Biochimica et Biophysica Acta, 983 (1989) 142–152
Elsevier

BBAMEM 74506

Cation sidedness in the phosphorylation step of Na^+/K^+-ATPase

Harry T.W.M. van der Hijden and Jan Joep H.H.M. de Pont

Department of Biochemistry, University of Nijmegen, Nijmegen (The Netherlands)

(Received 24 February 1989)

Key words: ATPase, Na^+/K^+-; Cation sidedness; Phosphorylation; Liposome

Na^+/K^+-ATPase, reconstituted into phospholipid vesicles, has been used to study the localisation of binding sites of ligands involved in the phosphorylation reaction. Inside-out oriented Na^+/K^+-ATPase molecules are the only population in this system, which can be phosphorylated, as the rightside-out oriented as well as the non-incorporated enzyme molecules are inhibited by ouabain. In addition, the rightside-out oriented Na^+/K^+-ATPase molecules have their ATP binding site intravesicularly and are thus not accessible to substrate added to the extravesicular medium. Functional binding sites for the following ligands have been demonstrated: (i) Potassium, acting at the extracellular side: with high affinity (stimulating the dephosphorylation rate of the E_2P conformation) and low affinity (inducing the non-phosphorylating E_2K complex). (ii) Potassium, acting at the cytoplasmic side with both high and low affinity. The latter sites are also responsible for the formation of an E_2K complex and compete with Na^+ for its binding sites. (iii) Sodium at the cytoplasmic side responsible for stimulation of the phosphorylation reaction. (iv) Sodium (and amine buffers) at the extracellular side enhancing the phosphorylation level of Na^+/K^+-ATPase where choline chloride has no effect. (v) Magnesium at the cytoplasmic side, stimulating the phosphorylation reaction and inhibiting it above optimal concentrations.

Introduction

In the mechanism of action of Na^+/K^+-ATPase the phosphorylation step plays a pivotal role [1–5]. It is assumed to be a crucial step in the conversion of chemical energy into transport. Nearly all studies on the phosphorylation reaction have been carried out in membrane preparations in which added ligands have simultaneous access to binding sites located at the extracellular and the cytosolic side of the plasma membrane. In those studies it has been established that a maximal level of phosphorylation can be obtained in the presence of both Mg^{2+} and Na^+ and in the absence of K^+ ions. The latter ion stimulates the rate of the dephosphorylation reaction considerably.

However, the presence of ions at both sides of the membrane precludes conclusions on the sidedness of these effects. Although it is very likely that the stimulatory effects of Na^+ on the phosphorylation reaction take place at the cytosolic and the stimulatory effects of K^+ on the dephosphorylation reaction at the extracellular side, additional effects of these ions at the opposite sides cannot be excluded. Although Mg^{2+} is supposed to be involved in the binding of phosphate at the cytosolic side, possible effects at the extracellular side have not been studied before.

We therefore studied steady-state phosphorylation of Na^+/K^+-ATPase reconstituted in proteoliposomes, in which only phosphorylation of inside-out oriented pump molecules is possible. By variation of the ion composition in- and outside the vesicles insight has been obtained on the presence, location and function of ion-binding sites on Na^+/K^+-ATPase.

Materials and Methods

Preparation of Na^+/K^+-ATPase

Na^+/K^+-ATPase from rabbit kidney outer medulla has been prepared according to the method described by Jørgensen [6]. About 340 mg microsomes (on protein base) were incubated for one hour at 20 °C in a medium containing 0.58 mg/ml sodium dodecyl sulfate and 25 mM imidazole-HCl (pH 7.4), 3 mM ATP and 2 mM EDTA (final protein concentration 145 mg protein per ml). After the extraction the microsomal suspension was centrifuged on a sucrose gradient (0–50%). The ATP of

Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminotetraacetic acid; CCCP, carbonylcyanide *m*-chlorophenylhydrazone. K_{cyt}, Na_{cyt} and Mg_{cyt} represent K^+, Na^+ and Mg^{2+} concentrations at the cytosolic side, respectively. K_{ext}, Na_{ext} and Mg_{ext} represent these concentrations at the extracellular side.

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

the pooled fractions of the gradient was removed by incubation at 37°C in presence of Na^+, Mg^{2+} and K^+ and subsequent washing. The obtained membrane fragments, enriched in Na^+/K^+-ATPase, were stored in imidazole buffer (25 mM, pH 7.4) containing 10% sucrose. The specific Na^+/K^+-ATPase activity of the preparations ranged from 1.0 to 1.6 mmol P_i formed per mg protein per h.

Preparation of liposomes

Liposomes have been prepared by a reversed phase evaporation method as described by Szoka and Papahadjopoulos [7]. Mixtures of cholesterol, phosphatidylcholine and phosphatidylserine (ratio indicated in text and legends) in chloroform were evaporated under a stream of nitrogen to remove the organic solvent. After repeated washing with diethyl ether, a 1:1 mixture of diethyl ether and buffer solution of different composition was added and the solution thoroughly mixed on a vortex mixer, while the diethyl ether was again slowly evaporated by a stream of nitrogen. The final lipid content was between 20 and 50 mg/ml. After all diethyl ether had disappeared the liposomes formed were sonicated for 30 min in a Branson sonicator bath at maximal output.

Reconstitution procedure

Purified Na^+/K^+-ATPase (5 mg/ml) in 20 mM imidazole buffer (pH 7.2) was partially solubilized by incubation with cholate (final concentration 0.91% w/v) during 1 min at room temperature. This mixture was then added to a 10-fold volume of liposomes, giving a lipid to protein ratio of 40 to 100 (on weight basis). After thorough mixing, the preparation was frozen in liquid nitrogen or in a mixture of dry ice and acetone and subsequently thawed at room temperature. This freezing and thawing procedure was repeated twice. Thereafter the vesicle suspension was sonicated for 6 min in a Branson sonicator bath (at maximal output). Detergent was removed from the proteoliposomes by centrifuging aliquots of the suspension over a 10-fold volume Sephadex G-25 (coarse) column (equilibrated with the appropriate buffer solution) in a syringe. This centrifugation step took 5 min (100 g) and was repeated once. More than 99.9% of the cholate was removed by this procedure [8]. Together with the removal of the detergent the extravesicular medium could be exchanged by a medium of choice [9].

ATP hydrolysis

The ATP hydrolysis was determined as the release of $^{32}P_i$ from [γ-^{32}P] ATP [10]. To 10 μl proteoliposomes containing Na^+, Mg^{2+} and K^+, in Tris buffer (pH 7.2), 190 μl of a medium containing Na^+, Mg^{2+}, K^+, Tris (pH 7.2), ouabain (0.2 mM) and 0.1 to 1.0 mM labeled ATP were added at room temperature. For blanc values the reconstituted Na^+/K^+-ATPase was denatured with trichloroacetic acid prior to incubation with the assay medium. The $^{32}P_i$ production was measured after stopping the reaction at a given time by addition of 0.4 ml 10% trichloroacetic acid followed by mixing with 0.4 ml 20% (w/v) aqueous charcoal suspension. The charcoal adsorbs the adenosine phosphates from the medium, but leaves P_i in solution. The suspension was mixed thoroughly during 10 s each 5 min (three times). Thereafter the charcoal was sedimented by centrifugation for 10 min at 2000 × g at 0°C. Aliquots (0.2–0.5 ml) were taken from the supernatant, mixed with 4.5 ml liquid scintillation fluid (Aqualuma Plus). Radioactivity was measured with a liquid scintillation counter.

Transport assay

For transport studies proteoliposomes loaded with 20 mM K^+, were equilibrated with a medium containing $^{86}Rb^+$ (0.1 mM) during 3 h at room temperature [11]. After loading with the tracer the proteoliposome suspension was incubated with a 10-fold volume of transport medium containing Na^+, K^+, Mg^{2+} and ouabain, with or without ATP. After the incubation at room temperature the transport was quenched by layering an aliquot of the suspension on a Dowex 50-X8 (Tris-form) column [12]. The proteoliposomes were then eluted with 1 ml of a solution of 250 mM sucrose and 25 mg/ml bovine serum albumin. The eluate, containing proteoliposomes devoid of the external Rb^+, was counted in a liquid scintillation counter by measuring Cerenkov radiation.

Phosphorylation

Phosphorylation of the reconstituted Na^+/K^+-ATPase was carried out at 22°C at pH 7.0. The labeled ATP concentrations varied between 0.2 and 20 μM (The Radiochemical Centre, Amersham, U.K., specific radioactivity 3000 Ci/mol). The reaction was started by rapid mixing of 10 μl proteoliposomes (preincubated with 0.2 mM ouabain and 2 mM Mg^{2+}, if not indicated otherwise) with 90 μl of the medium containing ATP and the other ligands. The reaction was stopped after 3 s by addition of 3 ml 5% (w/v) trichloroacetic acid, containing 100 mM phosphoric acid. The denatured phosphoprotein was filtered on a 1.2 μm pore width Selectron filter (Schleicher and Schuell, Dassel, F.R.G.), which was then washed three times with 3 ml of the stopping solution. Incorporated $^{32}P_i$ was determined by liquid scintillation counting. For blanc values the proteoliposomes were mixed with the stopping solution prior to addition of ATP.

Dephosphorylation

After phosphorylation for 10 s at room temperature 900 μl of the dephosphorylation medium was added to the phosphorylation mixture (100 μl). The dephospho-

rylation mixture contained, apart from the buffer and cations, 1 mM unlabeled ATP in order to dilute the labeled ATP 1000-fold. Together with the 10-fold dilution of the volume of the medium the final dilution of the labeled ATP was 10000 which actually avoided further phosphorylation by the labeled ATP. After rapid mixing the dephosphorylation reaction was stopped (at the time indicated) by addition of 5 ml 5% (w/v) trichloroacetic acid containing 100 mM phosphoric acid. After stopping the reaction, the mixture was further treated as described in the phosphorylation procedure.

Materials

ATP and Tris were purchased from Boehringer, Mannheim, F.R.G. [γ-^{32}P]ATP and ^{86}Rb were obtained from Amersham, Buckinghamshire, U.K., phosphatidylcholine (egg) and phosphatidylserine (bovine brain) were purchased from Avanti Polar Lipids, Birmingham, AL, U.S.A., CCCP from Aldrich, Milwaukee, WI, U.S.A. and cholesterol, nigericin, monensin and valinomycin from Sigma, St. Louis, MO, U.S.A. All other chemicals were of reagent grade.

Results

Pump- and hydrolytic activities of the Na^+/K^+-ATPase containing proteoliposomes

Proteoliposomes, reconstituted by the freeze-thaw/sonication procedure were able to extrude Rb^+ actively upon addition of ATP to the medium. At 22°C the reconstituted Na^+/K^+-ATPase pumped $^{86}Rb^+$ out of the preloaded vesicles with an initial velocity of 0.5 mmol/mg protein per h (based on total protein content). This velocity deviated from linearity after 30 s (not shown). Hydrolysis of ATP was assayed in the presence of ouabain (0.2 mM). P_i release was linear in time for about one min. Addition of the ionophores valinomycin + CCCP and nigericin increased the linearity of the P_i release (Fig. 1), whereas monensin did not change the rate of hydrolysis at all. The velocity of ATP hydrolysis by the proteoliposomes was about 10 to 20% of that of the purified enzyme (at 20°C). The incorporation of the enzyme was about 50% inside-out and 50% rightside-out (determined by opening the vesicles with detergent [13]. The non-incorporated part of the enzyme population was less than 10 percent on protein base as determined by separation of the proteoliposomes from the fragmented enzyme by sucrose gradient [14]. These molecules were inactive in hydrolysis and phosphorylation because of the presence of ouabain. The rightside-out oriented Na^+/K^+-ATPase molecules were also silent because of the presence of the ATP site intravesicular, inaccessible to ATP, added to the medium. Only the inside-out oriented Na^+/K^+-ATPase molecules could be active in the different assays used: phosphorylation, ATP-hydrolysis and active transport.

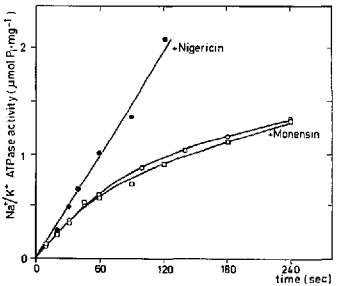

Fig. 1. ATP hydrolysis by reconstituted Na^+/K^+-ATPase. Na^+/K^+-ATPase reconstituted in liposomes (phosphatidylcholine 26, phosphatidylserine 2 and cholesterol 20 mg/ml) containing Tris (20 mM, pH 7.2), K^+ (10 mM) and Na^+ (100 mM) were preincubated with ouabain (1 mM; 60 min at room temperature). 10 μl of these proteoliposomes (protein concentration 1 mg/ml) were then incubated with 190 μl of the labeled ATP-containing medium (Mg^{2+}, 1 mM; K^+, 100 mM; Na^+, 10 mM; ATP, 1 mM and ouabain, 0.2 mM) at room temperature and the reaction was stopped with trichloroacetic acid at the indicated time (as described in Materials and Methods). Prior to incubation the proteoliposomes were preincubated with ionophores dissolved in ethanol (5 μl), to obtain a final concentration of 20 μM nigericin (●) or monensin (□). The control without ionophore (○) is also shown.

Ouabain sensitivity and phosphorylation level

The steady state level of phosphorylation of Na^+/K^+-ATPase containing membrane sheets was totally inhibited by ouabain ($I_{50} = 1$ μM), whereas only a partial decrease (< 25%) of the phosphorylation level of the Na^+/K^+-ATPase containing proteoliposomes was observed (Fig. 2). The decrease in phosphorylation level at low ouabain concentrations (< 10 μM) is due to the non-incorporated part of the enzyme. The resulting phosphorylation level at this ouabain concentration must be assigned to the inside-out oriented part of the pump molecules, which are not inhibited by ouabain, because they have their ouabain binding site intravesicularly. At higher ouabain concentrations a further decrease of the phosphorylation level could be seen, probably due to small amounts of ouabain diffusing into the proteoliposomes. In all further experiments the proteoliposomes were preincubated with 0.2 mM ouabain and 10 mM Mg^{2+} to make sure that the measured phosphorylation levels were due to inside-out oriented Na^+/K^+-ATPase molecules. The maximal level of phosphorylation for proteoliposomes containing 200 mM Tris with 100 mM Na^+, 5 mM Mg^{2+}, 15 μM ATP and 0.2 mM ouabain in the phosphorylation medium reached 30 to 40% (based on total protein content of the

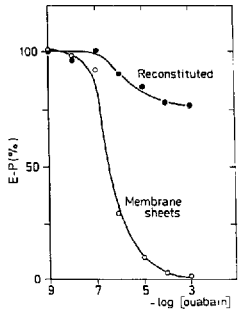

Fig. 2. Inhibition of reconstituted and fragmented Na^+/K^+-ATPase by ouabain. Proteoliposomes with the same lipid composition as described in Fig. 1, containing Tris (200 mM, pH 7.2) were preincubated with the indicated concentrations of ouabain in presence of Mg^{2+} (2.5 mM) at room temperature. After 60 min the phosphorylation reaction was carried out at the same temperature in a medium containing Na^+ (100 mM), Mg^{2+} (2.5 mM), Tris (100 mM; pH 7.0) and labeled ATP (1 μM) (●). Fragmented enzyme, preincubated with ouabain, was phosphorylated under the same conditions (○). In the absence of ouabain the steady-state phosphorylation level was 2.4 nmol/mg and 0.8 nmol/mg for the fragmented enzyme and the proteoliposomes, respectively.

proteoliposome suspension) of that of the purified enzyme under the same conditions, without ouabain.

Effects on the steady-state phosphorylation level by cytosolic and extracellular sodium

In proteoliposomes containing only intravesicular buffer solution (Tris 200 mM, pH 7.0) Na_{cyt} has a stimulating effect on the steady-state phosphorylation level. In a phosphorylation medium, containing 1 μM ATP and 5 mM Mg^{2+}, an enhancement of the phosphorylation level was according to the Michaelis-Menten formalism with a half-maximal stimulating concentration of 10.0 ± 2.5 mM (Fig. 3a). The $K_{0.5}$ values for Na^+ derived from this figure are represented in Table I. The $K_{0.5}$ value was increased by increasing K_{cyt} concentrations (Table II).

The Tris concentration at the cytoplasmic side had no effect on the steady-state phosphorylation level. In

TABLE I

$K_{0.5}$ Na_{cyt} values for reconstituted Na^+/K^+-ATPase

The $K_{0.5}$ values for Na^+ were derived from Fig. 3a after subtraction of the Na^+ insensitive level by Scatchard analysis.

ATP (μM)	$K_{0.5}$ Na_{cyt} (mM)
1.0	12
2.0	8.9
5.0	6.5
20.0	4.9

TABLE II

$K_{0.5}$ Na_{cyt} values for different K_{cyt} concentrations

Proteoliposomes, as described in the legend of Fig. 2, were phosphorylated in the presence of 5 mM Mg^{2+} and 1 μM ATP and K_{cyt} as indicated.

K_{cyt} (mM)	$K_{0.5}$ Na_{cyt} (mM)
0.0	10
0.1	12
1.0	20
10.0	23
200	100

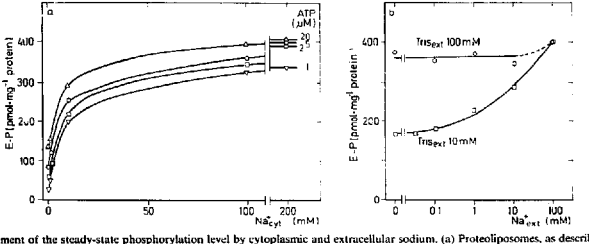

Fig. 3. Enhancement of the steady-state phosphorylation level by cytoplasmic and extracellular sodium. (a) Proteoliposomes, as described in Fig. 2 were phosphorylated in a medium containing Mg^{2+} (5 mM), Tris (20 mM, pH 7.0), choline chloride (to adjust osmolarity) and Na^+ (as indicated). Four different ATP concentrations were used for the phosphorylation: 1 μM (▽), 2 μM (□), 5 μM (○) and 20 μM (△). (b) The same phosphorylation experiment (ATP concentration 1 μM) was carried out with proteoliposomes with intravesicularly increasing Na^+ content and different Tris concentration: 10 mM (□) and 100 mM (○). Choline chloride was added intravesicularly in order to keep the osmolarity constant at 380 mosM. $K_{0.5}$ values were obtained after subtraction of the Na^+-independent level from Scatchard plots.

the absence of added Na_{cyt} the phosphorylation level was already 10 to 20% of the maximal level (with 200 mM Na_{cyt}). Increasing the ATP concentration from 1 to 20 μM increased this level to 35% of the maximum (Fig. 3a). The phenomenon, that ATP, Mg^{2+} and buffer were sufficient to give a (submaximal) level of phosphorylation indicates that Na^+ is not a unique cation to induce the phosphorylation. This level, which was reached in the absence of added Na^+, must be due to buffer stimulated phosphorylation as described by Schuurmans Stekhoven et al. [15,16]. Extracellular sodium increased the steady-state phosphorylation level in presence of 20 mM extracellular Tris, whereas with 100 mM extracellular Tris no further increase of the phosphorylation level could be observed (Fig. 3b). With 100 mM extracellular Tris the same phosphorylation level as with 100 mM Na_{ext} was obtained.

Cytoplasmic and extracellular Mg^{2+}

The role of Mg^{2+} in the reaction mechanism of Na^+/K^+-ATPase is rather complex [19]. Chelating agents have to be used in studies on the role of divalent cations in the reaction mechanism of Na^+/K^+-ATPase and have effects themselves, which cannot easily be explained [17,18]. In Na^+/K^+-ATPase-containing membrane sheets a maximal phosphorylation level could be reached in the presence of 50 mM Na^+ without adding any Mg^{2+} (Fig. 4). However, in the presence of 0.5 mM EDTA the steady-state phosphorylation level decreased and a dose-dependent increase in the phosphorylation level was found with a maximum between

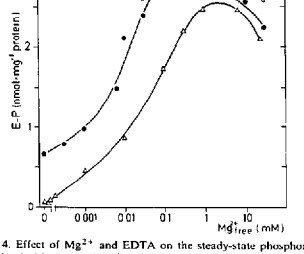

Fig. 4. Effect of Mg^{2+} and EDTA on the steady-state phosphorylation level of fragmented Na^+/K^+-ATPase. Purified enzyme was phosphorylated in a medium containing Na^+ (50 mM), Tris (70 mM, pH 7.0), choline chloride (30 mM), ATP (1 μM), EDTA and Mg^{2+} to get the indicated free Mg^{2+} concentration (0 mM (○), 0.5 mM (●) or 10 mM (▲)).

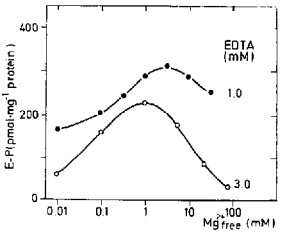

Fig. 5. Effect of cytoplasmic Mg^{2+} on the steady-state phosphorylation level in reconstituted Na^+/K^+-ATPase. Proteoliposomes as in Fig. 2, containing Tris (170 mM, pH 7.0) and choline chloride (30 mM) were preincubated with ouabain (0.2 mM) and Mg^{2+} (2 mM). After preincubation EDTA to a final concentration of 10 mM was added. Immediately thereafter the preincubated proteoliposomes were phosphorylated in a medium as described in the legend of Fig. 4. The EDTA concentration during the phosphorylation was diluted to 1 mM (●) and 3 mM (○), respectively.

0.1 and 1.0 mM Mg^{2+}. In the presence of 10 mM EDTA the steady-state phosphorylation level was nearly zero in the absence of added Mg^{2+} and the dose-response curve was shifted to the right. At higher Mg^{2+} concentrations there was a tendency to a lowering of the E-P level.

In the reconstituted system the Mg^{2+}-dependency can only be studied in the presence of EDTA, since Mg^{2+} is needed for the binding of ouabain to the non-incorporated and rightside-out oriented Na^+/K^+-ATPase. Fig. 5 shows that the phosphorylation level in the reconstituted system depended on the Mg_{cyt} concentration. An increase in the EDTA concentration lowered the E-P level at all Mg_{cyt} concentrations and shifted the dose-response curve to the left. The Mg_{cyt} concentration at which the maximal E-P level was reached was lower with higher EDTA concentrations too. In the reconstituted system the inhibitory effect at higher Mg^{2+} concentrations was much more pronounced than with the fragmented enzyme.

The Na_{cyt} dependency of the E-P level depended not only on the ATP concentration (Fig. 3a) but also on the Mg^{2+} concentration (Fig. 6). The E-P level at 1 mM Mg^{2+} was higher than at 5 mM. The effect of Mg^{2+}, however, was most pronounced in absence of Na_{cyt}. With 5 mM Mg^{2+} the steady-state phosphorylation level in absence of Na_{cyt} was less than 15% of the maximal level, whereas this was almost 50% with 1 mM Mg^{2+}.

The affinity for ATP was influenced by cytoplasmic Mg^{2+} as well as by cytoplasmic Na^+. An increase of the Mg^{2+} concentration increased the $K_{0.5}$ whereas increas-

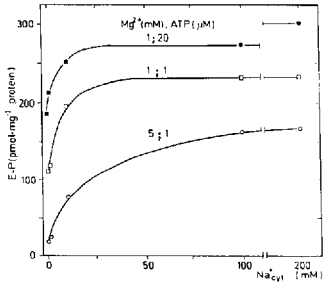

Fig. 6. Effect of cytoplasmic Mg^{2+} and ATP on the sodium dependency of the steady-state phosphorylation level. Proteoliposomes as described in Fig. 2 were phosphorylated in the presence of Tris (20 mM, pH 7.0), choline chloride (to maintain isoosmolarity) and either 5 mM Mg^{2+} and 2 μM ATP (○), 1 mM Mg^{2+} and 2 μM ATP (□), or 1 mM Mg^{2+} and 20 μM ATP (●).

ing the Na^+ concentrations decreased the $K_{0.5}$ (Table III).

Modification of the extracellular Mg^{2+} concentration had no effect on the steady-state phosphorylation level (Fig. 7). The variation in these experiments is due to the fact that for changes in the extracellular concentration the intravesicular composition has to be changed so that each point represents a separate proteoliposome preparation.

Cytoplasmic potassium

When the phosphorylation reaction was carried out with 1 μM ATP at 0 °C with at the extracellular side 150 mM Tris only a minor inhibitory effect of K_{cyt} on the steady-state phosphorylation level was observed at concentrations of K_{cyt} up to 10 mM (Fig. 8). At room temperature, however, K_{cyt} at low concentrations already considerably decreased the phosphorylation level.

TABLE III

ATP affinity for reconstituted Na^+/K^+-ATPase

Proteoliposomes, as described in the legend of Fig. 2, were phosphorylated in the presence of the indicated Mg^{2+} and Na^+ concentrations with increasing ATP concentrations. $K_{0.5}$ values were derived from Scatchard plots.

Na^+ (mM)	Mg^{2+} (mM)	$K_{0.5}$ ATP (μM)
0.2	1.2	1.25
1.0	5.0	1.54
20	5.0	0.77
20	1.2	0.40
100	5.0	0.24

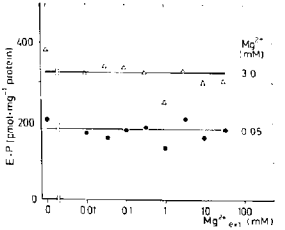

Fig. 7. Effect of extracellular Mg^{2+} on the steady-state phosphorylation level. Proteoliposomes (as in Fig. 2), loaded with Tris (170 mM, pH 7.0), choline chloride (to maintain isoosmolarity), EDTA (0.1 mM) and Mg^{2+} to obtain the indicated free Mg^{2+} concentrations were phosphorylated with two different cytoplasmic Mg^{2+} concentrations: 0.05 mM (●) and 3 mM (△).

Increase of the ATP concentration to 10 μM or replacement of the intravesicular Tris by Na^+ decreased this potassium sensitivity. Lowering the temperature to 15 °C under the latter conditions totally abolished the inhibitory effect of K^+.

An antagonism between Na_{cyt} and K_{cyt} on the steady-state phosphorylation level could be observed under conditions where the proteoliposomes were sensitive to low concentrations of K_{cyt}. At high Na_{cyt} concentrations (200 mM) the $K_{0.5}$ for K^+ was 0.6 mM whereas at low Na_{cyt} concentrations (10 mM) the $K_{0.5}$ was only 0.05 mM (Fig. 9).

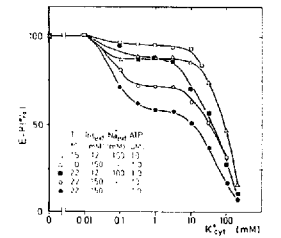

Fig. 8. Decrease of the steady-state phosphorylation level by cytoplasmic K^+. Proteoliposomes (phosphatidylcholine 20 mg/ml; cholesterol 6 mg/ml), containing Tris (150 mM, pH 7.0) were phosphorylated in the presence of Na^+ (20 mM), Mg^{2+} (5 mM), the indicated K_{cyt} concentrations and 1 μM ATP at 0 °C (○), at 22 °C (●) or at 22 °C with 10 μM ATP (○). Proteoliposomes with the same lipid composition, containing histidine (12 mM, pH 7.0) and Na^+ (100 mM) were phosphorylated with 1 μM ATP at 22 °C (■) or with 10 μM ATP at 15 °C (□).

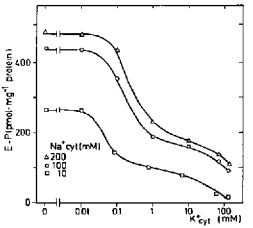

Fig. 9. Decrease of the phosphorylation level by cytosolic potassium at different cytosolic sodium concentrations. Proteoliposomes as described in the legend of Fig. 2 were phosphorylated in the presence of Mg^{2+} (5 mM), ATP (1 μM) and Na_{cyt} 10 mM (□), 100 mM (○) or 200 mM (△), with the K_{cyt} concentrations as indicated.

Under all conditions mentioned above a decrease in the steady-state phosphorylation level was observed at K_{cyt} concentrations above 10 mM. The I_{50} value for the latter effect was between 60 and 100 mM. This decrease is probably due to occupation of the Na^+ sites by K_{cyt} yielding an occluded E_2K conformation [20,21]. The decrease of the steady-state phosphorylation level was not due to an increase of the dephosphorylation rate, because the dephosphorylation rate in the presence of K_{cyt} was not significantly higher than in absence of this ligand (not shown).

Extracellular potassium

Fig. 10 shows, that K_{ext} reduced the steady-state phosphorylation level only for 15% at concentrations

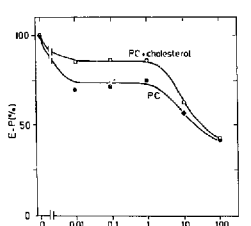

Fig. 10. Effect of extracellular K^+ on the steady-state phosphorylation level. Proteoliposomes with (○) and without (●) cholesterol (20 mg/ml, phosphatidylserine was 4 mg/ml and phosphatidylcholine 26 and 46 mg/ml, respectively) were prepared with different concentrations of extracellular K^+. The proteoliposomes contained additionally Tris (100 mM; pH 7.0) and choline chloride to maintain isoosmolarity. The phosphorylation medium was the same as in Fig. 2.

between 0.01 and 1 mM. At higher concentrations a further decrease in the E-P level was observed. The small decrease at low K_{ext} concentrations is unexpected since in fragmented enzyme preparations a nearly completely decrease in E-P is found. An explanation could be that in the reconstituted system the amount of E_2P is very small. Yoda and Yoda [22,23] have suggeted that part of the phosphorylated intermediate is in the E*P form (already proposed by Nørby and Klodos [28,29]) which only can be depsophorylated by K_{ext} after conversion into E_2P. The ratio of E_2P to E*P + E_1P has reported to be increased with decreasing cholesterol content of Na^+/K^+-ATPase-containing liposomes. The above experiment was therefore repeated with proteoliposomes containing no cholesterol, whereas in all other experiments the cholesterol content was 40%. At K_{ext} concentrations between 0.01 and 1 mM there was a significant further decrease in the steady-state phosphorylation level compared to that in cholesterol containing proteoliposomes. At K_{ext} concentrations above 10 mM the E-P level further decreased as was the case for the cholesterol containing proteoliposomes. An explanation for the latter inhibition could be the formation of an E_2K conformation which cannot be phosphorylated anymore.

Dephosphorylation

Since steady-state phosphorylation levels are the result of phosphorylation and dephosphorylation reactions, interpretation of the effects of added K^+ on the E-P level is complex. Therefore the phosphorylated intermediate was first prepared during 10 s, whereafter the radioactive ATP was diluted 10000-fold. The E-P level was measured at various time points. Dephosphorylation curves showed non-linear behaviour in a semilogarithmic plot. Fig. 11 indicates that the dephosphorylation rate in the first 3 s was faster than in the following 6 s. The steady-state phosphorylation level in proteoliposomes containing a high content of cholesterol (Fig. 11a) was only 5% reduced when 10 mM K_{ext} was present in the vesicles either at 37°C or at 0°C. At 37°C the steady-state phosphorylation level was about half of that at 0°C. The dephosphorylation rate in the first 3 s was, however, faster in vesicles containing K_{ext} than in vesicles without K_{ext}. The relative increase of the dephosphorylation rate was larger at 0°C than at 37°C.

The proteoliposomes without cholesterol (Fig. 11b) showed a larger difference in the steady-state phosphorylation level at 0°C between vesicles with and without K_{ext} (34%). The fast dephosphorylation rate in the first 3 s was for both preparations the same.

Addition of 1 mM ADP also increased the rate of dephosphorylation suggesting that part of the phosphorylated intermediate was ADP-sensitive. When the phosphointermediate was formed in the presence of

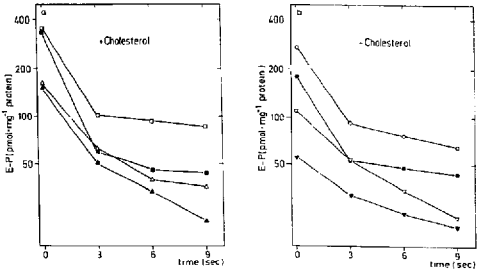

Fig. 11. Dephosphorylation of reconstituted Na^+/K^+-ATPase. (a) Proteoliposomes (as in Fig. 8) containing Tris (12 mM, pH 7.0), Na^+ (50 mM) and choline chloride (100 mM) (open symbols) or Tris (12 mM, pH 7.0), Na^+ (50 mM), choline chloride (90 mM) and K^+ (10 mM) (closed symbols) were phosphorylated during 10 s and dephosphorylated during the indicated time. Reactions were carried out at 0 °C (squares) and 37 °C (triangles). Proteoliposomes containing no Na^+ showed an identical dephosphorylation pattern. (b) The same dephosphorylation experiment was carried out with proteoliposomes without cholesterol (phosphatidylcholine was 26 mg/ml) loaded with Tris (200 mM, pH 7.0) (open symbols) or Tris (190 mM, pH 7.0) and K^+ (10 mM) (closed symbols). Dephosphorylation at 0 °C is represented by circles and at 37 °C by triangles.

K_{cyt}, the ADP-sensitive fraction was reduced to very low levels (not shown).

Discussion

Proteoliposomes as tools for sidedness studies

Na^+/K^+-ATPase reconstituted in lipid vesicles provides a good system to study such properties as transport and sidedness of ligand activation or inhibition on transport and overall or partial reactions. The composition of the intravesicular medium is totally under control and can be chosen during preparation of the proteoliposomes. Because it is possible to separate (or exchange) the extravesicular medium by gel filtration or ion exchange chromatography for any medium of choice, control of the extravesicular medium is also guaranteed. In studying a fast process as phosphorylation, where only short periods of incubation with ligands are necessary, the hazard of leakage of ligands in either direction is almost excluded. In the present study proteoliposomes were always preincubated with ouabain and Mg^{2+} at a concentration which inhibits more than 97% of non-incorporated ATPase molecules. The rightside-out oriented Na^+/K^+-ATPase has its ATP binding site inside the vesicle, which cannot be reached by externally added ATP. This leaves only a single population: the inside-out incorporated pump molecules as subject for our phosphorylation studies. The orientation of the latter pump molecules makes the cytoplasmic side accessible from the medium: hence variation in the composition of the extravesicular medium represents a variation at the cytoplasmic side of these ATPase molecules.

Na^+ and buffer cations

Titrating the Na^+ concentration in the phosphorylation medium (Na_{cyt}), which also contained Mg^{2+} and ATP, increased the phosphorylation level in a similar way as with fragmented Na^+/K^+-ATPase. The mechanism of this activation is probably the formation of the E_1 conformational state of the enzyme (Fig. 12), which can bind ATP and subsequently can be phosphorylated [24]. The maximal level of phosphorylation, however, was only attained when the extracellular (intravesicular) medium contained a minimal concentration of Tris. The latter buffer could be successfully replaced by sodium or by other buffers like imidazole, triethanolamine, triallylamine or histidine, but not by choline chloride or sorbitol. This indicates that the presence of either sodium or one fo the amine buffers at the extracellular side is a prerequisite for high phosphorylation levels. It im-

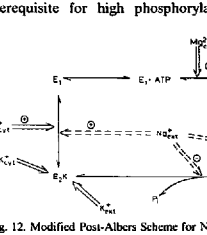

Fig. 12. Modified Post-Albers Scheme for Na^+/K^+-ATPase in which the sidedness of ligands is given. (+) indicates a stimulatory and (−) an inhibitory effect on the transitions (in a clockwise direction of the reaction cycle). ⇒⇒⇒ indicate the three possible sites of effects in the reaction cycle as discussed in the text.

plicitly proves the presence of extracellular binding sites for Na^+ and amine buffers. The mechanism of this extracellular effect of Na^+ or amine buffers is not clear but could be due to inhibition of the dephosphorylation rate (Fig. 12) as described by Schuurmans and Stekhoven et al. [25] and by Fukushima [26]. Retardation of the dephosphorylation reaction would lead to accumulation of phosphorylated intermediates. An alternative explanation is that the enzyme is partly in the E_2 conformation and that extracellular ligands induce an E_1 conformation, which can bind ATP and can subsequently be phosphorylated (Fig. 12). Such allosteric effect of Na^+ has been reported by Karlish and Stein [27]. A third explanation is that the extracellular ligands induce a shift to the left in the $E_1P \leftrightarrow E_2P$ equilibrium (Fig. 12). Such a shift would in itself not have an effect on the phosphorylation level, but if the hydrolysis of E_2P during the three seconds of the phosphorylation period is faster than that of E_1P, it would lead to an increase in the steady-state phosphorylation level [33]. Further study is required to establish which of these three alternatives is valid.

The effects of K^+

Extracellular K^+ is supposed to reduce the steady-state phosphorylation level by increasing the dephosphorylation rate (Fig. 12). However, under the standard conditions mentioned above K_{ext} reduces the steady-state phosphorylation level in proteoliposomes containing high cholesterol by only 15%. According to the third explanation mentioned above the reduction of the phosphorylation level by K_{ext} occurs through the E_2P part of the phosphorylated intermediates. This suggests that in the proteoliposomes containing high levels of cholesterol most of the phosphorylated intermediates were either in the E_1P or the E^*P form. The latter form is postulated by Nørby and Klodos [28,29]. In proteoliposomes containing no cholesterol low concentrations of K_{ext} were able to lower the steady-state phosphorylation level by 30% indicating that the amount of E_2P was increased. This finding is in agreement with the observation of Yoda and Yoda [23] that cholesterol shifts the $E^*P \leftrightarrow E_2P$ equilibrium to the left. The reduction of the steady-state phosphorylation level at high concentrations of K_{ext} is consistent with results of Karlish and Stein [31]. Blostein and Chu [32] however, found a total reduction by 100 μM K_{ext} of the steady-state phosphorylation level in erythrocyte ghosts without Na_{ext} and no reduction in ghosts with Na_{ext}. This might indicate, that our reconstituted system with high intravesicular buffer concentrations represents a system with properties in between the two extremes of the ghost preparations with and without Na_{ext}. Possibly the enzyme is mainly in an E^*P form and only a minor part in the E_2P form. A similar suggestion was made by Yoda and Yoda [33].

Both in cholesterol-containing and in cholesterol-free proteoliposomes K^+ concentrations above 1 mM gave a further decrease in the steady-state phosphorylation level. The high K^+ concentration needed for this effect makes it unlikely that the effect is due to an increase in the dephosphorylation rate. We suggest that an E_2K complex was formed which could not be phosphorylated anymore (Fig. 12).

Reduction of the steady-state phosphorylation level by low K_{cyt} concentrations could only be observed under special conditions. A high-affiity K^+ binding site is proposed by other authors [27,34,35]. Presence of 150 mM Tris, absence of Na_{ext}, low ATP concentration and an incubation temperature of 22°C resulted in a 40–50% reduction in the steady-state phosphorylation level at 1 mM K_{cyt}. Increase in the ATP or Na_{ext} concentration (or a decrease in the Tris concentration) and a lowering of the temperature led to a diminishment of this effect. This might be the reason, why others [23,32] observed that the phosphorylation in inside-out oriented Na^+/K^+-ATPase in proteoliposomes and erythrocytes was insensitive to K_{cyt}. Under the given conditions (high extracellular buffer concentration and 22°C the dephosphorylation rate of E_2P is considered to be high, but at the same time the intravesicular buffer blocks the transformational change from E^*P to E_2P [30]. These conditions make it possible to obtain a shift of E^*P to E_2P. The blockade of this conformational change by extracellular Tris was apparently overruled by K_{cyt} (Fig. 12) and a decrease in the phosphorylation level was observed as a consequence of the fast dephosphorylation. With low extracellular Tris or histidine concentrations the E^*P to E_2P conformational change was apparently not inhibited, but the dephosphorylation reaction was slow. As a consequence the change of E^*P to E_2P could not be observed as a decrease in the steady-state phosphorylation level. At 0°C K_{cyt} apparently was not able to induce the shift between E^*P and E_2P and therefore no decrease in the steady-state phosphorylation level could be observed. The latter result is in accordance with that of Blostein and Chu [32].

At higher K_{cyt} concentrations (10–200 mM) a decrease of the phosphorylation level was observed under all conditions mentioned above, as was the case with high K_{ext} concentrations. This reduction of the level can be explained by occupation of the low-affinity K^+-sites. Occupation of these sites by K^+ prevents the conversion of E_2 to E_1 which means that part of the enzyme cannot be phosphorylated anymore [31]. This is confirmed by the antagonism between Na_{cyt} and ATP on the one and K_{cyt} on the other hand. The increase by K^+ of the dephosphorylation rate of fragmented enzyme is in the light of our model the result of two simultaneous effects on the enzyme: (1) the shift of E_1P and E^*P towards E_2P, induced by K_{cyt} (which is probably very fast) and (2) the increase of the dephosphorylation rate of E_2P by

K_{ext}. A remarkable discrepancy between membrane sheets and reconstituted enzyme was found. In presence of 10 mM K^+ and 100 mM Na^+ the steady-state phosphorylation level of Na^+/K^+-ATPase in fragmented membrane sheets was practically zero [15]. With 10 mM K^+ and 100 mM Na^+ at both sides of the reconstituted enzyme we found phosphorylation levels which reached 26% of the maximum in absence of K^+ under otherwise the same conditions. The results obtained with the reconstituted enzyme, however, establish the presence of K^+-binding sites both at the extracellular and at the intracellular side of the membrane.

Effects of Mg^{2+}

Variation of the free extracellular Mg^{2+} concentration has no influence on the steady-state phosphorylation level. The cytoplasmic Mg^{2+} concentration however increases the steady-state phosphorylation level at low Mg^{2+} concentrations and lowers it at high Mg^{2+} concentrations. The magnitude of the effect depends similar to that in the fragmented enzyme on the EDTA concentration. This effect of EDTA, which can be observed even more clearly in fragmented enzyme [17,28], is not easy to explain. Possible explanations are direct inhibitory effects of EDTA, complexation of unknown divalent cations which function can be imitated by Mg^{2+} or a change in the lipid–protein interaction which can only be reversed by high Mg^{2+} concentrations. In the reconstituted enzyme the inhibitory effect of high Mg^{2+} concentrations is more pronounced than in the fragmented enzyme. In the proteoliposomes Mg^{2+} stimulates the phosphorylation in the presence of Na_{cyt} and low ATP concentrations. In the absence of Na_{cyt} however high levels of phosphorylation can only be obtained when the Mg^{2+} concentration is below 1 mM. Higher concentrations of Mg^{2+} strongly decrease the phosphorylation level. This antagonism has also been observed for the buffer stimulated phosphorylation of fragmented enzyme [15]. In the absence of Na_{cyt} and at relatively high ATP concentration (20 μM) the reconstituted enzyme is phosphorylated to 80% of the maximal level attained in presence of saturating Na_{cyt}. This level is obtained with extracellular imidazole, but also with (high concentrations of) Tris. With fragmented enzyme, where ligands can approach the enzyme from both sides, Tris does not stimulate phosphorylation in the absence of Na^+. This different behaviour of Tris can be explained by its inhibitory capacity at the cytoplasmic side, or by the different lipid environment of the reconstituted enzyme. The stimulating effect of buffers is only observed at the extracellular side, whereas Na^+ has a dual effect by enhancing phosphorylation from the cytoplasmic as well as from the extracellular side.

Conclusions

In conclusion our findings indicate that: (1) High buffer (or Na^+) concentrations are necessary at the extracellular side to obtain maximal phosphorylation levels. (2) Amine buffer-stimulated phosphorylation is suppressed by Mg^{2+} and low ATP concentration. (3) Cytoplasmic K^+ reduces the steady-state phosphorylation level at low concentrations by shifting the EP intermediates towards the E_2P conformation and at high concentrations via occupation of the low-affinity K^+-sites by yielding the K^+-occluded state of the enzyme. (4) Extracellular K^+ reduces the steady-state phosphorylation level partially, i.e. only those EP intermediates which are in the E_2P conformation will be hydrolysed. (5) Binding sites are demonstrated for: K_{cyt} (high and low affinity), K_{ext} (high and low affinity), Na_{cyt}, Na_{ext}, Mg^{2+} (cytoplasmic) and amine buffers at the extracellular side.

Acknowledgements

The authors would like to thank Dr. F.M.A.H. Schuurmans Stekhoven for his useful comments on this manuscript. This investigation was financially supported by the Netherlands Organization for Scientific Research (N.W.O.), through the Netherlands Biophysics Foundation.

References

1 Glynn, I.M. and Karlish, S.J.D. (1975) Annu. Rev. Physiol. 37, 13–55.
2 Schuurmans Stekhoven, F.M.A.H. and Bonting, S.L. (1981) in Membrane Transport, New Comprehensive Biochemistry, Vol. 2 (Bonting, S.L. and De Pont, J.J.H.H.M., eds.), pp. 159–182, Elsevier-North Holland Biomedical Press, Amsterdam.
3 Jørgensen, P.L. (1982) Biochim. Biophys. Acta 694, 27–68.
4 Robinson, J.D. (1983) in Current Topics in Membranes and Transport, Vol. 19 (Bronner, F., Kleinzeller, A., Hoffman, J.F. and Forbush, B. III, eds.), pp. 485–512, Acadamic Press, New York.
5 Skou, J.C., Norby, J.G., Maunsbach, A.B. and Esmann, M., eds. (1988) The Na^+,K^+-pump Part A: Molecular Aspects, Alan R. Liss, New York.
6 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36–52.
7 Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75, 4194–4198.
8 Skrabanja, A.T.P., Asty, P., Soumarmon, A., De Pont, J.J.H.H.M. and Lewin, M.J.M. (1986) Biochim. Biophys. Acta 860, 131–136.
9 Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891–2899.
10 Fu, Y.F., Schuurmans Stekhoven, F.M.A.H., Swarts, H.G.P., De Pont, J.J.H.H.M. and Bonting, S.L. (1985) Biochim. Biophys. Acta 817, 7–16.
11 Karlish, S.J.D. and Pick, U. (1981) J. Physiol. 312, 505–529.
12 Gasko, O.D., Knowles, A.F., Shertzer, H.G., Soulinna, E.M. and Racker, E. (1976) Anal. Biochemistry 72, 57–65.
13 Cornelius, F. and Skou, J.C. (1984) Biochim. Biophys. Acta 772, 357–373.
14 Skrabanja, A.T.P., Van der Hijden, H.T.W.M. and De Pont, J.J.H.H.M. (1987) Biochim. Biophys. Acta 903, 434–440.
15 Schuurmans Stekhoven, F.M.A.H., Swarts, H.G.P., De Pont, J.J.H.H.M. and Bonting, S.L. (1985) Biochim. Biophys. Acta 815,

16–24.
16 Schuurmans Stekhoven, F.M.A.H., Swarts, H.G.P., 't Lam, G.K., Zou, Y.S. and De Pont, J.J.H.H.M. (1988) Biochim. Biophys. Acta 937, 161–176.
17 Klodos, I. and Skou, J.C. (1975) Biochim. Biophys. Acta 391, 474–485.
18 Klodos, I. and Skou, J.C. (1977) Biochim. Biophys. Acta 481, 667–679.
19 Sachs, J.R. (1988) J. Physiol. 400, 575–591.
20 Post, R.L., Hegyvary, C. and Kume, S. (1972) J. Biol. Chem 247, 6530–6540.
21 Forbush, B. III (1987) J. Biol. Chem. 262, 11104–11115.
22 Yoda, S. and Yoda, A. (1987) J. Biol. Chem. 262, 103–109.
23 Yoda, S. and Yoda, A. (1988) J. Biol. Chem. 263, 10320–10325.
24 Rephaeli, A., Richards, D. and Karlish, S.J.D. (1986) J. Biol. Chem. 261, 6248–6254.
25 Schuurmans Stekhoven, F.M.A.H., Swarts, H.G.P., De Pont, J.J.H.H.M. and Bonting, S.L. (1986) Biochim. Biophys. Acta 855, 375–382.
26 Fukushima, Y. (1987) J. Biol. Chem. 262, 11000–11005.
27 Karlish, S.J.D. and Stein, W.D. (1985) J. Physiol. 359, 119–149.
28 Nørby, J.G., Klodos, I. and Christiansen, N.O. (1983) J. Gen. Physiol. 82, 725–759.
29 Klodos, I. and Nørby, J.G. (1987) Biochim. Biophys. Acta 897, 302–314.
30 Yoda, S. and Yoda, A. (1986) J. Biol. Chem. 261, 1147–1152.
31 Karlish, S.J.D. and Stein, W.D. (1982) J. Physiol. 328, 295–316.
32 Blostein, R. and Chu, L. (1977) J. Biol. Chem. 252, 3035–3043.
33 Yoda, A. and Yoda, S. (1987) J. Biol. Chem. 262, 110–115.
34 Matsui, H. and Homareda, H. (1982) J. Biochem. 92, 193–217.
35 Jørgensen, P.L. and Petersen, T. (1982) Biochim. Biophys. Acta 705, 38–47.