Preparation B contained 92% closed vesicles with the ATP-site at the outside (S2). Freeze drying resulted in opening of most of the vesicular structures. This fraction (C), however, also contained 27% vesicles with the ATP-site at in the inside (S3). The osmotic shock treated preparation (fraction D) contained only 25% open structures (S1) and 22% vesicles of type S3. Probably a reorientation occurs during resuspension of the enzyme.

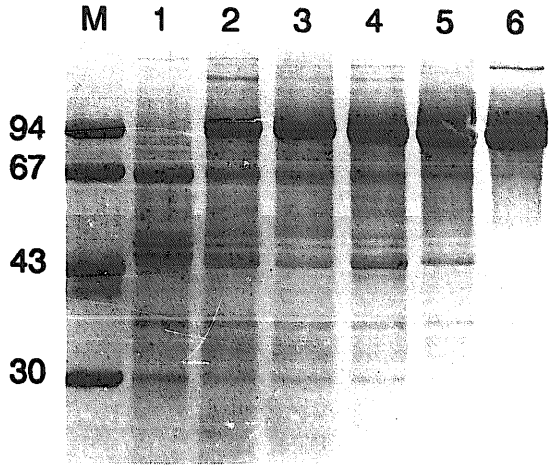

Fig. 5. SDS-PAGE pattern of the various H,K-ATPase preparations. 10 μg protein was electrophoresed on a 10% SDS-slab gel and stained with Coomassie Blue. Lane M, molecular weight markers (in kDa); lane 1, 100000× g supernatant; lane 2, microsomal fraction (fraction M); lane 3, fraction A (37% sucrose/Ficoll interface); lane 4, fraction B (0.25 M sucrose/Ficoll interface); lane 5, fraction C (freeze dryed) and lane 6, osmotic shock preparation (fraction D).

Further characterization of the preparation by electron microscopy and SDS-PAGE

The degree of purifity of H,K-ATPase preparation can be shown by SDS gel electrophoresis (Fig. 5). In lane 6 (fraction D) mainly the α-subunit of H,K-ATPase was observed, indicating a higher degree of purification, compared to fraction B (lane 4). Parallel with this the ATP phosphorylation level increased, from 1.19 to 2.39 nmol P per mg protein (see Table I).

The morphological apparence of the preparations (fractions A, B, C and D) were also investigated by electron microscopy (Fig. 6). Fraction A contains a mixture of various structures, vesicles, open membranes and endoplasmic reticulum. Fraction B shows mostly unilamellar vesicles but some multilamellar vesicles can be distinguished. The open membranes apparently do not contain much ATPase activity (Fig. 4 and Table I). Fraction C and D show more vesicular structures than fraction B, whereas the biochemical analysis indicated that less closed vesicles were present. This suggests that a large fraction of these vesicles is not ion-tight. In addition the number of multilamellar vesicles in fraction C and D are clearly higher than that in fraction B, which is in agreement with the larger percentage of S3 structures. The recentrifugation of fraction C and D apparently removed part of the membranes not containing H,K-ATPase resulting in the higher phosphorylation capacity of these fractions.

The ATP-phosphorylation capacity of H,K-ATPase

The data in Table I also show that we are able to isolate very highly purified H,K-ATPase. The density of the membranes (vesicles) isolated at the 0.25 M sucrose and the 7% Ficoll/ 0.25 M sucrose interface (fraction B) could be changed by diluting with low buffer concentrations, following centrifugation and resuspension. The obtained fraction could pass the 7% Ficoll/ 0.25 M sucrose cushion and could be collected as a pellet (fraction D). The degree of purification is

TABLE I

ATPase activity, ATP phosphorylation level and orientation of H,K-ATPase preparations

Preparation		M	A	B	C	D
n		4	7	8	7	6
ATP phosphorylation level (nmol P/mg protein)						
Control		0.55 ± 0.01	0.62 ± 0.20	1.14 ± 0.18	1.21 ± 0.21	1.86 ± 0.32
+ $C_{12}E_8$ (0.1 mg/ml)		0.67 ± 0.05	0.83 ± 0.23	1.19 ± 0.19	1.66 ± 0.26	2.39 ± 0.42
ATPase activity (μmol P_i /mg protein per h)						
Mg^{2+}-ATPase		8 ± 1	10 ± 1	7 ± 1	8 ± 2	6 ± 1
K^+-activated ATPase						
control		6 ± 1	16 ± 6	3 ± 1	71 ± 25	31 ± 9
+ Nigericin 10 μM		35 ± 7	36 ± 18	66 ± 16	72 ± 21	110 ± 29
+ $C_{12}E_8$ 0.03 mg/ml		27 ± 6	39 ± 19	59 ± 14	78 ± 29	115 ± 30
Orientation (%)	ATP-site					
S1: open structure		15 ± 4	37 ± 14	5 ± 2	71 ± 11	25 ± 11
S2: closed structure	outside	67 ± 9	36 ± 13	92 ± 6	2 ± 5	53 ± 12
S3: closed structure	inside	18 ± 6	27 ± 4	4 ± 7	27 ± 8	22 ± 2

The phosphorylation level and the ATPase activity were determined as described in Materials and Methods. From these data the orientation of H,K-ATPase in the membrane preparations in calculated, see Materials and Methods. The data are expressed as mean values ± S.D. for the number of experiments as indicated.

visualized by SDS-PAGE, see Fig. 5. Fraction D preparations reached in the presence of $C_{12}E_8$ (activation of 28%) an ATP phosphorylation level of 1.6–2.8 (2.39 ± 0.42, mean ± S.D.) nmol P per mg protein.

Discussion

Free fatty acids and H,K-ATPase

Im and Blakeman [17] were the first who reported

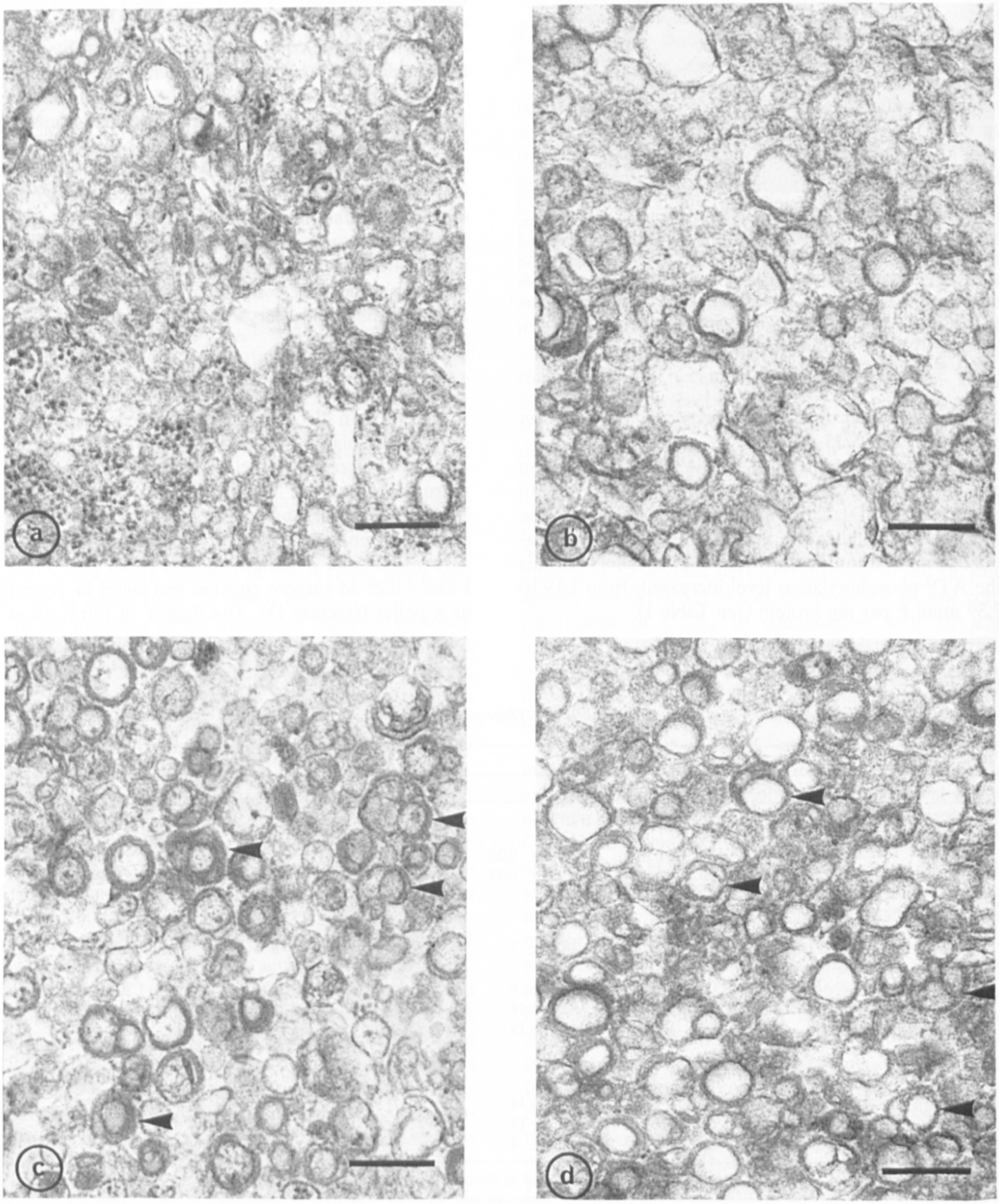

Fig. 6. Electron micrograph of fractions A, B, C, and D (see text). Multilamellar structures are indicated with arrow heads. Bar = 0.5 μm.

inhibition of the overall K^+-activated ATPase activity by unsaturated fatty acids. They found inhibition of the K^+-activated ATPase activity, the K^+-stimulated *p*-nitrophenylphosphatase activity and the phosphoenzyme formation. In their study no mechanistic explanation for the inhibitory effects of fatty acids was given. We can distinguish three different effects of unsaturated free fatty acids on H,K-ATPase. (i) Short-term inhibitory effects: decrease of the rate of the K^+-activated dephosphorylation of the phosphoenzyme. (ii) Long-term inhibitory effects: inactivation of the enzyme, depending on the enzyme concentrations, temperature and ligands present. (iii) Enhancement of the ATP phosphorylation level: increase of accessible ATP-sites by disturbance of vesicular structures. The inhibitory effects (i and ii) of free fatty acids on H,K-ATPase were comparable with the effects on Na,K-ATPase [18].

The orientation of H,K-ATPase in the membrane preparation

The observed increase in the steady-state ATP phosphorylation level by free fatty acids as well as by the detergents cholate, SDS and $C_{12}E_8$ of H,K-ATPase means that under 'normal' conditions not all ATP-sites are available for phosphorylation.

For determining maximal K^+-activated ATPase activity and orientation in inside-out vesicles (S2, Fig. 1) the use of ionophores is preferred. Detergents, alone or in combination with ionophores, cause inactivation even at the low concentrations needed for opening membrane vesicles because of the high temperature (37 °C) and the long incubation period needed for determination of enzyme activity. This could also explain the absence of a further stimulation by detergent of the K^+-activated ATPase activity [15,16]. In our study we combined the data of two different assays, phosphorylation (with and without detergents) and K^+-activated ATPase activity (with and without ionophores) and avoided the just mentioned difficulties of a combined use of detergent and ionophore.

Our results show that it is very difficult or even impossible to prepare membrane H,K-ATPase fractions which contained only one type of membrane structure. Only fraction B contained 92% closed vesicles with the ATP-site at the outside. Preparing open structures (S1, Fig. 1) by freeze-drying or by osmotic shock treatment was not possible. Pretreatment of the enzyme fractions, at high protein and rather high detergent (cholate, SDS or $C_{12}E_8$) concentrations for 60 min at room temperature, as described by Ray and Nandi [16], led also to loss of phosphorylation capacity (data not shown).

An alternative explanation for the increase in ATP phosphorylation level in the presence of $C_{12}E_8$ and the absence of stimulation by nigericin of the K^+-activated ATPase activity is the existence of multilamellar vesicle structures (S3b, Fig. 1). In the presence of the K^+-ionophore the ATP-site of an intravesicular structure is still inaccessible, but is set free in the presence of detergent and can be determined during 10 s phosphorylation, without inactivation. The presence of these multilamellar structures was confirmed by electron microscopy. This means that the vesicles determined as S3 may contain unilamellar structures (S3a), with the ATP-site at the inside, as well as multilamellar structures (S3b).

The phosphorylation capacity of H,K-ATPase

A simple purification method of H,K-ATPase leading to a very high phosphorylation capacity is described in this paper. Closed vesicles are isolated first. After treatment with low buffer concentrations these vesicles are opened resulting in a change in density to higher values. By repeated centrifugation over a 7% Ficoll in 0.25 M sucrose cushion the membranes containing H,K-ATPase are further purified.

In the presence of $C_{12}E_8$ these osmotic shock treated vesicles (fraction D) reached ATP phosphorylation levels up to 2.8 nmol P per mg protein. These values are expressed in Lowry protein values, which have to be corrected by 1.42 to compare them with amino acid data [21]. This results in a maximal ATP phosphorylation capacity of 3.9 nmol P per mg protein, which is equal to 1 phosphorylation site per 256 kDa H,K-ATPase. As has been deduced from their cDNA the α subunit of pig H,K-ATPase has a mol mass of 114 kDa [3], while the β subunit has a mol mass of 33 kDa on protein base [6]. Assuming a molecular ratio of α and β of 1 [28,29] our H,K-ATPase preparations could reach phosphorylation levels as high as 0.57 mol P per $\alpha\beta$ (0.52 ± 0.10, mean $\pm$ S.D.). This is only a minimal value, since it would increase with the degree of purity of the enzyme and absence of inactivated enzyme. These results indicate, as in case of Na,K-ATPase [9], one ATP phosphorylation site per α subunit H,K-ATPase.

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