COUPLED Na^+-K^+ TRANSPORT IN VESICLES CONTAINING A PURIFIED (NaK)-ATPASE AND ONLY PHOSPHATIDYL CHOLINE

Shirley Hilden and Lowell Hokin

Department of Pharmacology
University of Wisconsin, Medical School
Madison, Wisconsin 53706

Received January 23,1976

SUMMARY

Endogenous phospholipids of a purified (NaK)-ATPase were displaced by exogenous phosphatidyl choline. If vesicles were made from phosphatidyl choline and enzyme containing only phosphatidyl choline, coupled Na^+-K^+ transport could be demonstrated. This transport was inhibitable by ouabain. Therefore, the number of components necessary for Na^+-K^+ transport has been reduced to the purified (NaK)-ATPase and one phospholipid.

Several laboratories have recently demonstrated transport by inserting (NaK)- ATPase into phospholipid vesicles. In this laboratory, coupled Na^+-K^+ transport has been demonstrated using a purified enzyme from the dogfish shark rectal salt gland (1,2). With a purified enzyme from the dog kidney, Goldin and Tong (3) demonstrated Na^+ transport accompanied by Cl^- movement but no coupled transport of K^+. However, vesicles containing partially purified enzyme preparations from brain (4) and electric organ (5) showed coupled Na^+-K^+ pumps.

With vesicles containing purified NaK ATPase where Na^+-K^+ transport has been reconstituted, it becomes possible to study minimal requirements for transport. Purified (NaK)-ATPase preparations contain two major proteins and phospholipid. The four major phospholipids in the dogfish enzyme are phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine and sphingomyelin (Table I). Warren, *et. al.* (6,7) have developed a method for substituting endogenous phospholipid with exogenous phospholipid in membrane enzymes. A phospholipid, detergent and enzyme solution is

placed on a discontinuous sucrose gradient and centrifuged. The protein and associated phospholipid goes through the gradient while detergent and phospholipid not associated with protein stay at the top of the gradient. They have been able to obtain 98-99% phospholipid substitution in Ca ATPase and 90% substitution in β-hydroxylbutyrate dehydrogenase (6,7,8).

In this paper, we use this technique to demonstrate that (NaK)-ATPase with only one phospholipid, namely, phosphatidyl choline, is capable of coupled Na^+-K^+ transport.

MATERIALS AND METHODS

The phosphatidyl choline was the Sigma III E brand in hexane. This preparation gave only one spot on two dimensional thin layer chromatography. Immediately before use, the hexane was removed under a stream of nitrogen. Other materials were as previously reported (1,2).

Displacement of endogenous phospholipids with exogenous phosphatidyl choline. The endogenous phospholipids of the purified NaK ATPase were displaced by exogenous phosphatidyl choline using the method of Warren, *et al.* (6,7). The following solution was left on ice for one hour with frequent shaking using a vortex mixer: phosphatidyl choline, 20 mgm/ml; cholate, 20 mgm/ml; NaKATPase (stored at -70°C), 10 mgm/ml; 0.25 M sucrose; 1 M KCl; 0.12 M NaCl; 30 mM imidazole; 1 mM EDTA and 1 mM cysteine (pH 7). Five-tenths to one ml of this solution was added to a discontinuous sucrose gradient containing 0.7 ml 15% sucrose layered on top of 0.2 ml 50% sucrose in a 2 ml cellulose nitrate tube. When necessary, an overlay was placed above the sample to completely fill the tube. The overlay and sucrose solutions contained 1 M KCl, 20 mM NaCl, 30 mM imidazole, 1 mM EDTA and 1 mM cysteine. The tubes were capped, placed in Delrin adaptors and centrifuged in a fixed angle 65 rotor at 48K for 12-17 hours in a L2-65B centrifuge. The material at the 15-50% sucrose interface or the precipitate at the bottom of the tube was resuspended in dialyzing solution (volume equal to original sample volume) which contained 100 mM KCl, 20 mM NaCl, 30 mM imidazole (pH 7.0), 1 mM EDTA and 1 mM cysteine. Part of this suspension was dialyzed against 1000 volumes of dialyzing solution to remove the sucrose. This dialyzed material will be referred to as singly substituted enzyme. The rest of this solution was treated again with phosphatidyl choline and cholate, layered on a sucrose gradient, centrifuged, resuspended and dialyzed as described above. This second solution will be referred to as twice substituted enzyme. The protein concentration was measured by the Lowry method (9) and the phospholipid content of the purified enzyme (control), singly substituted and twice-substituted enzymes were analyzed by two-dimensional thin layer chromatography and P_i analysis (10).

Ion Transport in Vesicles Made From Enzyme of Differing Phospholipid Compositions. Vesicles were formed from the three different forms of enzyme by a modification of previously published methods (1,2). A solution containing phosphatidyl choline, 30 mgm/ml; cholate, 15 mgm/ml;

TABLE I

Phospholipid composition of purified NaK ATPase

singly and twice substituted with phosphatidyl choline

	number of substitutions					
	none		once		twice	
	μgm mgm protein	% of total	μgm mgm protein	% of total	μgm mgm protein	% of total
phosphatidyl choline	192	49	358	90	480	100
phosphatidyl ethanolamine	122	31	23	6		
phosphatidyl serine	43	11				
sphingomyelin	29	8	8	2		
all other phospholipids	6	2	7	2		
total phospholipid	392	101	396	100	480	100

Purified NaK ATPase (control) was prepared as described by Hokin, *et.al.* (11). The endogenous phospholipids of this enzyme were replaced by phosphatidyl choline and analyzed for phospholipid content as described in Materials and Methods. The singly substituted enzyme was derived from the control enzyme. The twice substituted enzyme was derived from the singly substituted enzyme. A typical experiment is shown.

and enzyme, 0.5 mgm/ml was prepared so that the final concentrations were 100 mM KCl, 95 mM NaCl, 30 mM imidazole, 1 mM EDTA and 1 mM cysteine (pH 7) and dialyzed for 2 days at 4°C against 100 mM KCl, 20 mM NaCl, 30 mM imidazole, 1 mM EDTA and 1 mM cysteine (pH 7). ^{22}Na (0.5 to 0.7 μC/ml) and ^{42}K (25 to 50 μC/ml) were added to this vesicle solution and left overnight at 4°C. These vesicles preloaded with isotope were then incubated at 25°C for 10 minutes in the presence of 5 mM $MgCl_2$ ± 5 mM Tris ATP. Transport of Na^+ and K^+ was measured as described previously (1,2).

RESULTS

Substitution of endogenous phospholipid with exogenous phosphatidyl choline.

Table I reports phospholipid content of the original enzyme, enzyme which had gone through one phospholipid substitution and enzyme which had gone

TABLE II

Ion transport by vesicles made from

control enzyme, singly and twice substituted enzyme

enzyme	isotope	isotope content-(%) +ATP	isotope content-(%) -ATP	net transport-(%) in	net transport-(%) out
control	^{22}Na	0.75	0.19	0.56	
	^{42}K	0.84	1.02		0.18
singly-substituted enzyme	^{22}Na	1.56	0.20	1.36	
	^{42}K	1.02	1.38		0.36
twice-substituted enzyme	^{22}Na	1.35	0.22	1.11	
	^{42}K	0.86	1.29		0.43

Vesicles were prepared as described in Materials and Methods. After incubation in the presence or absence of ATP, vesicles were passed over a Sephadex G-50 column to separate vesicles from free isotope. Isotope content (%) is the percent of the total counts of ^{42}K or ^{22}Na which elutes in the void volume in the vesicles. Net transport is the difference between the isotope content of vesicles incubated in the presence of ATP (ie. transporting vesicles) and the isotope content of vesicles incubated in the absence of ATP (ie. non-transporting vesicles).

through two phospholipid substitutions. After the first substitution, there was no detectable phosphatidyl serine. After two substitutions, the only detectable phospholipid was the exogenous phospholipid, phosphatidyl choline. There was no detectable phosphatidyl serine in any twice-substituted enzyme which was analyzed. In one of three preparations, there was detectable phosphatidyl ethanolamine (2%).

Transport by enzyme containing only phosphatidyl choline. The singly- and twice substituted enzymes were used to prepare vesicles. Table II shows that both the singly-and twice-substituted enzymes were capable

TABLE III

Ouabain inhibition of transport by vesicles made with control or twice substituted NaK ATPase

enzyme	ouabain	isotope	isotope content-(%) +ATP	isotope content-(%) -ATP	net transport-(%) in	net transport-(%) out
control	-	^{22}Na	0.75	0.19	0.56	
		^{42}K	0.84	1.02		0.18
	+	^{22}Na	0.10	0.10	0.00	
		^{42}K	0.24	0.20		0.04
twice substituted enzyme	-	^{22}Na	1.35	0.22	1.11	
		^{42}K	0.86	1.29		0.43
	+	^{22}Na	0.15	0.14	0.01	
		^{42}K	0.20	0.21		0.01

Vesicles were prepared in media $\pm 10^{-4}$M ouabain. Other procedures are described in Materials and Methods. The enzyme used in making these vesicles is the same as that used in Table II.

of both Na^+ and K^+ transport. In fact, transport by the substituted enzymes was higher than transport by the control enzyme. Net transport of 1.11% by the twice-substituted enzyme is equivalent to 400 nmol Na^+/mgm/10 min. Transport by the control vesicles is low compared to previously reported results (1,2). Net transport by control vesicles of 0.56% is equivalent to 224 nmol Na^+/mgm/10 min which compares to our previously reported value of 1090 nmol/mgm/10 min (2, Table IX), when vesicles were prepared with a more complex egg lecithin.

Table III shows that transport by the control enzyme and the twice-

substituted enzyme is ouabain-inhibitable. In order to inhibition transport in vesicles, it is necessary to have ouabain inside the vesicles, which is accomplished by preparing vesicles in the presence of 10^{-4}M ouabain.

DISCUSSION

These experiments reduce the number of components necessary for coupled Na^+-K^+ transport. Previous studies used vesicles with a more complex phospholipid preparation or a less pure enzyme. In earlier papers from this laboratory, we demonstrated coupled transport in vesicles made from purified (NaK)-ATPase and a crude preparation of egg lecithin. Endogenous phospholipids were not removed. Coupled Na^+-K^+ transport has also been demonstrated in vesicles containing a simple phospholipid, endogenous phospholipids, and a partially purified (NaK)-ATPase from brain or electric organ (4,5). This paper shows that vesicles containing a purified (NaK)-ATPase and only one phospholipid (phosphatidyl choline) are capable of transport. This system may not be the minimal system capable of transport, since no function has been demonstrated yet for the glycoprotein of the purified (NaK)-ATPase, although antibodies against the glycoprotein partially inhibit (NaK)-ATPase activity (12) and reconstituted Na^+-K^+ transport in vesicles containing the purified (Na^+-K^+)-ATPase (13).

The conclusion of this paper is in conflict with many but not all studies which suggest that phosphatidyl serine is essential for (NaK)-ATPase activity in native membranes (14). In most of the former studies, reactivation of the (NaK)-ATPase by phosphatidyl serine followed inactivation by detergents or phospholipase treatment. Perhaps the specificity for phosphatidyl serine was not for (NaK)-ATPase activity but for some other process such as combination of dilipidated protein and phospholipid in aqueous solution. Evidence against the essentiality of phosphatidyl serine was provided by de Pont *et.al.* (15), who converted

more than 99% of the original phosphatidyl serine to phosphatidyl ethanolamine, by treating the (NaK)-ATPase with phosphatidyl serine decarboxylase without affecting enzyme activity.

ACKNOWLEDGEMENT

The authors would like to thank Mrs. Mary Lochner and Mrs. Connie Capacio for preparing the enzyme, and Mr. John Dixon for carrying-out the phospholipid analyses.

REFERENCES

1. Hilden, S., Rhee, H.M. and Hokin, L.E. (1974) J. Biol. Chem. 249, 7432-7440.
2. Hilden, S. and Hokin, L.E. (1975) J. Biol. Chem. 250, 6296-6303.
3. Goldin, S.M. and Tong, S.W. (1974) J. Biol. Chem. 249, 5907-5915.
4. Sweadner, K.J. and Goldin, S.M. (1975) J. Biol. Chem. 250, 4022-4024.
5. Racker, E. and Fisher, L.W. (1975) Biochem. Biophys. Res. Commun. 67, 1144-1150.
6. Warren, G.B., Toon, P.A., Birdsall, N.J.M., Lee, A.G. and Metcalfe, J.C. (1974) FEBS Letters 41, 122-124.
7. Warren, G.B., Toon, P.A., Birdsall, N.J.M., Lee, A.G. and Metcalfe, J.C. (1974) Proc. Nat. Acad. Sci. U.S. 71, 622-626.
8. Houslay, M.D., Warren, G.B., Birdsall, N.J.M. and Metcalfe, J.C. (1975) FEBS Letters 51, 146-151.
9. Lowry, O.H., Rosebrough, N.J., Fan, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
10. Perrone, J.R., Hackney, J.F., Dixon, J.F. and Hokin, L.E. (1975) J. Biol. Chem. 250, 4178-4184.
11. Hokin, L.E., Dahl, J.L., Dixon, J.F., Hackney, J.F. and Perdue, J.F. (1973) J. Biol. Chem. 248, 2593-2605.
12. Rhee, H.M. and Hokin, L.E. (1975) Biochem. Biophys. Res. Commun. 63, 1139-1145.
13. Rhee, H.M. and Hokin, L.E. (1975) personal communication.
14. Dahl, J.L. and Hokin, L.E. (1974) Ann. Rev. Biochem. 43, 327-356.
15. de Pont, J.J.H.H.M., Van Prooijen-Van Eeden, A. and Bonting, S.L. (1973) Biochim. Biophys. Acta 323, 487-494.