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Na^+-Na^+ exchange mediated by ($Na^+ + K^+$)-ATPase reconstituted into liposomes. Evaluation of pump stoichiometry and response to ATP and ADP

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($Na^+ + K^+$)-ATPase from shark rectal glands reconstituted into lipid vesicles and oriented inside out catalyses an ouabain-sensitive Na^+-Na^+ exchange in the absence of intravesicular K^+ when ATP is added extravesicularly. Intravesicular ouabain inhibited the exchange completely. This was also the case with digitoxigenin added to the vesicles. Intravesicular oligomycin inhibited the Na^+-Na^+ exchange partly in a fashion which was ATP dependent. The exchange is accompanied by a net hydrolysis of ATP with an apparent K_m of 2.5 μM. ADP was found to give no stimulation of the Na^+-Na^+ exchange, contrarily, ADP inhibited the ATP-dependent exchange of Na^+ both at optimal and supraoptimal ATP concentrations. When initial influx and efflux of ^{22}Na was measured and the hydrolysis of ATP concomitantly determined a coupling ratio of 2.8:1.3:1 was found, i.e. 2.8 moles of Na^+ were taken up (cellular efflux) and 1.3 moles of Na^+ extruded (cellular influx) for each mole of ATP hydrolyzed. The electrogenic Na^+-Na^+ exchange generated a transmembrane potential which was measured with the fluorescent probe ANS (8-anilino-1-naphthalenesulfonic acid) to be 60 mV positive inside the liposomes (extracellular).

Introduction

Experiments with red blood cells have shown that with Na^+ but with no K^+ in the external medium there is a Na^+-Na^+ exchange which besides ATP, which is not hydrolyzed, required ADP and which presumably is electroneutral [1–4]. With Na^+ but no K^+ on the original extracellular side of everted red blood cells and in the absence of ADP there is a presumably one-for-one Na^+-Na^+ exchange which is accompanied by a hydrolysis of ATP [5,6].

In experiments with vesicles reconstituted with ($Na^+ + K^+$)-ATPase from kidney there is an electrogenic ATP hydrolysis-dependent Na^+-Na^+ exchange with netto 0.5 Na^+ transported from the cytoplasmic to the external side per ATP hydrolyzed [7,8].

There seems thus to be at least two different types of Na^+-Na^+ exchange: One which is not accompanied by ATP hydrolysis and one which is. Since they ar both ouabain sensitive they are probably attributed to the Na,K-pump, the ($Na^+ + K^+$)-ATPase.

The present paper deals with the Na^+-Na^+ exchange in vesicles reconstituted with purified ($Na^+ + K^+$)-ATPase from shark. The experiments were planned to investigate which of the Na^+-Na^+ exchange reactions were operative in this preparation, and furthermore to see, if it is possible to measure the stoichiometry between ATP hydro-

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; ANS, 8-anilino-1-naphthalenesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; $C_{12}E_8$, octaethyleneglycoldodecyl monoether.

lyzed, the Na^+ transported from cytoplasmic to external side and the Na^+ transported in the opposite direction.

The experiments showed that there was no (ATP + ADP)-dependent Na^+-Na^+ exchange but an ATP hydrolysis-dependent Na^+-Na^+ exchange with a $Na^+_{cellular\ out}$: $Na^+_{cellular\ in}$: ATP stoichiometry of 2.8 : 1.3 : 1 which generates a membrane potential of about 60 mV positive to the extracellular side of the transport system. A preliminary report of the results has been published [9].

Methods

Materials. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) were obtained from Avanti Polar Lipids. Cholesterol was from Sigma. Octaethyleneglycoldodecyl monoether ($C_{12}E_8$) was from Nikko Chemicals, Tokyo, Japan. Bovine serum albumin was from Behring Institute. $^{22}NaCl$ was obtained from the Radioactive Center, Amersham. ATP-regeneration was provided for by the phosphocreatine/creatine phosphokinase system obtained from Sigma. Polystyrene beads, Bio-Beads were from Bio-Rad. 8-Anilino-1-naphthalenesulfonic acid (ANS) was from Fluka, AG. Digitoxigenin was from Sigma. When ADP-free ATP was needed ATP was purified and converted to the Tris-salt in a DEAE-Sephadex column.

Preparation of membrane bound and solubilized enzyme. The membrane bound ($Na^+ + K^+$)-ATPase from rectal glands of the spiny dogfish (*Squalus acanthias*) was prepared as described by Skou and Esmann [10]. The specific activity at 37°C was 1200 μmol P_i/mg per h. The enzyme was stored at −20°C in 20 mM histidine, 25% glycerol (pH 7.0).

The membrane bound enzyme was solubilized using the nonionic detergent $C_{12}E_8$ essentially according to Esmann et al. [11]. 1 vol. of enzyme was mixed with 1/4 vol. containing 4 mg $C_{12}E_8$/mg enzyme in the presence of 150 mM NaCl at 0°C. The mixture was centrifuged for 1 h at 280 000 × *g* at 10°C and the solubilized enzyme collected from the supernatant. This leads to a purificatin and the specific activity was 600–900 μmol P_i/mg per h at 22°C.

ATPase assay. ATPase activity of membrane-bound enzyme was determined in a medium containing (mM): 130 NaCl, 20 KCl, 4 $MgCl_2$, 3 ATP, 0.2 EGTA, 20 histidine, pH 7.4 at 37°C and 0.33 mg/ml bovine serum albumin. For solubilized enzyme the albumin concentration was 0.66 mg/ml and the assay medium contained 0.1 mg/ml $C_{12}E_8$.

The activity of solubilized enzyme was determined at 22°C, pH 7.0. The hydrolysis of ATP was initiated by the addition of enzyme and terminated by the addition of 50% trichloroacetic acid 0.5 to 2.0 min later. Inorganic phosphate was determined by the method of Fiske and SubbaRow [12].

The activity of the enzyme after reconstitution was determined as described above for solubilized enzyme at 22°C, pH 7.0 except that inorganic phosphate was determined with the more sensitive method of Baginski et al. [13] with the addition of 5% sodium dodecyl sulfat to the arsenite-citrate reagent.

Protein. Protein was determined as described by Peterson [14]. Bovine serum albumin was used as a standard.

Preparation of proteoliposomes. Liposomes with incorporated ($Na^+ + K^+$)-ATPase were prepared as previously described, Cornelius and Skou [15]. In essence the lipids are solubilized by addition of $C_{12}E_8$ to a concentration of 1.2 mg $C_{12}E_8$/mg lipid followed by addition of an appropriate amount of solubilized enzyme (usually a protein to lipid weight ratio of 1 : 20 was used). Usually the medium contained 130 mM NaCl, 0.1 mM $MgCl_2$, 30 mM histidine (pH 7.0). After formation of the mixed protein/lipid/detergent micelle solution the detergent was removed by adsorption to polystyrene beads (Bio-Beads) by incubation overnight at 4°C followed by 1 h at 22°C. The lipids used were PC/PE/PI/cholesterol in a weight ratio of 60 : 14 : 2 : 24. The beads were sedimentated by a mild centrifugation and the proteoliposomes collected by using a constriction pipette. After preparation the liposomes could be stored at −70°C for several months.

Determination of sidedness. The orientation of the Na^+,K^+-pump molecules after reconstitution was determined by the 'ouabain/ionophore method' described previously (Cornelius and Skou, Fig. 5 [15]). In proteoliposomes with a 1 : 20 pro-

tein/lipid weight ratio about 15% of the enzyme is incorporated inside-out (i/o), 65% right-side-out (r/o) and 20% with both intravesicular and cytoplasmic side exposed (n-o).

Flux measurements. ATP was added to the extravesicular medium which means that in all measurements of Na^+-fluxes only enzyme oriented inside-out gives rise to vectorial transport. Influx and efflux of ^{22}Na in the proteoliposomes were measured under initial equilibrium exchange conditions with Na^+ in electrochemical equilibrium across the liposome membrane: Usually, 130 mM NaCl was present both internally and externally at the start of the experiment. In experiments without ADP, ATP was regenerated via the phosphocreatine/creatine phosphokinase system. The concentration of phosphocreatine and creatine phosphokinase were 1.5 mM and 5 IU/ml, respectively.

Influx (equivalent to cellular efflux) measurements were carried out by adding proteoliposomes to a flux medium with the same composition as used for preparing the liposomes and MgATP (variable concentration) and ^{22}Na (specific activity approx. $2 \cdot 10^{11}$ cpm/mol). At given time intervals samples (50 µl) were withdrawn and layered over a cationic resin cartridge which was quickly flushed with about 1 ml flush medium (260 mM sucrose). The radioactivity of the eluate was determined directly in a gamma counter. Control experiments with ^{22}Na but without proteoliposomes showed that an elution time of 10 s was adequate to remove practically all radioactivity. These samples served as blanks. The 'apparatus' is constructed from two disposable 1 ml syringes (Fig. 1). The resin cartridge contained a stopper of glass wool and about 0.5 ml Bio-Rex 70 (Bio-Rad). The resin was converted from the H^+-form to the Tris-form by eluting with 1 M Tris followed by 150 mM Tris until pH stabilized at 7.0. Before use the columns were cooled to 4°C, and eluted with 2 ml 260 mM sucrose containing 3 mg/ml bovine serum albumin. A careful convertion and pH-equilibration is essential in order to obtain reproducible results.

Efflux was measured using proteoliposomes prepared in the presence of ^{22}Na. The efflux was measured in the tracer equilibrated liposomes by diluting 20-fold the external ^{22}Na specific activity at a constant external concentration of Na^+, and start the raction with the addition of ATP. For both influx and efflux experiments a parallel experiment with the i/o-orientated enzyme inhibited with digitoxigenin added to the flux medium is run. Digitoxigenin is lipid soluble and penetrates the proteoliposomes readily. The ATP-dependent influx or efflux is expressed as the difference between measurements with and without digitoxigenin (see Fig. 2).

For both influx and efflux experiments the change in liposome associated ^{22}Na-activity seems to follow simple monoexponentials where

$$(1)\ A_t = A_\infty \cdot (1 - \exp(-kt)) \qquad \text{(influx)}$$

and

$$(2)\ A_t = A_\infty + (A_0 - A_\infty) \cdot \exp(-kt) \qquad \text{(efflux)}$$

where A_t, A_0 and A_∞ are the liposome associated ^{22}Na activities at time t, zero and infinite, respectively. k is the observed initial rate coefficient.

A_∞ was measured after 24 h, however, isotope equilibrium was attained after 5–6 h and the liposome associated activity remained constant thereafter. A_∞ for influx was found to be identical with A_0 for efflux in liposomes prepared to the same specific ^{22}Na-activity. Although digitoxigenin-sensitsive influx was greater than efflux (see Results) the total influx and efflux were equal. It threfore seems that the proteoliposome volume is constant during the flux experiments. for a more detailed discussion of the problems in measurements of A_∞ see Gunn and Frohlich [16].

Therefore the initial rate coefficients for influx and efflux were calculated from the slopes of the graphs of $\ln(A_\infty/(A_\infty - A_t))$ and $\ln(A_0 - A_\infty)/(A_t - A_\infty)$ versus time, respectively, by linear regression, and the initial influx and efflux calculated from $k \cdot A_\infty$ and $k \cdot (A_0 - A_\infty)$, respectively. Fluxes are given as mole Na^+ per min per mg i/o-protein.

Fluorescence measurements. The fluorescence of ANS was measured using a Perkin-Elmer MPF 44A spectrofluorometer at 22° C. The excitation wavelength was 365 nm and the emission wavelength 480 nm. For measurements of electrogenic, ATP-depenent membrane potential 200 µl proteoliposomes containing 130 mM Na^+, 4.0 mM Mg^{2+} and 30 mM histidine, pH 7.0 was added to a 3 ml

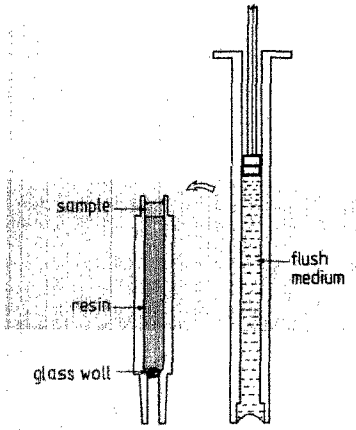

Fig. 1. The assembly used to remove extravesicular ^{22}Na in influx experiments. To the left is shown the resin cartridge filled with 0.5 ml Bio-Rex 70 in the Tris-form. To the right the plunger device composed of the upper part of a 1 ml disposable syringe filled with flush medium. 50 μl of the sample is layered on top of the resin and after assembling the device the sample is expelled by the flush medium into a counting vial.

cuvette containing the same buffer. 2 μl 1.5 mM ANS and 100 μl 10 mM ouabain was added and the relative increase in fluorescence ($\Delta F/F$) measured upon addition of 1 μl 30 mM ATP.

A stop-flow apparatus connected to the spectrofluorometer was used for calibration of the fluorescence signal [17]. The proteoliposomes contained 1 mM K^+ 129 mM Na^+, 4 mM Mg^{2+} and histidine 30 mM, pH 7.0. The external K^+-concentration was varied up to 130 mM by isoosmotic replacement of NaCl with KCl and the liposomes were made K^+-permeable by addition of 3 μM valinomycin.

Results

When proteoliposomes are equilibrated in 130 mM Na^+, and K^+ is absent, the i/o oriented enzyme molecules engage in an exchange of intravesicular (extracellular) Na^+ for extravesicular (cytoplasmic) Na^+, in the presence of Mg^{2+} and ATP in the extravesicular medium.

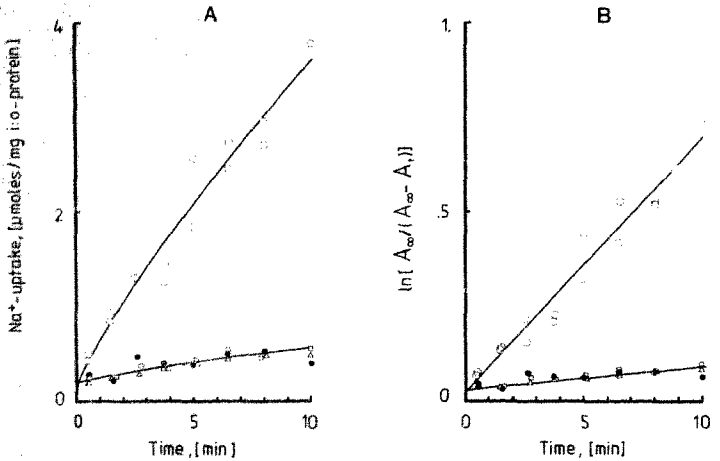

Fig. 2. Panel A shows Na^+ uptake by reconstituted vesicles in the absence of K^+ and ADP. Proteoliposomes were prepared with 130 mM Na^+, 100 μM Mg^{2+} and 30 mM histidine-HCl, pH 7.0 at both sides. At zero-time the vesicles were added to a flux medium with the same composition and 10 μM ATP, 1.5 mM phosphocreatine, 5 IU creatine phosphokinase and ^{22}Na at a specific activity of about $1 \cdot 10^{11}$ cpm/mol (○). On parallel batches the flux medium contained digitoxigenin (10 μM) (●), or no ATP (□). Alternatively vesicles with 1 mM ouabain present intravesicularly were used (△). The Na^+ uptake was determined from the ^{22}Na trapping after passage of the vesicles through cationic exchange resins as shown in Fig. 1. Panel B shows the same experiments with the results presented in a logarithmic (ln) plot.

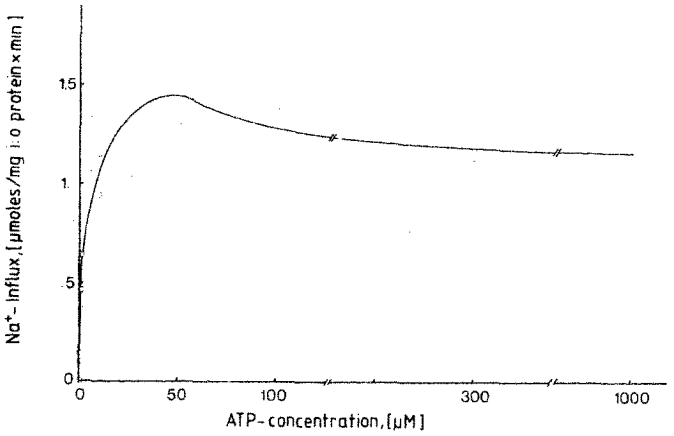

Fig. 3. Na^+ influx as a function of the ATP concentrations. Results from two series of experiments are shown. Each point represents the influx calculated from experiments as depitched in Fig. 2. The K_m value for ATP is approx. 2.5 μM.

ATP

Fig. 2 shows a typical $^{22}Na^+$-influx experiment at 22°C with proteoliposomes. In panel A the upper curve represents the uptake of Na^+ in the presence of 130 mM Na^+ on both sides of the proteoliposomes and with 25 μM ATP extravesicular. Below is the uptake of Na^+ in proteoliposomes (i) with digitoxigenin (10 μM) present in the flux medium (○), (ii) without ATP added to the flux medium (□) and (iii) in the presence of 25 μM ATP using proteoliposomes prepared with ouabain (1 mM) intravesicularly (△). Panel B represents the logarithmic transformations when $\ln(A_\infty/(A_\infty - A_t))$ is plotted against time. According to equation (1) straight lines should result with a slope equal to the observed rate constant.

As seen from Fig. 2 the three lower curves are identical giving the leak of Na^+ into the vesicles. Ouabain does not penetrate the liposomes and has to be present intravesicularly in order to inhibit i/o-enzyme molecules. Digitoxigenin penetrates the liposomes so quickly that inhibition of ATP-dependent Na^+ influx is almost instant. This makes digitoxigenin an ideal quenching agent in these flux measurements.

Fig. 3 shows how the influx of Na^+ depends on the ATP substrate concentration. Each point in the curve is the initial influx calculated from an influx experiment as the one shown in Fig. 2 consisting of seven experimental points performed at that particular ATP-concentration. In order to avoid shortage of ATP, an ATP regenerating system consisting of phosphocreatine/phosphokinase was included. The K_m value for ATP calculated from Lineweaver-Burk plots is about 2.5 μM at 22°C, pH 7.0. With the highest ATP-concentrations used (0.3 and 1 mM) a slight depression of the influx was observed in accordance with experiments with non-sided enzyme preparations where a decrease in hydrolytic activity is also seen at the higher substrate concentrations.

ADP

In red blood cells Na^+-Na^+ exchange is found not to be accompanied by net ATP-hydrolysis [2] and the exchange rate is stimulated by ADP [3]. In the present study no stimulation of ADP was observed, contrarily ADP was found to depress the ATP-dependent Na^+-Na^+ exchange. In Fig. 4 the effect of ADP at both optimal (25 μM) and supraoptimal (1 mM) ATP concentrations is shown. A very pronounced inhibition with 250 μM ADP was observed at both ATP-concentrations studied. In these experiments an ATP regeneration system was not included for obvious reasons. However, it was controlled that he influx in the absence of ADP with and without regeneration was the same for up to about 10 min. After this time a decrease was observed in the rate of influx if phosphocreatine/creatine phosphokinase was omitted.

Oligomycin

Oligomycin (10 μM) added to the extravesicular

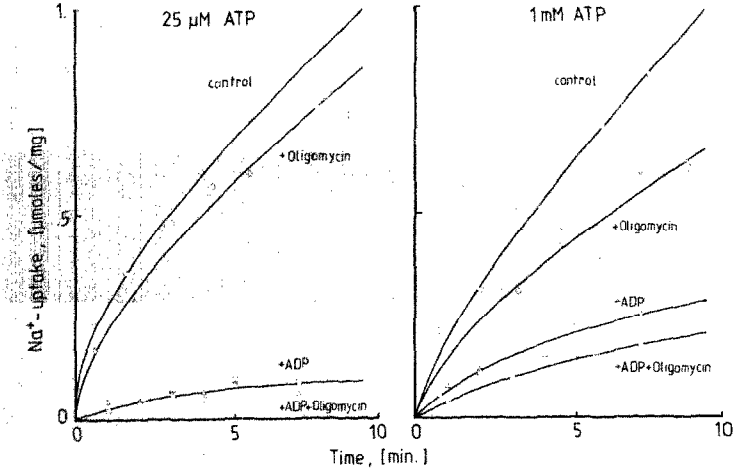

Fig. 4. Na^+ influx measured in the presence of 250 μM ADP at optimal (25 μM) and supraoptimal (1 mM) ATP concentration. Also shown are results using reconstituted vesicles with oligomycin (10 μg/ml) with the same adenine nucleotide composition. In all experiments the phosphocreatine/creatine phosphokinase-regeneration system was omitted.

medium of the proteoliposomes only inhibits Na^+ influx to a minor degree. However, if the oligomycin was present inside the vesicles thus acting on the extracellular side of the transporting enzyme molecules there is an inhibition but only partial (up to 50%). The inhibitory effect of extracellular oligomycin depends on the ATP concentrations. As seen in Fig. 5 the fractional inhibition of Na^+-influx increases with the ATP concentration just as has been observed for the ADP-dependent Na^+-Na^+ exchange in red cells [18] and by the oligomycin inhibition of ATP-hydrolysis with Na^+ and K^+ in unsided preparations [17]. Thus, at optimal ATP concentration (25 μM) the inhibition with oligomycin was small.

Pump stoichiometry

Fig. 6 shows the influx and efflux measured on parallel batches of proteoliposomes. Furthermore is shown the rate of ATP hydrolysis in the presence of extravesicular ouabain in order to inhibit the activity of the n-o oriented enzyme. All three parameters are thus related to activity from enzyme molecules oriented in the i/o mode. Calculation of the initial influx gave a value of about 1.6 μmol Na^+/mg i/o-protein per min and the efflux 0.73 μmol Na^+/mg i/o-protein per min. Compared with the rate of ATP hydrolysis which was 0.57 μmol/mg i/o-protein per min, 2.8 molecules of Na^+ are transported into the liposomes (from the cytoplasmic to the extracellular side) and 1.3

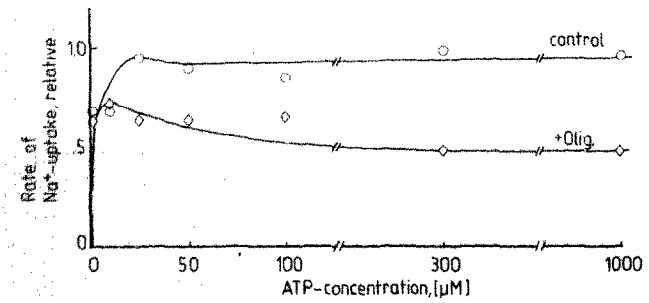

Fig. 5. Na^+ influx using reconstituted vesicles prepared either in the presence of 10 μg/ml oligomycin or without oligomycin (control) as a function of ATP concentration. Each point represents an influx experiment as depicted in Fig. 2.

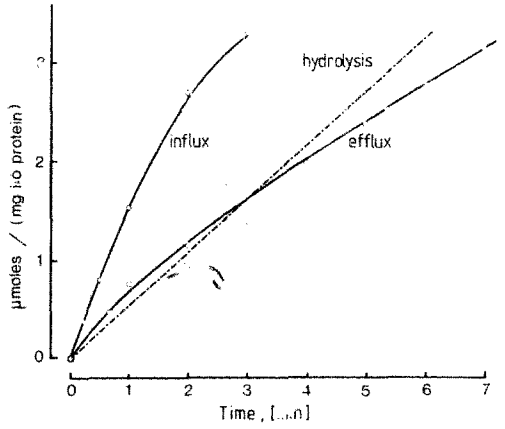

Fig. 6. Influx, efflux and ATP hydrolysis determined on parallel batches of reconstituted vesicles. From the initial slopes the influx was calculated to 1.6 µmol Na^+/mg i/o-protein per min, the efflux 0.73 µmol Na^+/mg i/o-protein per min and the rate of ATP hydrolysis 0.57 µmol P_i/mg i/o-protein per min thus giving the stoichiometry of 2.8 : 1.3 : 1.

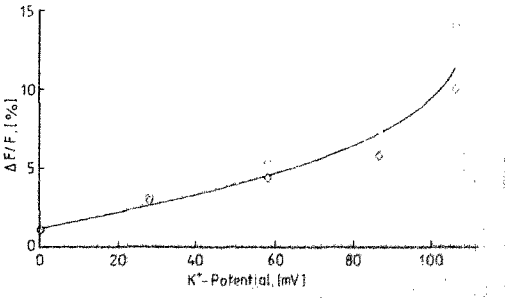

Fig. 7. Calibration curve for ANS fluorescence as a function of transmembrane K^+-diffusion potential. The relative enhancement of ANS fluorescence, $\Delta F/F$ was measured using a stopped flow apparatus at different gradients of K^+ in the presence of valinomycin. One syringe contained the reconstituted vesicles with 1 mM K^+, 129 mM Na^+, 1.5 mM ANS and 3 µM valinomycin. The other syringe contained solutions with increasing K^+ concentrations and the same concentrations of ANS and valinomycin as in syringe one. The temperature was 22°C, pH 7.0.

out of the liposomes (from the extracellular to the cytoplasmic side) per ATP molecule hydrolyzed. The ATP-hydrolysis accompanying the Na^+-Na^+ exchange was about 6% of the ATP hydrolysis measured in experiments with Na^+-K^+ exchange (not shown).

Electrogenicity

The result depicted in Fig. 6 indicates that the Na^+-Na^+ exchange is electrogenic – unless some charge is being carried with the pump in order to balance electrically the excess of Na^+. An electrogenic transport should generate a transmembrane potential unless the liposomes are very leaky to Cl^-.

The effect of Na^+-Na^+ exchange on transmembrane potential was measured with the fluorescent probe ANS [19]. The fluorescent response from ANS due to a membrane potential was calibrated using proteoliposomes produced in 1 mM K^+ and 129 mM Na^+ and varying the external K^+ concentration at constant osmolarity in the presence of the K^+ ionophore valinomycin. However, the time constant for the fluorescence response was so high that in order to resolve it, it proved necessary to use a stop-flow apparatus (cf. Ref. 20). By plotting the calculated K^+-diffusion potential versus the relative increase in fluorescence the calibration curve shown in Fig. 7 was constructed.

In Fig. 8 is shown the fluorescence enhancement when the Na^+-Na^+ exchange is started by the addition of 10 µM ATP. The Na^+ concentration is 130 mM on both sides of the membrane. Addition of ATP results in a slowly increasing fluorescent signal which again declines towards the base line. The event is repeated by readdition of ATP. The response is equivalent to a membrane potential of 65 ± 13 mV (mean ± S.D., $n = 6$), positive inside the proteoliposomes. The response is eliminated by addition of 2 mM Ca^{2+} which

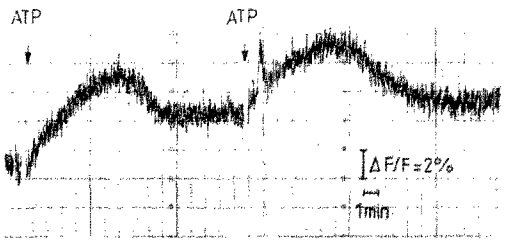

Fig. 8. The fluorescence signal from ANS (1.5 mM) in reconstituted vesicles containing 130 mM Na^+, 0.1 mM Mg^{2+}, 30 mM histidine, pH 7.0 upon addition of 10 µM ATP.

inhibits the $(Na^+ + K^+)$-ATPase activity. In proteoliposomes containing ouabain no potential developed after ATP addition. Substitution of 30 mM of the very lipid soluble anion SCN^- for Cl^- caused the membrane potential to collapse indicating a short circuiting of the membrane potential. This concentration of SCN^- was without deleterious effect on the ATPase activity as tested on solubilized enzyme in solution.

Discussion

In the present experiments using reconstituted rectal gland $(Na^+ + K^+)$-ATPase from shark a Na^+-Na^+ exchange associated with ATP hydrolysis is characterized. Addition of ADP did not stimulate this exchange, only inhibition was observed. Na^+-Na^+ exchange accompanied by an ATP hydrolysis has also been observed in experiments on everted red blood cells [5,6]. In these experiments about 3 Na^+ are transported out per ATP hydrolyzed and the Na^+-Na^+ stoichiometry appears to be 1:1. In the present experiments there are also about 3 Na^+ transported out per ATP hydrolyzed while fewer (one or two) Na^+ are taken up. The electrogenicity of the Na^+-Na^+ transport measured in the present experiments indicates that no other cations or anions accompany the Na^+-Na^+ exchange. A similar electrogenic effect of a Na^+-Na^+ exchange associated with ATP hydrolysis has been observed in vesicles reconstituted with $(Na^+ + K^+)$-ATPase from kindey. In these experiments there was a net accumulation of 0.5 moles of Na^+ per mole ATP hydrolyzed [7,8]; the unidirectional fluxes were not measured.

Phosphorylation from ATP in the presence of Na^+ leads to an occlusion of Na^+ [212], which probably means a transfer of Na^+ from the cytoplasmic to the membrane phase. Since the phosphoenzyme with ADP bound exist in insignificant amount in steady state [22], the occlusion of Na^+ is probably measured on the following phosphoform with no ADP bound, and it is therefore not possible to decide if it is the phosphorylation, or as it is shown in Fig. 9 the phosphorylation and the following release of ADP which leads to the occlusion, $E_1 \sim P \cdot ADP \cdot Na_3$ to $E_1' \sim P(Na_3)$. Kinetic experiments suggest that besides the phosphoenzyme with ADP bound, the reaction with ATP in the presence of Na^+ leads to the formation of at least three consecutive phosphoenzymes [22]. One is ADP sensitive and is in Fig. 9 the phosphoenzyme with 3 Na^+ occluded, $E_1' \sim P(Na_3)$. Another, $E_1'' \sim PNa_3$, has a fast rate of conversion to the ADP-sensitive phosphoenzyme which means that it is so to say ADP sensitive, and finally E_2-P, the ADP-insensitive, K^+-sensitive phosphoenzyme.

The transfer of Na^+ from the membrane phase to the external medium must involve a deocclusion i.e. a transition to a conformation which can exchange Na^+ with the external medium. In the ADP stimulated Na^+-Na^+ exchange this conformation must be ADP sensitive and can therefore not be E_2-P but must be a phosphoenzyme which precedes formation of E_2-P and which follows formation of the Na^+ occluded form; this phosphoform is in Fig. 9 denoted $E_1'' \sim PNa_3$ and is the phoshoform which in the kinetic experiments [22] has a fast rate of disappearance when ADP is added. It is denoted E_1 because it is K^+ insensitive.

In the Na^+-Na^+ exchange associated with ATP hydrolysis the conformation which releases Na^+ to the external medium must be the same as in the ADP-dependent Na^+-Na^+ exchange. However, an cellular outward transport of 3 Na^+ with a cellular inward transport of one or two Na^+ suggests that the phosphoform which is responsible for the inward transport of Na^+ has a conformation which is different from $E_1'' \sim PNa_3$. The K^+ behaviour of extracellular Na^+ in the ATP hydrolysis-dependent Na^+-Na^+ exchange suggests that the phosphoform which accepts Na^+ for the inward transport is the same as the one which accepts K^+ for inward transport in the Na^+-K^+ exchange reaction, E_2-P. According to this reasoning E_1 and E_2 is not related to sidedness but to site number for the transported cations.

$E_1'' \sim PNa_3$ must be at a lower free energy level than $E_1' \sim P(Na_3)$, but as it is 'ADP sensitive' while E_2-P is not it seems likely that the major free-energy change is at the step from $E_1'' \sim PNa_3$ to E_2-P Na_{1-2}. For this reason $E_1'' \sim PNa_3$ is shown with $\sim$ P.

Whether a Na^+-Na^+ exchange stimulated by ADP or associated with ATP hydrolysis is predominant must depend on the steady-state distri-

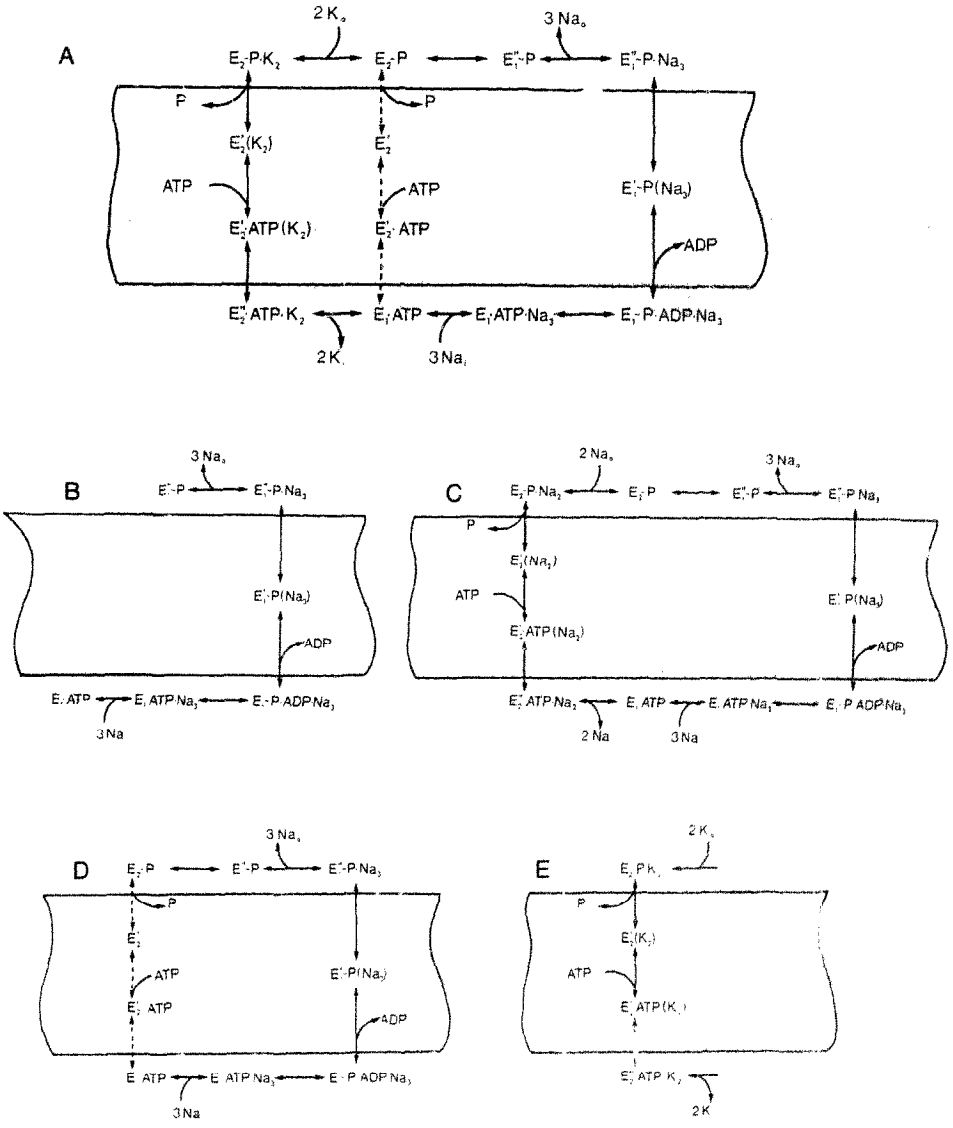

Fig. 9. A consecutive model for the mechanism of the Na^+,K^+-pump which is based on the Albers-Post scheme [24,25], the modifications of this scheme by Karlish et al. [30], and on the scheme for formation of the phosphoenzymes by Nørby et al. [22]. The symbols E_1 and E_2 refer to different conformations of the enzyme; E_1 representing the form with three cation sites (the Na-form) and E_2 the form with two cation sites (the K-form). E, E' and E'' represent different enzyme conformations. E ~ P represents the high energy bonded and E-P the low energy bonded phosphoenzyme. Parenthesis indicate that the ions are occluded. (A) The Na^+-K^+ exchange. (B) The ADP-dependent Na^+-Na^+ exchange. (C) The ATP hydrolysis-dependent Na^+-Na^+ exchange. (D and E) The uncoupled Na^+ efflux (D) and the K^+-K^+ exchange (E) respectively.

bution between the ADP sensitive phoshoenzymes and E_2-P. With the shark enzyme which has been used in the present experiments, phosphorylation from ATP in the presence of Na^+ by enzyme free in solution leads to formation of purely the E_2-P form, i.e. the distribution between E_1 P-forms and E_2-P is poised towards E_2-P. This may explain that no ADP sensitive Na^+-Na^+ exchange is seen upon reconstitution using this enzyme preparation. An increase in the Na^+ concentration increases the steady-state fraction of the phosphoenzyme in the E_1 forms [23]. However, the Na^+ concentration necessary to see such an effect with the shark enzyme is so high that the isotop dilution makes the flux experiments impractical.

The ratio between the E_1 phosphoforms and the E_2-P form varies for different enzyme sources. For example with brain enzyme in the presence of ATP and 150 mM Na^+ a certain fraction of the enzyme molecules at steady state is on the E_1 phosphoform. It is unknown how this distribution is for enzyme from red blood cells, which has the ADP-stimulated Na^+-Na^+ exchange [1–4].

The rate of the turnover of the enzyme, the hydrolysis of ATP, in the Na^+-Na^+ exchange reaction measured in the present experiments is 34 μmol ATP hydrolyzed per mg inside-out protein per h (22°C, pH 7.0). With about 4 nmol enzyme molecules per mg protein this gives a turnover rate of about 2 s^{-1}. With K^+ instead of Na^+ on the intravesicular side of the membrane the turnover rate is about 32 s^{-1} (22°C, pH 7.0) [15]. Assuming that the Na^+-Na^+ exchange and the Na^+-K^+ exchange is on the same pathway [24,25] (for alternative suggestions see Refs. 26 and 27) the lower rate of turnover of ATP hydrolysis-dependent Na^+-Na^+ exchange than of the Na^+-K^+ exchange must be due to a lower rate of transfer of Na^+ from the intravesicular to the cytoplasmic side of the membrane. Since the rate of dephosphorylation of E_2-P in the presence of Na^+ is very low this is probably the rate-limiting step in the ATP hydrolysis-dependent Na^+-Na^+ exchange reaction.

In the present experiments oligomycin decreases the rate but does not inhibit completely the ATP hydrolysis-dependent Na^+-Na^+ exchange. Oligomycin occludes Na^+ to an E_1 form of the enzyme and decreases the rate of deocclusion [28]. As oligomycin inhibits the ADP stimulated Na^+-Na^+ exchange but not the ADP-ATP exchange [29] this suggests that oligomycin decreases the rate of deocclusion of Na^+ from $E_1' \sim P(Na_3)$ i.e. the transition to $E'' \sim PNa_3$. The partial inhibition by oligomycin of the ATP hydrolysis-dependent Na^+-Na^+ exchange then suggests that in the presence of oligomycin it is no longer the dephosphorylation of E_2-P wlhich is rate limiting but the transition from $E_1' \sim P(Na_3)$ to $E_1'' \sim PNa_3$. The low or no inhibition by oligomycin at a low ATP concentration suggests that under these conditions the rate of deocclusion of Na^+ in the presence of oligomycin is comparable to the rate of the rate-limiting step without oligomycin. The increase in the fractional inhibition by oligomycin by an increase in the ATP concentration suggests that without oligomycin ATP increases the rate of the rate-limiting step.

The inhibition of the ATP hydrolysis-dependent Na^+-Na^+ exchange by higher concentrations of ATP (Fig. 3) is not accounted for in the model in Fig. 9, this suggests a low affinity ATP effect on the exchange reaction.

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