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RIGHT-SIDE-OUT PUMPING Na,K-ATPase-LIPOSOMES: A NEW TOOL TO STUDY THE ENZYME'S RECEPTOR FUNCTION

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The technology to prepare right-side-out pumping Na,K-ATPase-liposomes is described. The 50% right-side-out oriented pumps of ATP-containing liposomes are then activated by the addition of external Rb ions, leading to a ouabain-sensitive Rb-influx which is the mirror-image of the inside-out transport. The resulting internal Rb concentration is 4 to 10 fold larger than the external concentration. Finally, the accumulated Rb ions can be extruded by driving the 50% inside-out oriented pumps by external ATP.

The Na,K-ATPase molecule (EC 3.6.1.37) contains three coupled functional components: i) an ATPase on the intracellular side, ii) a transmembraneous Na:K exchange system, and iii) an extracellular receptor for cardioactive steroids (1-3) as well as for their putative endogenous analogues: 'endodigitalis' or 'ouabain like factor' (3,4), the natriuretic hormone (5-7) and the inhibitory factors appearing in hypertension (8).

At the present time, the chemical nature of the hormone-like receptor-ligands is unkown. Besides the chemical analysis, an assay-system is useful to study their effect on actively pumping Na,K-ATPase molecules. As we are able to form a population of unilamellar 100 nm Na,K-ATPase-liposomes with a narrow size distribution (9), a defined number of Na,K-ATPase molecules per vesicle and a strictly symmetrical 50% inside-out, 50% right-side-out orientation of the reconstituted pump molecules (10), we thought that an entrapped ATP reservoir

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may phosphorylate the right-side-out oriented pump molecules and so activate the receptor exposed on the external liposome surface.

However, to achieve this goal a large number of technical problems had to be solved. The present paper describes the technology to construct an in vitro tool where the 50% right-side oriented pumps are activated by internal ATP so that the potential ligands can be added directly to the fully activated receptor on the external liposome membrane. In addition, it is possible to activate also the 50% inside-out oriented pumps.

MATERIALS AND METHODS

Purified phosphatidylcholine was type V-E from Sigma, purified bovine brain phosphatidylserine and cholestyramine-resin were purchased from Serva. L-cysteine, NaCl, RbCl, MgCl2, EDTA, histidine, ouabain and Na-cholate were purchased from Merck in the highest purity grade availabe; Na2ATP was grade I from Boehringer; ^{86}RbCl was from Amersham. The pH of all solutions was adjusted with Tris-HCl. Na,K-ATPase was purified from the outer medulla of fresh rabbit kidneys by the SDS method (11) to a specific activity of 25 to 35 U/min. Tris-ATP was prepared according to Hudgins and Bonds (12).

A lipid solution was prepared by drying 16 mg phosphatidylcholine and 4 mg phosphatidylserine (13) and dissolving them in 1 ml of a solution containing 30 mM histidine, 1 mM EDTA, 1 mM L-cysteine, 5 mM MgCl2, pH 7.15 (solution A) and in addition 23 mM Na-cholate.

Three g of cholestyramine-resin are suspended in 10 ml of solution A, the suspension is well mixed and then centrifuged for 3 min at 1,000 x g. The resin is resuspended in 10 ml of solution A and recentrifuged. The procedure is repeated 3 times. The last supernatant is carefully and completely removed and the washed cholestyramine-resin is stored at 4° for up to 2 weeks.

ATP-containing Na,K-ATPase-liposomes are formed as follows: an aliquot of the enzyme suspension containing 100 µg protein is put into a Beckman Airfuge tube and sedimented for 15 min at about 100,000xg at 4°. To the pellet, 50 µl of a solution containing 30 mM histidine, 1 mM EDTA, 1 mM L-cysteine, 5 mM MgCl2, 25 mM Na2ATP, 25 mM Tris-ATP, pH 7.15 (solution B) plus 23 mM Na-cholate are added and the enzyme pellet is suspended. The undissolved enzyme is sedimented by a 10 min Airfuge centrifugation at about 100,000 x g. To the supernatant an aliquot of the lipid solution is added at a 1:1 (v/v) ratio and the mixture is is transferred to a dialysis bag that has been prepared as previously described (13).The bag is then put into a transparent recipient which contains about 300 mg washed cholestyramine-resin and 5 ml of solution B. The container is immersed in a ice-water bath and the solution is stirred vigorously so that the cholestyramine-resin is well suspended and

constantly moving around the dialysis bag. Tight, transport-active liposomes are formed within 3 hours. The dialysis can be continued for at least 14 more h without modifying the transport properties or trapped volume of the Na,K-ATPase liposomes.

After dialysis, the liposomes are transferred to an Airfuge centrifugation tube and centrifuged for 60 min at about 100,000xg in a coldroom at 2°. The supernatant is rapidly and carefully removed and the pellet is resuspended in the same volume that was present before centrifugation of a solution containing 50 mM NaCl, 5 mm MgCl2, 30 mM histidine, 1 mM EDTA, 1 mM L-cysteine , pH 7.15 (solution C), recentrifuged for 60 min and resuspended in the same volume of the same solution.

The washing procedure was controlled after hydrolysing the ATP to Pi and ADP by the addition of 5 µg Na,K-ATPase protein and 10 mM KCl for 30 min at 37°. The Pi was determined by a highly sensitive colorimetric method which detects Pi down to 1 µM concentration (14). After two centrifugation and resuspension steps, the external Pi concentration was below 50 µM. The same result was obtained by washing the liposomes by filtrating them rapidly through 1 ml syringues filled with Sephadex-G-50 gel in a centrifuge (15). However, with small samples the latter procedure was less reproducible with regard to liposome recovery and volume.

Right-side-out transport assay: About 10 µCi (10 µl) of the original ^{86}Rb-solution are dried and dissolved in 30 µl of solution A to yield a final RbCl concentration of 1.5 mM. In general, 7.5 µl samples of liposomes are put to the bottom of a conical plastic tube kept in ice. About 2 mm above the liposome level, 1.5 µl of the ^{86}Rb solution are put on the plastic wall. The radioactive droplet with the liposomes and the tube is then immediately transferred to a water bath at 25°C to start the active transport (= time 0). To determine the ouabain-sensitive Rb-transport, 0.9 µl of a 1 mM ouabain solution are added to the liposomes in ice before they are mixed with the ^{86}Rb solution. At the end of the desired incubation time, the tubes are put in ice and 4 µl liposomes are immediately put on the ice-cooled gel columns which have been washed before for at least 1 h with a solution containing 50 mM Tris-Cl, 30 mM histidine, 1 mM L-cysteine, 1 mM EDTA, pH 7.2; fractions are collected and counted as previously described (13). From the 5 µl unwashed liposomes remaining in the tube, 2 x 2 µl are counted to know the total (=external) ^{86}Rb. The ^{86}Rb taken up by the liposomes is expressed as % of external ^{86}Rb (13). In agreement with results obtained in inside-out transporting liposomes (16), the same transport pattern is observed whether KCl or RbCl is used as a carrier for the ^{86}Rb ions.

Inside-out transport: The right-side-out pumps are blocked and the inside-out pumps are activated by the addition of 0.9 µl 1 mM ouabain and of 1 µl 100 mM Tris-ATP to the 9 µl Rb labeled liposomes by the mixing techniques described above.

RESULTS

Right-side-out pumping liposomes.

The active transport of the 50% right-side-out oriented Na,K-ATPase molecules is activated by the addition of external RbCl to the liposomes and by increasing the temperature from 0°

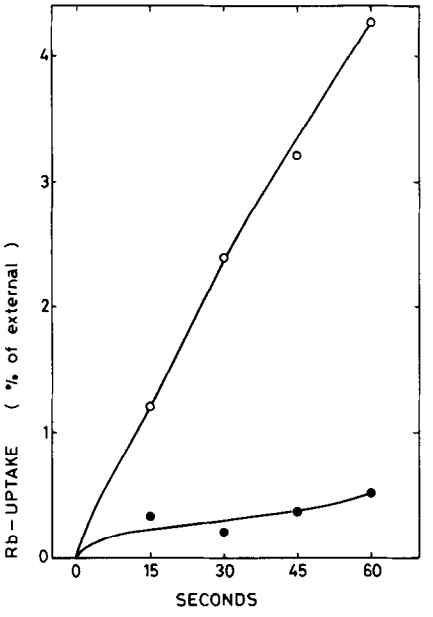


Fig. 1. Rb-uptake by right-side-out pumping Na,K-ATPase-liposomes. The ^{86}Rb incorporation was measured in the absence (○) and in the presence (●) of external ouabain as described under Materials and Methods. A typical experiment is shown.

to 25°. A linear, ouabain-sensitive uptake of Rb ions appears (Fig. 1) which presents a mirror-image of the active transport performed by the previously characterized inside-out oriented Na,K-ATPase molecules (compare with Fig. 2 of ref. 16). The ouabain-insensitive flux is about 10% of the ouabain-sensitive flux.

The Na,K-ATPase-liposomes at the protein:lipid ratio selected in the present work contain on the average 2 to 3 randomly oriented Na,K-ATPase molecules per vesicle and an entrapped volume of about 1% or 10 µl/ml (10). Thus, an inside Rb-gradient is built up as soon as more than 1% of the external Rb ions are accumulated. Fig. 1 shows that 1% of the external Rb ions are incorporated within 15 s. The Rb-uptake is close to 4% within 60 s of incubation time in the presence of external Rb ions which means that, at this moment, the internal Rb concentration is 4-fold larger than the external one.

As the Rb-uptake in the right-side-out pumping liposomes is limited by the internal Na and ATP concentrations, we enclose a large reservoir of Na ions and ATP within the liposomes. The low external Rb concentration of 250 μM favors receptor studies with the right-side-out pumping Na,K-ATPase-liposomes as it is well known that external K or Rb ions stabilize a dephosphoform of the pump which has low affinity for cardioactive steroids (1,2,3).

The ouabain-sensitivity of the Rb-transport.

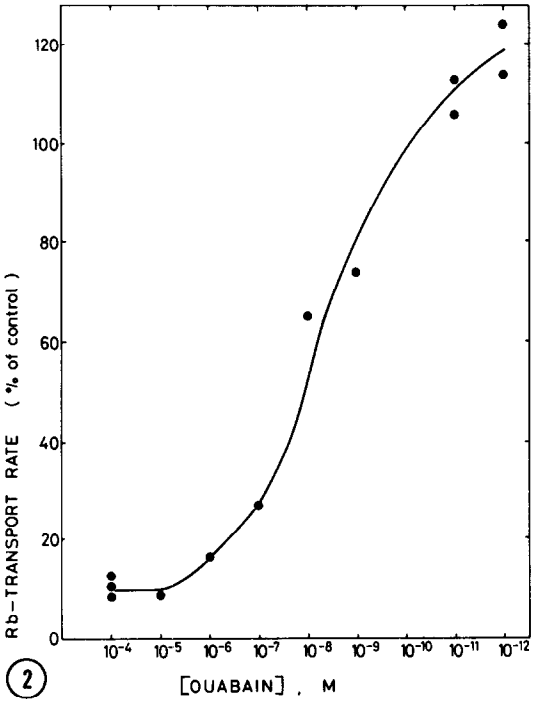
Fig. 2 shows an example for the use of the right-side-out pumping liposomes as a tool for studying the Na,K-ATPase receptor function. The active Rb-influx mediated by the purified rabbit kidney Na,K-ATPase is decreased by 50% by about 2×10^{-8} M ouabain.

Successive activation of both pump populations.

Fig. 3A shows the ouabain-sensitive Rb-influx mediated by the 50% right-side-oriented Na,K-ATPase molecules. The Rb-uptake reaches a maximum when 8% of the external Rb ions have been incorporated, i.e. when the internal Rb concentration is approximately 8-times larger than the external one. Then, the internal Rb concentration decreases at a slow rate, dictated presumably by the passive flux rate.
When external ATP is now added, the internal Rb ions are extruded rapidly by the inside-out pumps (Fig. 3B) in the same manner as previously described with solely inside-out pumping Na,K-ATPase-liposomes (compare with Fig. 1 of ref. 16).

DISCUSSION

Up to now, the 50% right-side-out oriented pumps of Na,K-ATPase-liposomes remained silent because the lipid membrane is relatively impermeable to ATP. However, an



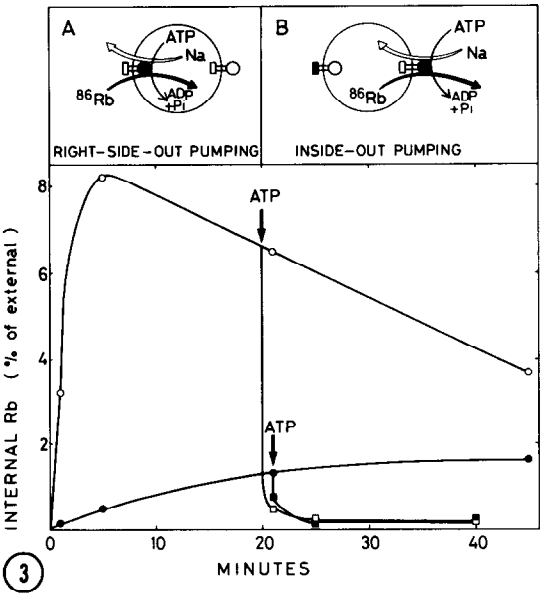


Fig. 2. Ouabain-sensitivity of the active Rb-uptake mediated by purified rabbit kidney Na,K-ATPase in right-side-out pumping liposomes. The ^{86}Rb-uptake occuring in the absence of ouabain was determined in the linear part of the uptake phase at 15 min following the addition of 250 μM external [^{86}Rb]Rb ions as described under Materials and Methods. Immediately before the Rb addition, the ouabain solutions in the concentration range indicated at the abcissa were added to the liposome samples as described under Materials and Methods and the transport inhibition was expressed in % of ouabain-free control.

Fig. 3. A. Ouabain-sensitive, i.e. active (o) and ouabain-insensitive, i.e. passive (●) Rb-uptake by the 50% right-side-out pumping Na,K-ATPase-lipsomes. B. Rb-extrusion of the actively (□) and of the passively (■) incorporated Rb ions by the 50% inside-out oriented pumps following the addition of external ATP and ouabain as described under Materials and Methods.

exclusive inside-out pumping system where the receptor component of the 50% actively pumping Na,K-ATPase molecules is within the liposomes is not suitable to study the molecular pharmacology of the Na,K-ATPase molecule as the receptor is only fully active when the pump is phosphorylated.

The present work shows that internal ATP activates the 50% right-side-out pump molecules so that the receptor is accessible at the external liposome surface. The right-side-

out transport is the mirror-image of the well described transport pattern (16) of the 50% inside-out oriented pumps.

The right-side-out pumping system could be used, e.g., for the following purposes: i) analysis of the effect of diverse ions known to modulate the receptor affinity, e.g. K or Ca ions for cardioactive steroids (1,2,3) or Na ions for the hypothalamic Na,K-ATPase inhibitor (17), ii) study of the transport pattern of Na,K-ATPase with low affinity receptors produced by mutation in vivo, in vitro or by insertion of the ouabain resistance gene (reviewed in ref. 18), and finally, iii) test of future antagonistes of the inhibitors appearing in hypertension.

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