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Characterization of $(Na^+ + K^+)$-ATPase-liposomes. III. Controlled activation and inhibition of symmetric pumps by timed asymmetric ATP, RbCl, and cardiac glycoside addition

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Inside-out as well as right-side-out oriented $(Na^+ + K^+)$-ATPase molecules reconstituted in liposomes are activated successively by timed asymmetric addition of ATP to the internal and external liposome compartment; this presents the first functional confirmation of the symmetric pump-orientation in cholate-dialysed preparations revealed previously by the equal distribution of intramembrane particles on the concave and convex surface of freeze-fractured $(Na^+ + K^+)$-ATPase-liposomes. The initial transport rates of the symmetrically oriented pump populations are regulated by varying the bilateral K or Rb ion concentrations; ATP, ouabain, digoxin or vanadate are used to activate or block selectively the right-side-out, inside-out or both $(Na^+ + K^+)$-ATPase populations. Finally, these liposomes of the second generation present a new tool to evaluate the membrane-permeability as well as the effects of receptor-ligands or other probes in a single preparation.

Introduction

The $(Na^+ + K^+)$-ATPase (EC 3.6.1.37) or sodium pump is a polyvalent membrane system catalyzing at least five modes of transmembranous exchange of Na and K ions coupled to reversible phosphorylation-dephosphorylation reactions located on the α-subunit [1–3]. In addition, the Na^+/K^+ exchange mode is electrogenic and contributes to the membrane potential [4,5]. Further, the system contains a receptor for cardiac glycosides and for their presumptive endogenous analogues (for a recent short review see Ref. 6).

Recently, the primary structure of the protein part of the system has been published [7–9]. However, the relationship between the transport function and the structure is not yet understood, mainly because appropriate tools to measure both parameters in a single preparation containing only transport-active $(Na^+ + K^+)$-ATPase molecules in inside-out and right-side-out orientation are lacking. Purified enzyme preparations are often membrane fragments without closed compartment to measure transport [10]. On the other hand, inside-out red blood cell vesicles [11] or right-side-out renal membrane vesicles containing 'caged' ATP [12] are useful tools for investigating the transport function, but the presence of numerous contaminating proteins in these crude membrane preparations does not allow to relate the transport mechanism directly to the molecular structure of the $(Na^+ + K^+)$-ATPase. Obviously, the investigation of transport-structure relations requires a

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model system with pure, transport-active ($Na^+ + K^+$)-ATPase as, for instance, ($Na^+ + K^+$)-ATPase-liposomes.

Following the initial description by Goldin and Tong [13] and Hilden, Rhee and Hokin [14], different types of such ($Na^+ + K^+$)-ATPase-liposomes are now formed by several groups (reviewed in Refs. 15, 25). Some preliminary attempts to establish transport-structure relations in ($Na^+ + K^+$)-ATPase-liposomes have been made by comparing the transport pattern of selectively proteolysed ($Na^+ + K^+$)-ATPase and control enzyme after reconstitution [16,17]. However, in these previous ($Na^+ + K^+$)-ATPase-liposomes as well as in recent preparations described by other groups [18–22] only the inside-out oriented pump molecules are activated by the addition of external ATP. As the right-side-out oriented pumps remain silent in the presence of external ATP, the immediate effects of extracellular enzyme modification on active transport are not measurable. Therefore, knowing the structure and properties of our ($Na^+ + K^+$)-ATPase-liposomes [23,24], we decided to develop a model where both, inside-out and right-side-out oriented ($Na^+ + K^+$)-ATPase molecules are working.

Our previous work had established that the intramembranous particles in freeze-fractured ($Na^+ + K^+$)-ATPase-liposomes are randomly distributed on the concave and convex fractured liposome-surface at all lipid/protein ratios and enzyme activities tested [23,24]. Such a symmetric particle distribution indicates random orientation of the ($Na^+ + K^+$)-ATPase molecules if the fracture plane is assumed to move asymmetrically around the pump molecules [24,25]. In consequence, we thought to exploit this presumptive random orientation as follows: in a first step, the right-side-out oriented pump-population of ATP-filled liposomes is activated by external Rb^+ (in a Na-Mg medium) and then, in a second step, the internal Rb^+ that has been accumulated by the right-side-out pumps is used to drive the inside-out oriented pumps upon external ATP addition, the right-side-out pumps being blocked simultaneously by external ouabain. A preliminary report showed that this model was feasible [26].

In the present work, we develop a method to monitor rapidly the initial transport rates of the symmetrically oriented pump populations. Further, the turnover of the right-side-out pumps was reduced by lowering the external RbCl concentration to obtain a linear Rb-accumulation phase lasting 5 to 10 min, i.e. long enough to monitor alterations of the transport kinetics by receptor-ligands or other probes interacting with the extracellular aspect of the sodium pump. The unilateral presence of the hydrophilic ouabain or vanadate leads to selective inhibition of the symmetric pump populations, while the bilateral presence of the hydrophobic cardiac glycoside digoxin inhibits both populations, illustrating that the ATP-filled ($Na^+ + K^+$)-ATPase-liposomes are a useful new tool for testing, in a single preparation, the sidedness, membrane-permeability, and transport-modification of various agents interacting with pure ($Na^+ + K^+$)-ATPase.

Materials and Methods

Materials

Salts (KCl, NaCl, RbCl) were of Suprapure quality from Merck; histidine, Tris, imidazole, EDTA, L-cysteine, ammonium heptamolybdate, L-(+)-ascorbic acid, were analytical grade Merck products. Na_2ATP (special quality) was purchased from Boehringer; its P_i content was controlled by the highly-sensitive malachite-green assay [27] and was found to be negligible (2.5 μM per 100 mM ATP). Purified egg phosphatidylcholine was obtained from Sigma (product No. P-5763). Cholestyramine-resine was from Serva. The ^{86}Rb and ^{42}K were purchased from the National Institute of Radio-elements, B-622 Fleurus, Belgium. The ($Na^+ + K^+$)-ATPase was purified from the freshly dissected outer medulla of rabbit or pig kidneys by the SDS-treatment procedure [28]. The specific activities were determined by the linked pyruvate kinase/lactate dehydrogenase assay [29] as described in detail elsewhere [30] and ranged between 18 and 28 μmol/min per mg protein at 37 °C. The Pierce BCA protein-assay reagent was used to determine the protein concentrations with the Bio-Rad bovine serum albumin as standard.

Colorimetric measurement of the ($Na^+ + K^+$)-ATPase activity

A highly-sensitive colorimetric P_i-assay [31,32]

was adapted to determine the specific enzyme activity in transport-conditions at varying RbCl and KCl concentrations. 3–4 μg purified pig kidney ($Na^+ + K^+$)-ATPase-protein (18 μmol/min per mg protein specific activity) were incubated for 5 to 45 min at 25 °C in 50 μl of a solution containing 50 mM NaCl, 5 mM $MgCl_2$, 2.5 mM ATP, 30 mM histidine, 1 mM Tris-EDTA, 1 mM cysteine, 0 to 10 mM KCl or RbCl, pH 7.10 (transport-solution); the reaction was stopped by adding 40 μl 3 M trichloroacetic acid; 20–30 μl of the incubation-mixture were transferred to a tube containing 300 μl of 1 M HCl and 700 μl of a freshly prepared mixture of 1.43 mM ammonium heptamolybdate and 57 mM L-(+)-ascorbic acid in 0.10 M H_2SO_4. The mixture was incubated for 20 min at 45 °C and the absorbance was read at 820 nm wavelength; the absorbance was linear between 0.1 and 0.8; the molar absorption coefficient, determined in 14 measurements, was 21 136 (±297; S.E.). The absorbance of transport-solution containing either ATP but no enzyme or enzyme but no ATP as well as the ATPase activity in the absence of KCl or RbCl or $MgCl_2$ or in the presence of ouabain represented consistently less than 5% of the maximal ATPase activity and were subtracted from the values measured in the presence of increasing concentrations of RbCl or KCl. The contaminating KCl concentration in the transport solution, determined by a Varian atomic absorption spectrophotometer, was below 5 μM.

Preparation of ATP-containing ($Na^+ + K^+$)-ATPase-liposomes

The method for preparing sodium-pump-liposomes containing an entrapped ATP-reservoir was described in detail elsewhere [26]. Briefly, 300 μg ($Na^+ + K^+$)-ATPase protein are treated at 0 °C with 100 μl transport-solution (pH 7.10) containing 23 mm sodium cholate and 50 mM Na_2ATP plus 50 mM Tris-ATP or 100 mM Na_2ATP. The solubilized enzyme is recovered in the supernatant after a 15 min centrifugation at about 100 000 × *g* in a Beckman Airfuge and mixed with the same volume of lipid-solution. The lipid-solution was prepared as previously described [33] and contained 20 mg phosphatidylcholine in 1 ml solution (23 mM sodium cholate, 5 mM $MgCl_2$, 30 mM histidine, 1 mM cysteine chloride, 1 mM Tris-EDTA (pH 7.10)). The cholate is removed by a 4 to 20 h dialysis at 0 °C in 4 ml transport-solution containing in addition either 25 mM Tris-ATP plus 25 mM Na_2ATP or 50 mM Na_2ATP as well as 400 to 500 mg cholestyramine-resin (washed as previously described [26]). The external ATP is removed by sedimenting the liposomes twice at about 100 000 × *g* in the Airfuge at 0 °C and resuspending them in the same volume of ATP-free transport-solution containing 50 mM NaCl when 25 mM Tris-ATP plus 25 mM Na_2ATP are entrapped within the liposomes or 100 mM NaCl when 50 mM Na_2ATP are entrapped; these two conditions gave identical transport results. The ATP-containing liposomes are transport-active for at least 24 h when they are stored at 0 °C in the absence of ATP, indicating that the pump turns over so slowly at 0 °C in the absence of K or Rb ions and in the presence of 50 mM ATP that the internal ATP-reservoir is preserved within the time period required to perform the experiments. The specific activity of unreconstituted ($Na^+ + K^+$)-ATPase incubated in the same solution used for the liposome preparation measured by the malachite green assay [27] during an incubation time of 20 h at 0 °C, was 0.63 μmol P_i/h per mg protein; considering that the enzyme turnover is reduced by an additional factor of about 10 due to the phosphatidylcholine required to form the artificial membrane [33], it can be calculated that at most 4% of the ATP entrapped within the liposomes is hydrolysed within 20 h, i.e. the ATP/ADP ratio is at least 25 at the moment of the active transport experiments.

Radio-flux assay

The active transport of the right-side-out oriented pump is started by adding [^{86}Rb]RbCl or [^{42}K]KCl to the liposome suspension. The ^{86}Rb or ^{42}K containing solutions are prepared as follows: an aliquot of the radioactive stock solution containing 40 nmol of [^{86}Rb]RbCl or [^{42}K]KCl is dried and then dissolved in 20 μl of transport-solution, yielding a RbCl or KCl concentration of 2 mM. Dilutions of this solution to yield final RbCl or KCl concentrations down to 1 μM are made by adding transport-solution.

3 μl of liposomes are put on the wall near to the bottom of a small conical plastic tube and in

front of the vesicle-droplet 3 μl of the radioactive solution are deposited. The two droplets are then mixed rapidly and the tube is immediately incubated at 25°C (= time 0 for transport). To activate the inside-out oriented pump, 10 to 50 mM external ATP are added together with 100 μM ouabain to block the right-side-out oriented pumps. At the end of the incubation time, 125 μl of ice-cold stop-solution containing 50 mM KCl, 1 mM Tris-EDTA, 1 mM L-cysteine, 30 mM histidine or imidazole buffer (pH 7.2) are added and 10 μl are transferred in duplicate to scintillation vials to determine the total radioactivity. The remaining 100 μl diluted liposomes are eluted in ice-cooled Sephadex G-50 columns as previously described [33] and the washed liposomes were collected in the void volume. The radioactivity was counted in 8 ml Kontrogel scintillation fluid.

The calculation of the internal concentration is performed as follows: the average entrapped volume of our liposome preparation, determined by a minimal 12 h incubation of the liposomes in the presence of external $^{86}Rb^+$ and in the absence of ATP consistently amounts to 1% of the external solution [23,24,30,33–35]; this ratio means that, in 1 ml undiluted liposome suspension, 10 μl solution is entrapped by the tight, transport-active liposome population. It follows that isotope-entrapping reaching values above 1% indicates active accumulation. To illustrate the detection-limit of the assay, the uptake was expressed in some figures as the average number of ions per liposome by measuring, firstly, the internal Rb ion concentration, secondly, calculating the average volume entrapped by a single 100 nm liposome, thirdly, multiplying the mM entrapped by the ratio of the single liposome volume to 1 liter (to obtain the amount of Rb^+ ions entrapped in mol per liposomes), and, finally, by converting this amount to the number of ions per liposomes by means of the Avogadro's number.

The results of the present paper were collected during 3 years on about 200 separate liposome-preparations. As the transport-rates vary slightly between different liposomes batches, typical experiments are shown in the figures of the present paper with the exception of Fig. 3 which represents normalized experiments. The TRS-80 Statistical Analysis Program of S.W. Hebber, licensed to Tandy Corporation (1983), was used for the statistics.

Results and Discussion

The $(Na^+ + K^+)$-ATPase-liposomes of the second generation described in the present study were developed on the basis of their ultrastructure, in particular on the basis of the average liposome size, the average number of $(Na^+ + K^+)$-ATPase molecules per liposome and the orientation of the reconstituted pumps. From the ratio of the water space entrapped by a single 100 nm liposome to the water space entrapped by a known volume of liposome suspension, it turns out that 1 ml suspension contains $2.62 \cdot 10^{13}$ tight liposomes presenting 8230 cm^2 external membrane surface. The liposome center to center distance is 337.5 nm, i.e. each liposome finds itself 237.5 nm apart from each other.

At maximum pump turnover, the concentrations within 100 nm liposomes change so rapidly [35] that precise initial flux kinetics are difficult to measure with conventional filtration techniques. It is well known that extracellular K^+ is a sensitive regulator of the pump activity [1–5]. Therefore, we lowered the turnover of the $(Na^+ + K^+)$-ATPase by reducing the externally added K^+ or Rb^+ concentrations as much as possible to get linear Rb^+-uptake velocities on a minute-scale. The lower limit was set, firstly, by the affinity of these ions for the external binding sites and, secondly, by the specific radioactivity of the isotope available. Unfortunately, the only tracer for K is the short-lived ^{42}K. Thus, the ^{86}Rb ion must be used as a K^+ substitute to get enough internal radioactivity at micromolar external Rb^+ concentrations in the 3 μl liposome sample we use per gel filtration step, i.e. per experimental point. However, it was not yet known whether K^+ and Rb^+ ions were equivalent in activating ATP hydrolysis and transport at micromolar concentrations at 25°C. Therefore, we designed experiments to determine, firstly, the K^+ or Rb^+ concentrations which produce the lowest detectable turnover rate of the pump, and, secondly, to examine whether Rb^+ is a precise substitute for K^+ at such low concentrations.

In the first set of experiments, the K^+ or Rb^+ activation of the ATP hydrolysis catalyzed by

unreconstituted rabbit renal $(Na^+ + K^+)$-ATPase in transport conditions was determined. Fig. 1A illustrates the effect of Rb^+ on enzyme turnover measured in the same solution and at the same temperature used for the transport assay of the reconstituted enzyme shown in Fig. 1B. Half-maximal stimulation of the ATP hydrolysis catalyzed by unreconstituted enzyme at 25 °C is attained around 120 μM RbCl (Fig. 1A). The inset in Fig. 1A illustrates that Rb^+ and K^+ are equipotent with regard to ATPase stimulation at concentrations as low as 10, 50 or 100 μM.

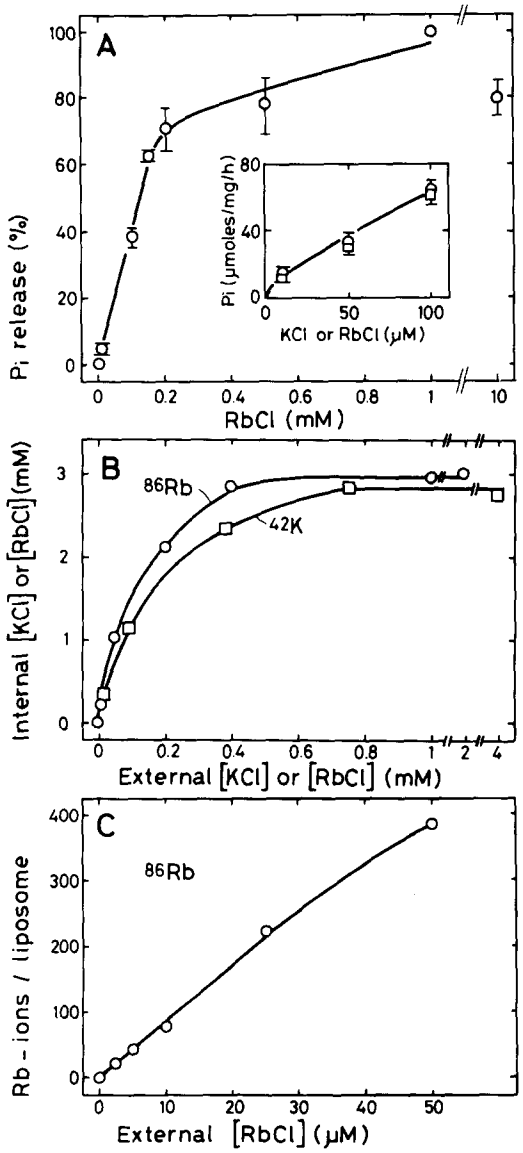

Fig. 1B represents the active $^{42}K^+$ or $^{86}Rb^+$ uptake by the right-side-out oriented pump into the ATP-containing $(Na^+ + K^+)$-ATPase-liposomes. The internal Rb^+ or K^+ concentrations reached within a 5 min incubation period at 25 °C are plotted as a function of the externally applied Rb^+ or K^+ concentration. Clearly, the transport activation by the two ions is quite similar (Fig. 1B). Further, the concentration for half-maximal activation is also between 100 and 150 μM, i.e. the affinity of the external binding sites for Rb^+ or K^+ ions of the reconstituted right-side-out $(Na^+ + K^+)$-ATPase (Fig. 1B) is in the same range as the affinity of the extracellular binding sites involved in the ATPase-stimulation of the unrecon-

Fig. 1. (A) Relationship between the $(Na^+ + K^+)$-ATPase enzyme turnover and the RbCl (○) or KCl (□) concentrations in the medium. 3–4 μg of purified, unreconstituted pig-kidney $(Na^+ + K^+)$-ATPase protein were incubated in 50 μl transport-solution (50 mM NaCl, 5 mM $MgCl_2$, 5 mM ATP, 30 mM histidine, 1 mM Tris-EDTA, 1 mM cysteine, pH 7.10) for 30 min at 25 °C. The enzyme turnover was stopped by adding 40 μl of a 3 M trichloroacetic acid solution and the P_i concentration was determined by the colorimetric assay described under Materials and Methods. The inset shows the equivalency of Rb^+ and K^+ ions at concentrations used in the active transport assays. Vertical bars denote S.E. of the mean for 3–7 separate determinations. (B) Relationship between the pump turnover and the RbCl (○) or KCl (□) concentrations in the medium. The active transport rate of the right-side-out oriented pumps was measured by incubating the ATP-containing $(Na^+ + K^+)$-ATPase-liposomes for 5 min at 25 °C in the presence of external $^{86}RbCl$ or ^{42}KCl. The $^{86}Rb^+$ uptake was measured at constant specific radioactivity using a source of about 6 mCi/mg; the $^{42}K^+$ uptake at low concentrations was measured immediately at arrival of the isotope and the uptake at higher concentrations between 15 to 20 h after the arrival. The ratio of internal to external concentrations indicates the inside-out gradient built-up by the right-side-out pump across the liposome-membrane within 5 min. The ATP-containing liposomes were prepared, analysed and the results calculated per liposome as described under Materials and Methods. Each experimental point represents single or duplicate determinations in a typical liposome preparation. (C) Detection limit of the $^{86}Rb^+$ influx (○) in individual liposome samples. The active Rb^+ accumulation was determined as explained for Fig. 1B. To perform such highly sensitive assays, the ^{86}Rb was ordered at the date of its fabrication when its specific activity was 18 mCi/mg. The external $^{86}RbCl$ concentrations were gradually reduced to the lowest concentration yielding a minimum of 300 cpm within a 5 min incubation in a 3 μl liposome aliquot. Each experimental point represents one or two determinations in typical preparations.

stituted enzyme (Fig. 1A), indicating that the reconstituted enzyme is not denatured.

The experiment in Fig. 1C was performed to determine the lowest possible external Rb^+ concentration which yields a significant amount of internal radioactivity within a 5 min incubation period in a 3 μl liposome sample. Fig. 1C shows the linear decrease of the transport velocity below 50 μM external Rb^+. The lowest Rb^+ concentration yielding significant ^{86}Rb uptake (from a fresh ^{86}Rb source) within 5 min in a 3 μl filtered sample is 1 μM. By calculating the number of ions entrapped per liposome on the basis of the average liposome diameter, it turns out that we are able to detect the entry of 10 to 20 ^{86}Rb ions per liposome in a 3 μl sample of our liposome suspension, thus, we have developed a miniaturized assay that can be performed with a minimal amount of pure enzyme and radioactivity. By increasing the volume of the liposome aliquot used per experimental point, the sensitivity augments in proportion to the volume.

Fig. 2 shows, firstly, the active Rb^+ uptake by the right-side-out pump population and, secondly, the successive extrusion of the accumulated Rb^+ or K^+ ions by the inside-out oriented pump population; the right-side-out pump activity is labeled phase 1 and the inside-out pump activity phase 2. The resulting triangles are composed of three parts: an ascending part (1), a descending part (2) and the baseline (time). The ascending phase corresponds to the active Rb^+ uptake catalyzed by the right-side-out pump upon addition of 1 mM external $^{86}RbCl$ (Fig. 2A) or ^{42}KCl (inset of Fig. 2A) or by 10 μM external $^{86}RbCl$ (Fig. 2B). Each experimental point represents the measurement of the internal ^{86}Rb or ^{42}K content of a 3 μl liposome aliquot determined after gel-filtration. The Rb^+ or K^+ incorporation proceeds linearly for 2 min at 1 mM external RbCl and for 3 min at 10 μM external RbCl (Figs. 2A, B). When the external RbCl concentration is below 10 μM, perfectly linear transport rates are observed within 5 to 10 min after the Rb^+ addition (not shown).

As soon as a significant amount of radioactivity has been reached within the liposomes, the descending phase 2 is started. In practice, to activate the inside-out oriented pump 2, a portion of the suspension incubating in the presence of external

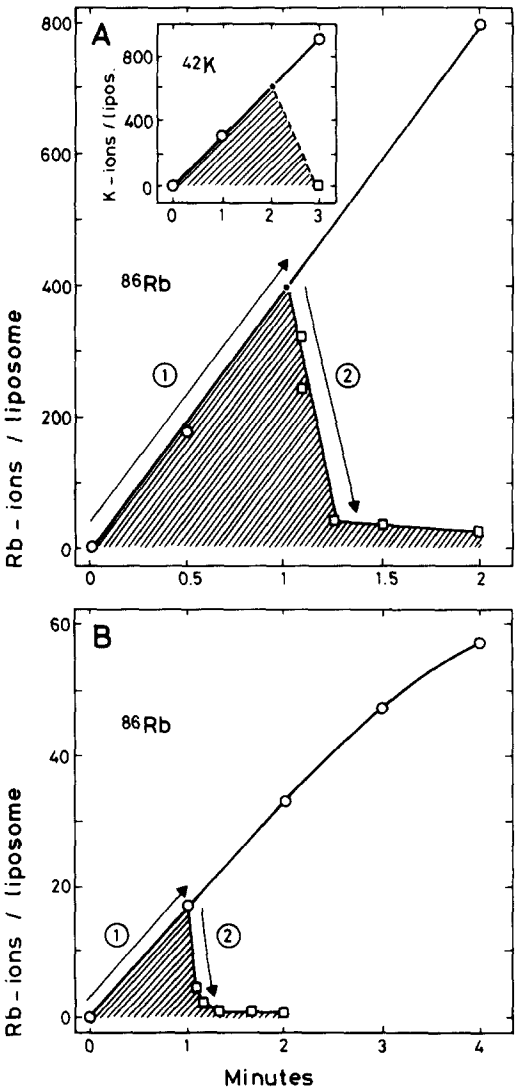

Fig. 2. (A) Typical triangles obtained at saturating RbCl or KCl concentrations by successive activation of the right-side-out oriented pump population 1 (○) and the inside-out oriented pump population 2 (□). At time zero, 1 mM external $^{86}RbCl$ or ^{42}KCl (inset) is added to aliquots of the ATP-containing liposomes to start the turnover of the right-side-out pumps (1). In phase 2, 10 mM ATP and 100 μM ouabain are added to activate the inside-out oriented pumps (2) and to inhibit the right-side-out oriented pumps. In the absence of external ATP, the right-side-out pump continues to accumulate Rb^+ or K^+ ions (○). Each experimental point represents one or two measurements in a typical preparation. (B) Typical triangle seen at suboptimal external RbCl (10 μM). The accumulated internal RbCl (○) accelerates the relative pumping rate of the inside-out (□) oriented pumps. The technology for low-turnover experiments is described in the legend for Fig. 1C. Each experimental point represents 1 measurement or an average of two measurements in a representative experiment.

^{86}Rb is transferred to a tube containing a mixture of ouabain and ATP. The ATP-activated inside-out pump then rapidly extrudes the accumulated Rb^+ ions (Figs. 2A, B) or K^+ ions (inset of Fig. 2A). The baselines of the triangles shown in Fig. 2 are determined by the time interval selected between the external K^+ or Rb^+ addition (right-side-out pump activation) and the external ATP addition (inside-out pump activation). Thus, the shape of the triangle can be selected at will by varying the external RbCl concentration to settle the Rb^+-uptake rate (slope of the ascending phase) as well as the internal RbCl concentration, which depends on the time elapsed before pump 2 is started, to condition the Rb^+-extrusion rate (slope of the descending phase).

In general, the Rb^+ extrusion by the inside-out pump is faster than the Rb^+ uptake by the right-side-out pump (Fig. 2, Table I). It is obvious, that, at non-saturating RbCl concentrations, the pump rate of the inside-out oriented pump is determined by the internal Rb^+ concentrations. When, at the moment of the external ATP addition, the internal Rb^+ concentration is higher than the external one, the inside-out pump runs about 10-times faster than the right-side-out pump (Table I). However, the observation that the extrusion rate is still about 4-times faster than the uptake rate at saturating external Rb^+ concentrations (Fig. 2A, Table I) infers that the inside-out pump, which has its catalytic center bathing in the external solution containing 10 mM ATP, seems to be in a better position for active transport than the right-side-out pump which has its catalytic center within the internal liposome space containing 50 mM ATP. Conversely, in mirror-symmetric conditions, i.e., when the external ATP concentration is made equal to the internal one, the inside-out pump works roughly at the same rate as the right-side-out pump (Table I).

Thus, the comparison of the transport rates observed with reconstituted pumps suggested that the increase of the ATP concentration from 10 mM to 50 mM lowers the turnover of the pump by a factor of 3 to 4. Experiments with unreconstituted enzyme confirmed this finding: the enzyme turnover was reduced by a factor of 3.9 ± 0.34 ($n = 4$; S.E.) when the ATP concentration was raised from 10 to 50 mM (Table I). As the free ADP is continuously regenerated to ATP in the pyruvate kinase/lactate dehydrogenase assay [29], the ATP/ADP : P_i ratio is infinitely high and product inhibition of the ATPase reaction is excluded. Control experiments ruled out that the inhibition was due to the increased osmolarity caused by the additional $Tris^+$ or Na^+ ions added to neutralize the high ATP concentration.

The 50 mM ATP concentration was used to entrap 11 500 ATP molecules per average liposome in order to maintain a virtually constant internal ATP/ADP : P_i ratio during the linear phase of Rb^+ uptake (Fig. 2B) where at most 200 internal ATP molecules are hydrolyzed at the 1–2 Rb : 1 ATP coupling ratio found in liposomes [15]. Another advantage of the excess ATP concentration lies in its inhibitory effect on the enzyme turnover which helps to prevent ATP hydrolysis

TABLE I

EFFECT OF BILATERAL RbCl AND ATP CONCENTRATIONS ON THE TRANSPORT RATES OF SYMMETRICALLY ORIENTED PUMPS

The 50 mM ATP-containing ($Na^+ + K^+$)-ATPase-liposomes were incubated at 25 °C in the presence of increasing external $^{86}RbCl$ concentrations to drive ^{86}Rb accumulation via the right-side-out pumps. The linear phase was determined by measuring the ^{86}Rb uptake in 1-min intervals and at the times indicated; external ATP was added to extrude the captured ^{86}Rb via the inside-out pumps as illustrated in Figs. 2, 3, 4 and 5 of the present paper. The ratio, R, of the ^{86}Rb-extrusion rate to the uptake rate was determined by dividing the descending slope by the ascending slope at suboptimal and at saturating external and internal RbCl concentrations. The internal RbCl concentration was calculated from the % isotope incorporated by the liposomes as indicated under Materials and Methods.

Prepn. No.	Incubation conditions				Ratio R
	Time (min)	Concentrations (mM)			
		external		internal	
		ATP	^{86}Rb	^{86}Rb	
333	2	10	0.005	0.044	12
313	1	10	0.010	0.072	10
314	1	10	0.010	0.074	10
314	1	10	1.0	1.54	4
366	3	10	2.0	2.1	4
367	3	10	2.0	2.08	4.5
367	3	50	2.0	1.48	1.5
368	3	10	2.0	2.18	4.3
368	3	50	2.0	2.18	1.6

TABLE II

PUMP-DISTRIBUTION BETWEEN CONVEX AND CONCAVE LIPOSOME-HALVES AND PUMP-NUMBER PER LIPOSOME

The number of intramembrane particles (IMP) on freeze-fractured convex (CV) and concave (CC) $(Na^+ + K^+)$-ATPase-liposome halves that had been prepared at the same lipid/protein ratio as in the present work were extracted from a previous data collection (Refs. 20, 21, 23 and 24) and listed below. Each value represents the mean of the number of intramembrane particles counted on 250 CV and 250 CC halves; the S.E. ranged between 0.10 and 0.15 in the 10 listed data collections.

	Prepn. No.					Average ±S.E.
	83	97	98	133	142	
CV	1.8	2.5	1.8	1.4	2.6	2.02±0.23
CC	1.8	2.1	1.7	1.6	2.5	1.94±0.55
CV/CC	1	1.2	1	0.9	1	1.04±0.05
CV+CC	3.6	4.6	3.5	3.1	5.1	3.96±0.38

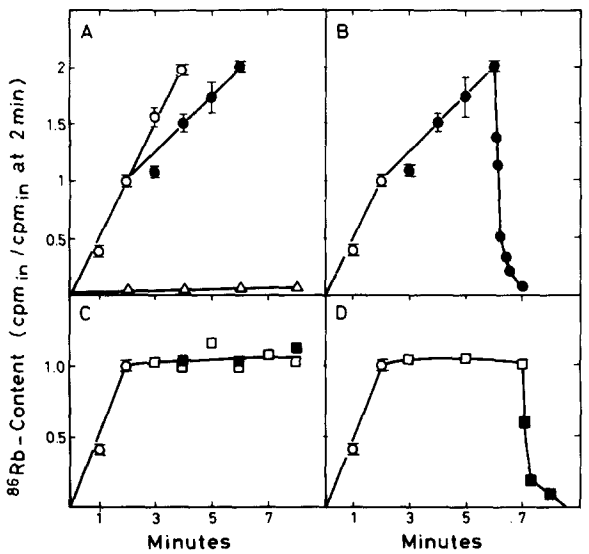

Fig. 3. (A) Control experiment to test the effect of 10% solvent (dimethyl sulfoxide) used to dissolve the cardiac glycosides. Active Rb^+ uptake (○) by ATP-containing $(Na^+ + K^+)$-ATPase-liposomes was initiated for each experimental point by the addition of 4 μl 20 μM ^{86}RbCl in transport-solution to 4 μl ATP-containing liposomes and incubating and processing them at 25 °C (see Materials and Methods for details). The average $^{84}Rb^+$ incorporation at 2 min was 4.52±0.19% (S.E.) of external (about 45 μM) and was set at 1.0 in all experiments to simplify the presentation of the results. 1 μl of dimethyl sulfoxide was mixed with 1 μl transport-solution and added to 8 μl incubation mixture at 2 min leading to a 50% reduction of the active Rb^+ uptake rate (●) after correction of the dilution factor. Vertical bars denote S.E. of the mean for 3–5 separate determinations. Ouabain was added (in a dimethyl sulfoxide solution) (△) to demonstrate inhibition of the right-side-out pump. (B) Control experiment to test the effect of dimethyl sulfoxide on the Rb^+ extrusion catalyzed by the inside-out pump. As described for Fig. 3A, for each experimental point, 2 μl of the 50% dimethyl sulfoxide solution were added to 8 μl ATP-containing liposomes which had been incubated for 2 min in the presence of 10 μM external ^{86}RbCl (○). 4 min after the addition of solvent 2 μl of a 50 mM ATP stock solution were added to demonstrate that the $^{86}Rb^+$ extrusion by the inside-out pump was not blocked by 10% solvent (●). (C) Block of both the right-side-out and the inside-out oriented pumps by the hydrophobe cardiac glycoside digoxin. The drug (10 mM) was dissolved in dimethyl sulfoxide. For each experimental point, one volume of this solution was mixed with 1 volume transport-solution immediately before use; 8 μl of the incubation mixture containing the liposomes and 10 μM ^{86}RbCl were incubated for 2 min at 25 °C as described for Figs. 3A and B. 2 μl of the 5 mM digoxin solution were added and the $^{86}Rb^+$ content was determined at 1 min intervals during 6 min after the digoxin addition without (□) and with (■) 10 mM external ATP; both pump populations are blocked within 1 min by the externally applied digoxin at 1 mM external digoxin. The figure represents the data collected in three separate preparations after a series of preliminary experiments performed in nine preparations. (D) Selective block of the right-side-out oriented pump population by the externally added lipophobe cardiac glycoside ouabain. The experiment was performed as described for Fig. 3C, except that for each experimental point 2 μl of a 5 mM ouabain solution were added to 8 μl of the $^{86}Rb^+$-liposomes. Ouabain blocks the $^{86}Rb^+$ uptake catalyzed by the right-side-out pumps (□) but not the $^{86}Rb^+$ extrusion by the inside-out pumps (■) as the drug does not permeate the liposomes within 5 min. The figure represents typical data collected in two separate preparations after a series of preliminary experiments performed in nine separate preparations.

during the preparation of the $(Na^+ + K^+)$-ATPase-liposomes.

The fact that the ratio of the transport rate displayed by the right-side-out pumps to the transport rate of the inside-out pumps is close to unity at identical bilateral ATP, Mg^{2+}, Na^+, and Rb^+ ion concentrations (Table I), confirms the symmetric distribution of the pumps determined on electron micrographs of freeze-fractured preparations where the ratio of the intramembrane particle density on convex surfaces is 1.04 at the protein/lipid ratio used in the present work yielding an average pump density of four per liposome (Table II).

The Rb^+ accumulation by the liposomes is impressive: within a 1 min incubation period in the presence of 10 μM external RbCl at 25 °C, the

internal Rb^+ concentration often reaches 70 μM (Table I), i.e. a 7-fold gradient is established across the liposome membrane. Despite this important Rb^+ accumulation, the internal ATP and Na^+ reservoirs remain virtually unchanged. In fact, at the classical 2 Rb : 1 ATP coupling ratio, the uptake of 40 μM Rb^+ ions consumes only 20 μM of the 50 mM internal ATP reservoir, i.e. less than 1‰. Similarly, not more than 1‰ of the 50 mM internal NaCl concentration are extruded in exchange for the 40 μM Rb^+ uptake. In consequence, the transport-rate of the reconstituted pumps depends on the only variable in the system, i.e. on the Rb^+ or K^+ concentration.

When the hydrosoluble cardiac glycoside ouabain is added together with the external ^{86}RbCl, only a slow ouabain-resistant or passive Rb^+ influx is seen (Fig. 3A). The average ouabain-resistant Rb^+-flux rate, determined in a series of seven different liposome preparations, amounts to 7% of the active Rb^+-uptake rate (Table III). When external ouabain is added to liposomes that have already accumulated Rb^+ ions for 2 min, the active uptake stops immediately and the internal Rb^+ concentration remains stable for at least 5 min (Fig. 3D); within this incubation period, ouabain does not cross the liposome membrane as the inside-out pump is not inhibited, i.e. external ATP extrudes the internal Rb^+ pool.

TABLE III

EFFECT OF RIGHT-SIDE-OUT PUMP INHIBITION OR INSIDE-OUT PUMP ACTIVATION ON THE $^{86}Rb^+$ CONTENT OF THE ATP CONTAINING $(Na^+ + K^+)$-ATPase-LIPOSOMES

The liposomes were incubated at 25 °C in the presence of 5 or 10 μM external ^{86}RbCl with or without 100 μM external ouabain for 3 (five experiments) or 15 min (two experiments) and the $^{86}Rb^+$ uptake was determined by the technique described under Materials and Methods. Control uptake was set at 100%. To extrude the accumulated $^{86}Rb^+$ ions, 10 mM ATP were added at 3 min or at 15 min after the ^{86}RbCl addition, the $^{86}Rb^+$ remaining in the liposomes was determined after 1 min and expressed as % of the value measured before the external ATP addition. The S.E. is indicated.

External additions	$^{86}Rb^+$ content (%)
$^{86}Rb^+$ ($n = 7$)	100
$^{86}Rb^+$ and ouabain ($n = 7$)	7.2 ± 1.7
$^{86}Rb^+$ and ATP ($n = 7$)	4.6 ± 0.8

In contrast, when the hydrophobic drug digoxin is added to the liposomes in the same conditions as the ouabain, the internal receptor site is occupied within 1 min as indicated by the block of the inside-out pump (Fig. 3C). Thus, the hydrophobicity as well as the inhibitory potency of a drug or endogenous factor can be tested in a single assay by measuring whether, in a given time interval, the substance binds to the external receptor population only or to both the internal and external populations as illustrated in Fig. 3 for the cardiac glycosides ouabain and digoxin.

Figs. 3C and 3D also show that the accumulated internal RbCl remains entrapped despite the arrest of the uptake process by the cardiac glycosides. The fact that the Rb^+ gradient is so stable is explained by the low passive Rb^+-permeability of the $(Na^+ + K^+)$-ATPase-liposomes. In the ATP-containing liposomes, the passive Rb^+ flux is 14-times (Table III) lower than the active flux rate.

The low cation-permeability of the reconstituted phosphatidylcholine-bilayer [25] explains why the ATP-containing liposomes described in the present work remain transport-active for about 2 days when they are stored at 0 °C in the absence of Rb^+ and K^+ ions (Fig. 4). The specific ATPase activity of the unreconstituted enzyme is only 0.63 μmol P_i/h per mg protein when it is incubated in the conditions used for the preparation and storage of the ATP-containing $(Na^+ + K^+)$-ATPase-liposomes. As the phosphatidylcholine addition further reduces the turnover [33], the pump is virtually arrested prior to the RbCl addition. Accordingly, the ATP reservoir of 50 mM or 11 500 molecules entrapped by a dialysed liposome remains available for active transport for at least 30 h after the liposome preparation (Fig. 4).

The activity of the inside-out pump is blocked if 100 μM vanadate is added prior to the addition of 10 mM external ATP (Fig. 5). In contrast, the uptake phase by the right-side-out pump is not affected by the presence of vanadate (Fig. 5). In the absence of vanadate, the external ATP extrudes the accumulated Rb^+-pool normally. Thus, the symmetrically reconstituted, actively transporting $(Na^+ + K^+)$-ATPase molecules can serve as mutual internal controls for the sidedness of chemical modification.

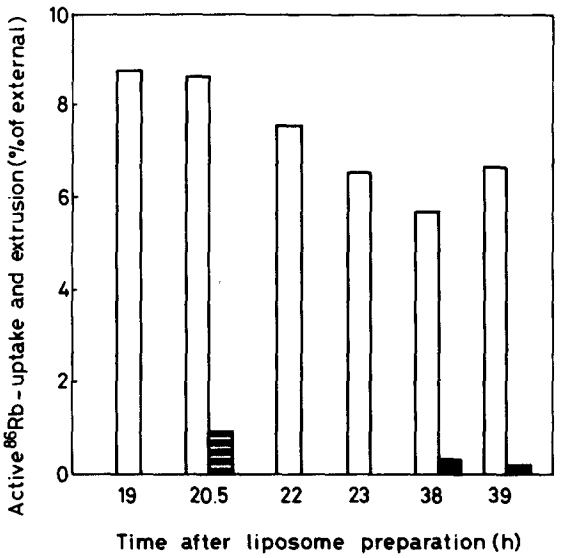

Fig. 4. Stability of a typical ATP-containing ($Na^+ + K^+$)-ATPase-liposome preparation. Liposomes No. 331 were dialysed for 16.5 h at 0 °C and the external ATP was removed by a repeated 60 min centrifugation and resuspension-step at 0 °C as described under Materials and Methods. The first active transport assay was run at 19 h after the beginning of the liposomes preparation. To test the active transport, 10 μM ^{86}RbCl in 4 μl transport-solution were added to 4 μl liposomes and the $^{86}Rb^+$ accumulation (white columns) was determined as described under Materials and Methods; the uptake in the presence of 100 μM external ouabain (striped columns) was 8.4% of control. The addition of 10 mM external ATP (black columns) activated the inside-out pumps and led to the extrusion of 95% of the accumulated Rb^+ ions even at 39 h after the liposome preparation. The liposome suspension contained 14837 ± 413 cpm $^{86}Rb^+$ ($n = 11$; S.E.) in 3 μl liposome-suspension and the average entrapment within 3 min was 1134 ± 92 cpm ($n = 6$; S.E.), i.e. 7.6% of the total radioactivity was entrapped within 3 min, yielding 38 μM internal ^{86}RbCl.

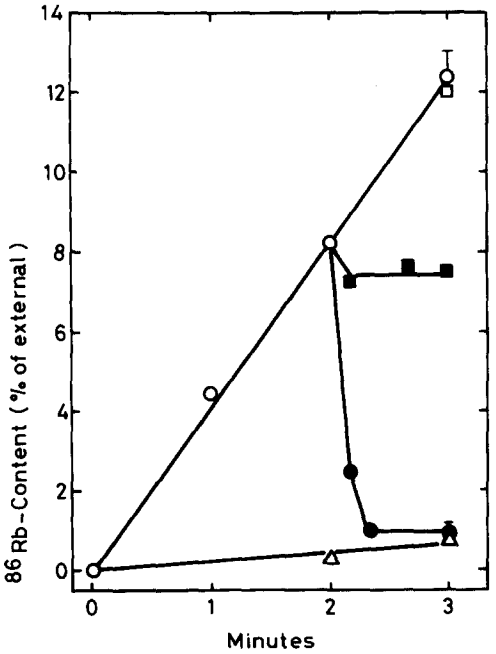

Fig. 5. Selective block of the inside-out oriented pump population by externally added vanadate. The active $^{86}Rb^+$ uptake (○) was initiated by the addition of 10 μM external ^{86}RbCl and incubation of the ATP-containing ($Na^+ + K^+$)-ATPase-liposomes at 25 °C as described under Materials and Methods. Upon addition of 10 mM external ATP, the inside-out pumps extruded over 90% of the accumulated Rb^+ ions (●). The addition of 100 μM vanadate immediately before the ^{86}RbCl addition prevents the activity of the inside-out pumps (■) without affecting the Rb^+ uptake catalyzed by the right-side-out pumps (□), while the presence of 100 μM external ouabain inhibits the active Rb^+ uptake (△). Vertical bars represent the S.E. of the mean of four separate determinations and the values without bars the average of two separate determinations collected in three different preparations.

In Table III, it is shown that the average amount of Rb^+ ions remaining in the liposomes despite the presence of the external ATP amounts to about 5%. This means that the liposome-population containing solely right-side-out pumps presents not more than 5% of the liposomes containing right-side-out as well as inside-out pumps. Alternatively, our transport-results may be consistent with a recent model of Zampighi et al. [36] proposing the artifactual creation of ($Na^+ + K^+$)-ATPase-dimers composed by symmetrically oriented $\alpha\beta$-monomers. If such units would reconstitute perpendicularly with respect to the plane of the liposome membrane, and if the $\alpha\beta$-monomer were the minimal functional transport-unit, a single reconstituted pump-unit may be capable to catalyze active Rb^+ uptake as well as Rb^+ extrusion in the ATP-containing liposomes.

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