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Phosphatase activity and potassium transport in liposomes with Na^+,K^+-ATPase incorporated

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We have used liposomes with incorporated pig kidney Na^+,K^+-ATPase to study vanadate sensitive K^+-K^+ exchange and net K^+ uptake under conditions of acetyl- and *p*-nitrophenyl phosphatase activities. The experiments were performed at 20°C. Cytoplasmic phosphate contamination was minimized with a phosphate trapping system based on glycogen, phosphorylase a and glucose-6-phosphate dehydrogenase. In the absence of Mg^{2+} (no phosphatase activity) 5–10 mM *p*-nitrophenyl phosphate slightly stimulated K^+-K^+ exchange whereas 5–10 mM acetyl phosphate did not. In the presence of 3 mM $MgCl_2$ (high rate of phosphatase activity) acetyl phosphate did not affect K^+-K^+ exchange whereas *p*-nitrophenyl phosphate induced a grater stimulation than in the absence of Mg^{2+}; a further addition of 1 mM ADP resulted in a 35–65% inhibition of phosphatase activity with an increase in K^+-K^+ exchange, which sometimes reached the levels seen with 5 mM phosphate and 1 mM ADP. The net K^+ uptake in the presence of 3 mM $MgCl_2$ was not affected by acetyl phosphate or *p*-nitrophenyl phosphate, whereas it was inhibited by 5 mM phosphate (with and without 1 mM ADP). The results of this work suggest that the phosphatase reaction is not by itself associated to K^+ translocation. The ADP-dependent stimulation of K^+-K^+ exchange in the presence of phosphatase activity could be explained by the overlapping of one or more step/s of the reversible phosphorylation from phosphate with the phosphatase cycle.

Introduction

Several transport modes and partial biochemical reactions catalyzed by the sodium pump have been crucial for establishing the steps of the Na^+,K^+-ATPase reaction cycle (see Ref. 1 for references). In the absence of intracellular Na^+, but in the presence of both intracellular and extracellular K^+, the pump effects a 1:1 exchange of K^+ across the membrane (K^+-K^+ exchange). Intracellular phosphate and ATP are required [2], but non-hydrolyzable analogues of ATP [3,4] or ADP [5] can substitute for ATP. On the other hand, in the presence of K^+ and Mg^{2+}, the enzyme hydrolyses various phosphoric anhydrides (e.g., *p*-nitrophenyl phosphate, umbelliferone phosphate, acetyl phosphate, etc.) [1]. Although the scheme for that catalytic activity is not yet fully understood, there are certain features that have been established. For instance, while phosphatase activity has some similarities with the reversible binding of inorganic phosphate [6,7], the reactivity, and particularly the sidedness of that reactivity, towards Na^+ and K^+ is different in phosphoenzymes formed from phosphatase substrates and that obtained from inorganic phosphate [8]. In addition, the existence of more than one phosphatase reaction pathway has been suggested depending on the substrate being used [9,10]. Although no cation transport process has been linked to this reaction [11] it is intriguing that the K^+ occluded $E_2(K)$ and K^+ deoccluded E_2PK could function as intermediates [7]; this might pave the way for an associated K^+ translocation if the K^+ sites were eventually exposed to inner and outer sides of the membrane. The present studies were undertaken to look into this problem. To that end, we have measured vanadate sensitive K^+-K^+ exchange and net K^+ uptake into liposomes with pig kidney Na^+,K^+-ATPase incorporated under conditions of acetyl- and *p*-nitrophenylphosphatase activities. The results indicate

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that the phosphatase reaction is not by itself associated with K^+ translocation. The stimulation of K^+-K^+ exchange by phosphatase substrates in the presence of Mg^{2+} and ADP can result from the E-P formation but not necessarily be coupled to the whole biochemical events leading to phosphatase activity.

Methods

Preparation of proteoliposomes. Na^+,K^+-ATPase was partially purified from pig kidneys [12] and stored at −85°C in a solution of 25 mM imidazole (pH 7.5 at 20°C)/1 mM EDTA-Tris/250 mM sucrose; before use, it was dialyzed overnight against 25 mM imidazole (pH 7.0 at 20°C)/1 mM EDTA-Tris. The usual activity was 18–22 units per mg total protein and was 99 percent ouabain-sensitive; this indicates a degree of purity of about 40 percent (see also Ref. 12). Proteoliposomes were prepared following the procedure described in Ref. 13 as adapted in our laboratory [14]. The phospholipid used was crude soybean phosphatidylcholine and the total protein/lipid ratio 1:40 [13,14]. Protein was determined by the method of Lowry et al. [15] as modified by Markwell et al. [16].

Measurement of potassium fluxes. Aliquots of 0.06 ml of proteoliposomes suspensions were added to 1.6 ml of cytoplasmic media; the uptake of $[^{86}Rb]K^+$ was measured at 20°C, in triplicate or quadruplicate, in the conditions indicated in the table legends. The cytoplasmic (extravesicular) solutions contained no phosphate compounds or 5–10 mM phosphate, acetyl phosphate or *p*-nitrophenyl phosphate in the absence and presence of 3 mM $MgCl_2$. In addition they had 140 mM Tris·HCl and 5 mM $[^{86}Rb]KCl$, 1 mM ouabain with and without 0.5 mM vanadate. 1 mM ADP was eventually added. Mg^{2+}-free solutions contained 2 mM CDTA. The intravesicular (extracellular) media had none or 100 mM KCl and 130–[KCl] mM of Tris-HCl. All solutions were Na^+-free. When required, and to minimize the inorganic phosphate contamination, the cytoplasmic media contained an inorganic phosphate trapping system. With *p*-nitrophenyl phosphate, the reaction was started after 60 min preincubation at room temperature with the phosphate trapping system in the cytoplasmic medium; when acetyl phosphate was the substrate, no preincubation with that system took place (see below). The $^{86}Rb^+$ incorporated was estimated from the radioactivity present in the effluent of short Sephadex columns as described previously [14]. In all cases the uptake of isotope was linear within the time of the assays. The absolute $[^{86}Rb]K^+$ flux was calculated as nmoles taken up per min per 10 μl liposomes suspension (nmol/min per 10 μl Lip.). In the presence of Mg^{2+}, the discrimination between the different K^+ fluxes was made as follows (see Ref. 17): (i) Ouabain acts on the extracellular phase; therefore, by adding ouabain to the extravesicular media we expected to inhibit all K^+ fluxes going via the right-side out pump units. (ii) Vanadate requires Mg^{2+} to bind and acts on the cytoplasmic side; hence, it was used as a tool to identify the $[^{86}Rb]K^+$ fluxes through the inside-out pumps, which were not attacked by extravesicular ouabain. Throughout this work all vanadate sensitive fluxes are considered as going by way of the Na^+/K^+ pump. (iii) We call basal flux the K^+ uptake through the Na^+/K^+ pump in the absence of the phosphate compounds being tested; its value under each conditions is indicated in the corresponding table legend. K^+transport insensitive to vanadate, which is taken as the K^+ leak [13], represented about 30–40 percent of the basal uptake. On the other hand, without Mg^{2+}, vanadate could not be used as before: under these conditions we estimated the flux difference in the presence and absence of a given phosphate compound.

Measurement of phosphatase activities. The hydrolysis of *p*-nitrophenyl or acetyl phosphate was estimated in experiments run in parallel with the transport determinations in the presence of 3 mM $MgCl_2$. To that purpose, 0.05 ml of proteoliposomes suspension were incubated for 2 to 5 min at 20°C in 0.2 ml final volume of cytoplasmic media. When the substrate was *p*-nitrophenyl phosphate, the rate of hydrolysis was calculated from the release of *p*-nitrophenol as in Ref. 18. For acetyl phosphate, the rate of hydrolysis was estimated as in Ref. 19 by the hydroxylamine method. The determinations were performed in duplicate or triplicate. In no case the difference between duplicate samples was more than 3 percent.

Materials and solutions. All solutions were made with de-ionized bi-distilled water. The chemicals were reagent grade. ADP (Na^+ salt), *p*-nitrophenyl phosphate (Tris salt), acetyl phosphate (Li^+ plus K^+ salt), ouabain, cholic acid, soybean phosphatidylcholine (Type II-S), Sephadex G-50–40, glycogen, NADP, glucose 1,6-diphosphate, glucose-6-phosphate dehydrogenase, phosphoglucomutase and phosphorylase a were obtained from Sigma, Chemical Co., USA. Vanadate was purchased from Fisher Co., USA. ^{86}Rubidium, as chloride salt, was provided by New England Nuclear, USA. Nucleotides and acetyl phosphate were transformed into Tris salts by passing them through Amberlite IR-120-P columns.

Counting was performed in a Beckman liquid counter with automatic quenching correction; when possible, the standard error of counting was less than 1 percent.

Results

Phosphate contamination and the use of a phosphate trapping system (P_iTS)

A crucial problem we had to deal with was phosphate contamination, because it was important to dis-

tinguish a possible action due to *p*-nitrophenyl phosphate or acetyl phosphate from that of phosphate simultaneously present. According to the experimental protocols, the two main sources of phosphate contamination could be (i) that coming with the phosphatase substrates, and (ii) that released from these compounds, either by spontaneous or enzymatic hydrolysis.

The test of possibility (i) depended on the substrate used. For *p*-nitrophenyl phosphate, a freshly made solution was completely hydrolyzed by incubation in 3 M HCl at 100°C for 3 h [20]. Aliquots were then used to measure *p*-nitrophenol [18] and phosphate [21] concentrations. For acetyl phosphate, aliquots of a freshly made solution were used to estimate total acetyl phosphate [19] and inorganic phosphate [21]. The results obtained (not shown) indicate that, within the resolution of the analytical determinations (0.8 percent for phosphate, 1.1 percent for *p*-nitrophenol and 2.1 percent for acetyl phosphate) the concentration of phosphate was equal to those of *p*-nitrophenol and acetyl phosphate. Therefore, if it existed, initial phosphate contamination could have been at the most about 3 percent. This number, which might look small, is large and potentially dangerous, for it means a possible phosphate concentration of 150 μM and 300 μM for 5 mM and 10 mM substrate concentrations respectively.

Spontaneous hydrolysis of 5 mM *p*-nitrophenyl phosphate and acetyl phosphate was determined at 20°C and pH 7.0 in 130 mM Tris-HCl. The two main findings, summarized in Table I, are: (a) as expected [20], a remarkable stability of *p*-nitrophenyl phosphate; the stable *p*-nitrophenol absorbance corresponded to 0.15 percent of the total *p*-nitrophenyl phosphate concentration; (b) acetyl phosphate is more stable than has been reported [20]; the anticipated hydrolysis of around 20 percent in 30 min reached only 15 percent in one hour (rate constant of 0.003 min^{-1}). Consequently, the predicted phosphate accumulation due to spontaneous hydrolysis of acetyl phosphate would amount to 15–30 μM after the usual 1–2 min incubation times.

TABLE I

Release of inorganic phosphate from the spontaneous hydrolysis of 5 mM p-nitrophenyl phosphate (pNPP) or acetyl phosphate (AcP) at room temperature

Incubations were carried out in 130 mM Tris-HCl, at 20±2°C and pH 7.0 . The concentrations of inorganic phosphate released from *p*-nitrophenyl phosphate were estimated from the absorbance of *p*-nitrophenol at 410 nm; those released from acetyl phosphate were calculated by the hydroxylamine reaction. The computed rate constant for the hydrolysis of acetyl phosphate was 0.003 min^{-1}. See Methods for more details.

Incubation time (min)	Inorganic phosphate (μM) released from	
	pNPP	AcP
0	7.3	-
10	7.6	43.9
20	7.8	249.3
40	7.6	610.8
60	7.1	771.4

TABLE II

Trapping of inorganic phosphate by a substrate-enzyme coupled system based of glycogen, phosphorylase a and glucose-6-phosphate dehydrogenase

Incubations were performed at 20°C in media of the following additional composition: 5 mM KCl; 3 mM $MgCl_2$; 135 mM Tris-HCl (pH 7.0 at 20°C); 3.5 mg/ml glycogen; 2 IU/ml phosphorylase *a*; 2 IU/ml glucose-6-phosphate dehydrogenase; 2 IU/ml phosphoglucomutase; 1 mM NADP; 0.016 mM glucose-1,6-diphosphate. Note: (i) unless otherwise indicated the initial concentration of inorganic phosphate (100%) was 50 μM; (ii) * the initial inorganic phosphate concentration was 200 μM; (iii) ** 5 mM *p*-nitrophenyl phosphate and *** 5 mM acetyl phosphate were added in the absence of unlabelled phosphate; (iv) in all cases 100000 cpm/tube of carrier free [^{32}P]phosphate were included; (v) the rate constant of radioactivity disappearance in the presence of 50 μM inorganic phosphate was 0.144 min^{-1}. At the times indicated in the table the ^{32}P activity was estimated by extraction with isobutanol-benzene [22].

Incubation time (min)	^{32}P activity (% of the cpm initially present)
0	100
1	74
5	53
10	27
20	5
20	12 *
20	15 **
20	34 ***
40	1 **

Enzymatic hydrolysis of the substrates. Examples of this kind are seen in Tables V and VI. According to that data, the phosphate accumulated by this mechanism after 1–2 min would be around 30–60 μM with acetyl phosphate and 5–10 μM with *p*-nitrophenyl phosphate.

From the results shown in this section, it is obvious that the phosphate accumulation per se could modify K^+ fluxes; i.e., stimulation of K^+-K^+ exchange in the presence of ADP. To minimize this problem we decided to use an enzymatic phosphate trapping system (P_iTS) based on glycogen degradation into glucose 6-phosphate via a pathway catalyzed by phosphorylase *a*, glucose-6-phosphate dehydrogenase and phosphoglucomutase. To that end, we included in the incubation solutions 3 mg/ml glycogen, 1 mM NADP, 2 IU/ml phosphorylase *a*, 2 IU/ml glucose-6-phosphate dehydrogenase, 2 IU/ml phosphoglucomutase and 16 μM glucose 1,6-diphosphate. The tests for the effectiveness of the P_iTS are shown in Table II. The actual procedure consisted of adding 100,000 cpm/tube of carrier free [^{32}P]phosphate to solutions containing 50 μM or 200 μM cold phosphate or 5 mM acetyl- or

p-nitrophenyl phosphate; after different incubation times at 20°C and pH 7.0 the remaining radioactivity was estimated as in Ref. 22. 20 min preincubation with P_iTS (without the proteoliposomes) resulted in a 95 percent disappearance of 50 μM phosphate in the media ($k = 0.114\ min^{-1}$), and 88 percent of 200 μM phosphate. On the other hand, in the absence of non-labelled phosphate, the ^{32}P activity was reduced to 1 percent after 40 min when there was 5 mM *p*-nitrophenyl phosphate and to 34 percent after 20 min with 5 mM acetyl phosphate. Finally, in control experiments (not shown) the inclusion of the P_iTS had no detectable effect on the phosphatase activity with either substrate used in this work.

A more convincing test of the effectiveness of the P_iTS is illustrated in Table III. One hour preincubation with the phosphate trapping system completely abolished the ADP plus inorganic phosphate stimulated K^+-K^+ exchange up to 200 μM phosphate; the exchange fluxes were reduced to one sixth at 0.5 mM and to one fourth at 1 mM initial phosphate concentrations.

Summarizing, the methodology used to reduced phosphate contamination to a minimum consisted of the following: (i) the working hematocrit was 5 percent; (ii) the transport incubation times lasted 1 or 2 min;

TABLE III

Effect of including an inorganic phosphate (P_i) trapping system (P_iTS) on the ADP- and (ADP + P_i)-supported K^+-K^+ exchange in liposomes with Na^+,K^+-ATPase incorporated

Potassium fluxes were estimated from the uptake of ^{86}Rb after 2 min incubation at 20°C. The compositions of solutions were as follows (mM): (a) extracellular (intravesicular): KCl, 100; Tris-HCl (pH 7.0 at 20°C), 30; (b) cytoplasmic (extravesicular): the phosphate concentrations indicated in the Table plus $MgCl_2$, 3; [^{86}Rb]KCl, 5; Tris-HCl (pH 7.0 at 20°C), 140; ouabain, 1. To each value, the average basal flux of 0.058 nmol/10 μl Lip. per min was subtracted. All values are the mean of duplicate determinations. The flux measurements were started by the addition of the proteoliposomes; previously to that, all tubes had been preincubated (with or without the phosphate trapping system) for 60 min at 20°C. Note: (i) the concentration of ADP was always 1 mM; (ii) the K^+-K^+ exchange observed in the presence of 0.5 mM vanadate was not affected by the assayed ligand; (iii) the (ADP + P_i)-supported K^+-K^+ exchange in absence of P_iTS was fitted to a Michaelian equation with a Km for inorganic phosphate of 280 μM and a V_{max} of 0.595 nmol/10 μl Lip. per min. For details see Methods.

Added P_i (mM)	Vanadate-sensitive [^{86}Rb]K^+-K^+ exchange (nmol/10 μl Lip. per min)	
	P_iTS absent	P_iTS present
None	0.110	0.109
0.03	0.134	0.110
0.1	0.243	0.110
0.2	0.323	0.130
0.5	0.452	0.170
1.0	0.573	0.258

TABLE IV

Effects of ADP, p-nitrophenyl phosphate (pNPP) and acetyl phosphate (AcP), in the absence or presence of Mg^{2+} ions, on K^+-K^+ exchange in liposomes with Na,K-ATPase incorporated

Potassium fluxes were estimated from the uptake of ^{86}Rb after 2 min incubation at 20°C. The compositions of solutions were as follows (mM): (a) extracellular (intravesicular): KCl, 100; Tris-HCl (pH 7.0 at 20°C), 30; (b) cytoplasmic (extravesicular): the ligand indicated in the Table plus CDTA, 2; [^{86}Rb]KCl, 5; Tris-HCl (pH 7.0 at 20°C), 140; ouabain, 1. In Experiment Nos. 2* and 3* there was 3 mM $MgCl_2$ and no CDTA in the extravesicular solution. Each entry is the mean ± S.E. of quadruplicate determinations. To each value, the average flux in the absence of any phosphate compound (0.118 ± 0.006 nmoles/10 μl Lip. per min) was subtracted. Note: (i) the flux in the absence of any phosphate compound is not the basal flux as defined in this work; (ii) the presence of Mg^{2+} ions in 2* and 3* did not modify the K^+ fluxes when there was no pNPP in the incubation solution; (iii) the letter on the right side column indicates the statistical significance according to the Student's *t*-test: (a) non significant, (b) $P < 0.002$, (c) $P < 0.001$. For details see Methods

Expt. No.	Ligand	(mM)	[^{86}Rb]K^+-K^+ exchange (nmol/10 μl Lip. per min)
1	ADP	1	0.096 ± 0.012 (c)
	pNPP	5	0.042 ± 0.008 (b)
	pNPP	10	0.080 ± 0.015 (c)
	ADP	1	
	+ pNPP	5	0.093 ± 0.011 (c)
	AcP	5	−0.008 ± 0.006 (a)
	AcP	10	0.007 ± 0.008 (a)
2	ADP	1	0.111 ± 0.014 (c)
	ADP	1	
	+ pNPP	5	0.104 ± 0.010 (c)
2*	ADP	1	0.109 ± 0.011 (c)
	ADP	1	
	+ pNPP	5	0.322 ± 0.023 (c)
3	ADP	1	0.125 ± 0.020 (c)
	ADP	1	
	+ pNPP	5	0.127 ± 0.021 (c)
3*	ADP	1	0.094 ± 0.012 (c)
	ADP	1	
	+ pNPP	5	0.281 ± 0.019 (c)

(iii) the substrate solutions were always freshly made and kept in ice until used; (iv) with *p*-nitrophenyl phosphate, the P_iTS was preincubated for one hour in the complete incubation media without the liposomes; with acetyl phosphate, and due to its instability, the P_iTS was only included during the flux measurement periods.

K^+-K^+ exchange fluxes in the absence of Mg^{2+}

Introductory experiments were performed in proteoliposomes lacking cytosolic Mg^{2+} ($MgCl_2$ was omitted and 2 mM CDTA was included). In this way, any possible effect of the ligand under investigation could be detected in the absence of phosphorylation and phosphatase activity. As expected, ADP stimulated K^+-

K^+ exchange [17,23,24]; in this case (see Table IV) that increase was about 1.8 fold. Considering that *p*-nitrophenyl phosphate can mimic ATP in its regulatory role [6], we anticipated a stimulation of the exchange by this compound; in fact, 5 mM and 10 mM *p*-nitrophenyl phosphate augmented K^+-K^+ exchange by 1.3- and 1.7-fold, respectively. Also, and within the experimental errors, addition of 1 mM ADP on top of 5 mM *p*-nitrophenyl phosphate resulted in K^+ fluxes similar to those seen with 1 mM ADP or 10 mM

TABLE V

Effects of ADP, acetyl phosphate (AcP) and p-nitrophenyl phosphate (pNPP), alone and in combination, on vanadate-sensitive K^+-K^+ exchange and phosphatase activity in liposomes with Na^+,K^+-ATPase incorporated in the presence of cytoplasmic Mg^{2+} ions

Potassium fluxes were estimated as indicated in the legend to Table III. Besides what is shown in the table the solutions had the following composition (mM): (a) extracellular (intravesicular): KCl, 100; Tris-HCl (pH 7.0 at 20°C), 30; (b) cytoplasmic (extravesicular): $MgCl_2$, 3 (unless its omission is indicated); [^{86}Rb]KCl 5; Tris-HCl (pH 7.0 at 20°C), 140; ouabain, 1. The entries are the mean ± S.E. differences between the fluxes obtained in the presence and absence of a given ligand. Each experiment was carried out in triplicate. The last column shows the phosphatase activities measured in parallel experiments under identical conditions. Here, each entry is the mean of duplicate determinations. Note: (i) * the cytoplasmic medium was free of $MgCl_2$ and contained in addition 2 mM CDTA; (ii) ** 2 mM inorganic phosphate were added with the liposomes; (iii) the K^+-K^+ exchange observed in the presence of 0.5 mM vanadate was not affected by the assayed ligand; (iv) the tubes containing *p*-nitrophenyl phosphate were preincubated for one hour with the phosphate trapping system (P_iTS), whereas in those with acetyl phosphate the P_iTS was added with the liposomes; (v) each experimental number refers to a different batch of proteoliposomes; (vi) the letter on the right side column indicates the statistical significance of the fluxes according to the Student's *t*-test: (a) non significant, (b) $P < 0.05$, (c) $P < 0.01$, (d) $P < 0.005$, (e) $P < 0.001$. The basal fluxes in the different experiments had the following values (nmol/10 μl Lip. /min): (1) 0.058 ± 0.002; (2) 0.042 ± 0.004; (3) 0.069 ± 0.004; (4) 0.059 ± 0.005; (5) 0.067 ± 0.014, (6) 0.057 ± 0.011; (7) 0.054 ± 0.004; (8) 0.046 ± 0.005. See Methods for details.

Expt. No.	Cytoplasmic ligand			Vanadate-sensitive [^{86}Rb]K^+-K^+ exchange (nmol/10 μl Lip. per min)	Phosphatase activity (nmol/10 μl Lip. per min)	
	ADP	AcP (mM)	pNPP			
1	1	–	–	0.117 ± 0.006	–	(e)
	–	5	–	0.004 ± 0.007	n.d.	(a)
	1	5	–	0.228 ± 0.010	n.d.	(e)
2	1	–	–	0.070 ± 0.023	–	(b)
	–	5	–	0.005 ± 0.006	n.d.	(a)
	1	5	–	0.223 ± 0.012	n.d.	(e)
3	– **	–	–	0.003 ± 0.006	–	(a)
	1	–	–	0.125 ± 0.015	–	(d)
	–	5	–	0.006 ± 0.007	11.20	(a)
	1	5	–	0.352 ± 0.012	7.52	(e)
	1 **	–	–	0.393 ± 0.025	–	(e)
4	1	–	–	0.117 ± 0.018	–	(d)
	1	5	–	0.287 ± 0.006	n.d.	(e)
5	1	–	–	0.139 ± 0.028	–	(c)
	1	5	–	0.354 ± 0.016	n.d.	(e)
6	1	–	–	0.105 ± 0.025	–	(b)
	–	–	5	0.111 ± 0.022	n.d.	(c)
	1	–	5	0.338 ± 0.028	n.d.	(e)
7	1	–	–	0.096 ± 0.010	–	(e)
	–	–	5	0.071 ± 0.007	1.80	(e)
	–	–	5*	0.046 ± 0.006	0.0	(d)
	1	–	5	0.295 ± 0.020	0.87	(e)
8	–	–	5	0.076 ± 0.009	2.32	(d)
	–	–	10	0.098 ± 0.006	3.53	(e)
	1	–	5	0.230 ± 0.012	0.83	(e)
	1	–	10	0.244 ± 0.011	1.64	(e)
	–	5	–	−0.015 ± 0.009	13.71	(a)
	–	10	–	−0.010 ± 0.007	16.83	(a)
	1	5	–	0.218 ± 0.021	8.56	(e)
	1	10	–	0.282 ± 0.011	11.06	(e)
	1	–	–	0.102 ± 0.006	–	(e)
	1 **	–	–	0.384 ± 0.024	–	(e)

TABLE VI

Comparative effects of acetyl phosphate (AcP), p-nitrophenyl phosphate (pNPP) and inorganic phosphate (P_i) on vanadate-sensitive net potassium uptake in the presence and absence of cytoplasmic Mg^{2+} ions and nucleotides in liposomes with Na^+,K^+-ATPase incorporated lacking intravesicular potassium

Net potassium uptake was estimated from the ^{86}Rb activity incorporated after 1 min at 20°C. The composition of solutions was as follows: (a) extracellular (intravesicular): 140 mM Tris-HCl (pH 7.0 at 20°C); (b) cytoplasmic (extravesicular): The ligand indicated in the table plus 130 mM Tris-HCl (pH 7.0 at 20°C). The $MgCl_2$ concentration was routinely 3 mM unless otherwise indicated. The concentration of [^{86}Rb]KCl was 5 mM in experiments 1 to 3 and 2.5 mM in 4 to 8. The reaction was started by adding the proteoliposome suspension. The entries are the mean ± S.E. differences between the vanadate-sensitive fluxes obtained in the presence and absence of a given ligand. Each experiment was carried out in triplicate. The last column shows the phosphatase activities measured in parallel experiments under identical conditions: here, each entry is the mean of duplicate determinations. Note: (i) * $MgCl_2$ in the cytoplasmic medium was 0.050 mM. (ii) ** The cytoplasmic medium was $MgCl_2$-free and contained in addition 2 mM CDTA. (iii) Each experimental number refers to a different batch of proteoliposomes. (iv) The vanadate-sensitive K^+ uptake in the absence of phosphate compounds had the following values (nmol/10 μl Lip. per min): (1) 0.064 ± 0.005; (2) 0.062 ± 0.008; (3) 0.060 ± 0.009; (4) 0.051 ± 0.009; (5) 0.031 ± 0.004; (6) 0.034 ± 0.005; (6*) 0.033 ± 0.005; (6**) 0.032 ± 0.004; (7) 0.050 ± 0.005; (8) 0.023 ± 0.003. (v) the letter on the right side column indicates the statistical significance of the fluxes according to the Student's *t*-test: (a) non significant, (b) $P < 0.05$, (c) $P < 0.02$, (d) $P < 0.005$. For other details see Methods.

Expt. No.	Cytoplasmic ligand (mM)				Net [^{86}Rb]K uptake (nmol/10 μl Lip. per min)	Phosphatase activity (nmol/10 μl Lip. per min)	
	AcP	pNPP	ADP	P_i			
1	–	–	0.06	–	0.016 ± 0.006	–	(a)
	–	–	–	0.14	−0.021 ± 0.018	–	(a)
	–	–	0.06	0.14	−0.010 ± 0.019	–	(a)
	5	–	–	–	0.010 ± 0.015	10.5	(a)
2	5	–	–	–	0.002 ± 0.012	–	(a)
3	5	–	–	–	0.020 ± 0.013	–	(a)
	–	5	–	–	0.003 ± 0.011	1.6	(a)
4	–	–	–	5	−0.044 ± 0.010	–	(c)
	–	5	–	–	0.013 ± 0.013	2.5	(a)
	–	–	1	–	−0.028 ± 0.011	–	(a)
	–	–	1	5	−0.022 ± 0.009	–	(a)
	–	5	1	–	−0.019 ± 0.010	–	(a)
5	–	5	–	–	0.018 ± 0.014	2.2	(a)
	–	5	1	–	−0.016 ± 0.007	–	(a)
6	–	–	–	5	−0.031 ± 0.005	–	(d)
	–	5	–	–	0.004 ± 0.011	1.6	(a)
	–	–	–	5	0.021 ± 0.007 *	–	(b)
	–	5	–	–	−0.016 ± 0.009 *	–	(a)
	–	–	–	5	0.009 ± 0.014 **	–	(a)
	–	5	–	–	−0.012 ± 0.007 **	–	(a)
7	–	5	–	–	−0.018 ± 0.009 *	–	(a)
8	5	–	–	–	0.063 ± 0.004 *	–	(a)
	–	–	–	1	−0.001 ± 0.004 *	–	(a)

p-nitrophenyl phosphate alone. This finding is relevant to the results obtained in the presence of Mg^{2+} (see Expts. 2* and 3* in this table as well as those in the following section). On the other hand, under Mg^{2+}-free conditions the sole addition of acetyl phosphate (either 5 or 10 mM) showed no effect on the K^+-K^+ exchange fluxes.

K^+-K^+ exchange fluxes and phosphatase activity in the presence of Mg^{2+}

Preliminary results can be seen in Table IV. In that table we can compare, in the same batch of liposomes, the effect of a phosphatase substrate in the absence and presence of Mg^{2+}; in that way we could avoid possible bias due to the variability in different preparations. The data from Expts. 2-2* and 3-3* show that, in the presence of the divalent cation and ADP, addition of *p*-nitrophenyl phosphate increases by 2–3-fold the K^+ exchange fluxes.

A complete set of experiments on vanadate-sensitive K^+-K^+ exchange in the presence of Mg^{2+} is illustrated in Table V; as additional information, parallel estimations of phosphatase activities are also included in this table. Each number in the first column identifies an experiment where a different batch of proteoliposomes was used. For fluxes, the entries are the mean ± S.E.

differences between the vanadate-sensitive values obtained in the absence and presence of the tested ligand. The phosphatase figures correspond to the total activity in the presence of 5 mM cytoplasmic K^+, all of which was inhibitable by 0.5 mM vanadate. Control experiments (not shown) reproduced most of the observations previously reported [17]: (i) a small stimulation in the exchange rate due to 1 mM ADP; (ii) no effects of millimolar concentrations of phosphate; (iii) a large increase when phosphate and ADP were present; (iv) in the absence of phosphate compounds, Mg^{2+} had no effect on the vanadate-insensitive K^+ fluxes, but we consistently failed to detect the small inhibition of the fluxes sensitive to vanadate.

In the absence of ADP and under conditions of high phosphatase activity, 5 mM or 10 mM acetyl phosphate did not alter the K^+-K^+ exchange; on the other hand, with 5 mM *p*-nitrophenyl phosphate there was a small stimulation; that stimulation was about 50 percent higher than in Mg^{2+}-free solutions (identified with a single asterisk in Table V). On the other hand, the simultaneous presence of 1 mM ADP and 5–10 mM *p*-nitrophenyl phosphate or acetyl phosphate markedly stimulated K^+-K^+ exchange to values sometimes comparable to those observed with mM [phosphate]. In addition, 1 mM ADP reduced phosphatase activity by 30–40 percent with acetyl phosphate and 50–60 percent with *p*-nitrophenyl phosphate.

Net potassium uptake in the presence and absence of cytoplasmic Mg^{2+}

In the absence of Na^+ and ATP, phosphatase activity is stimulated by potassium acting only on the cytoplasmic side [25,26]. In addition, although the $E_2(K)$ occluding form is a likely intermediate in the cycle, the most abundant form of the enzyme during phosphatase turnover is E_2, where K^+ ions are not occluded [7]. Furthermore, a phosphoenzyme is formed, at least with acetyl- [7,8] and β-(2-furyl)acryloyl phosphate [27]. Therefore, transitions between the E_1K, $E_2(K)$ and E_2K (in this case E_2PK) states might occur; this may lead to a net transport of the stimulating K^+ ions from the cytosol to the extracellular side. This possibility was investigated in the experiments described in the present section.

The actual experimental design consisted in following the entry (extrusion from the cell) of [^{86}Rb]K^+ in proteoliposomes without intravesicular (extracellular) K^+, the incubation solutions contained 5 mM *p*-nitrophenyl- or acetyl phosphate and different concentrations of $MgCl_2$ (none, 0.05 mM and 3 mM). In some cases 1 mM ADP was included; in others, the phosphatase substrates were absent while 0.14 mM, 1 mM or 5 mM phosphate (with and without ADP) was present. Phosphatase activity at 3 $MgCl_2$ was assayed in parallel experiments. Table VI shows that, in the presence of phosphatase substrates, the net K^+ uptake was the same regardless of the existence of phosphatase activity. In addition, there were two effects of inorganic phosphate [28] that phosphatase substrates could not mimic: (i) inhibition of net K^+ transport in the presence of 3 mM $MgCl_2$ (see row 1 in Expts. 4 and 6), and (ii) small stimulation with 0.05 mM $MgCl_2$ (see row 3 in Expt. 6).

It is important to point out that phosphatase activity depended only on cytosolic potassium, and was not influenced by the presence or absence of K^+ in the extracellular phase (not shown).

Discussion

According to the data described in the first part of Results it is unlikely that the stimulation of K^+ exchange fluxes by phosphatase substrates in the presence of Mg^{2+} is a consequence of phosphate contamination. Leaving phosphatase aside, an alternative biochemical basis for that stimulation could be a phosphoryl group exchange between water and *p*-nitrophenyl phosphate or acetyl phosphate. We think that possibility is unlikely: on the one hand, there is no experimental evidence for an exchange reactions of that sort; on the other, the $K_{1/2}$ for phosphate is between 100–300 μM (Refs. 7 and 29, Berberián and Beaugé, unpublished) for E-P formation and 250 μM for the (P_i + ADP)-dependent K^+-K^+ exchange (this work). Therefore, if these phosphoryl group exchanges exist they would be negligible under our experimental conditions. Consequently, we think it is safe to conclude, particularly with *p*-nitrophenyl phosphate, that in the presence of ADP the observed stimulation of K^+ exchange fluxes occurs because there is a simultaneous phosphatase activity.

Which are then the possible relationships between phosphatase reaction and K^+ translocation? To help in the analysis, a diagram showing the K^+-K^+ exchange pathways through the Na^+,K^+-ATPase reaction and hypothetical phosphatase cycles is depicted in Fig. 1. The meanings of enzyme forms, steps and ligand are detailed in the legend to the figure. There is a small fraction of K^+ fluxes (exchange and net) occurring via the Na^+ pump in the absence of any ligand [17,30]; in this case the pump units act as facilitated diffusion carriers, and the conformational transitions involved are

$$K^+_{(i)} + E_1 = E_1K = E_2(K) = E_2 + K^+_{(e)}$$

$E_2(K)$ and E_1K open up spontaneously towards the outside and inside of the cell, respectively. In order to keep Fig. 1 as clear as possible this parallel path has not been included.

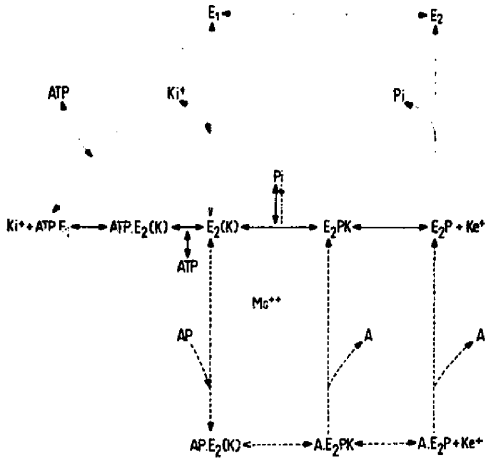

Fig. 1. Diagrammatic scheme indicating possible overlapping between the reaction steps involved in the K^+-K^+ exchange associated with reversible phosphorylation from phosphate and the phosphatase reaction carried out by the Na^+,K^+-ATPase in the presence of ATP (or ADP). The meaning of symbols and complexes are the following: K_i^+ and K_e^+ are the internal and external potassium ions, respectively; E_1 is the enzyme form with high affinity for ATP and low affinity for K^+; E_2 is the enzyme form with low affinity for ATP and high affinity for K^+; $E_2(K)$ is the K^+ occluding dephosphoenzyme; P_i is the inorganic phosphate which is intracellularly bound to, or released from, the enzyme; AP is the phosphoric anhydride that acts as phosphatase substrate; Mg^{2+}, ATP (or ADP) and AP are all intracellular. Note: (i) each possible pathway and enzyme complex in that part of the Na^+,K^+-ATPase reactions is included; (ii) the feasibility of more than one path for the phosphatase activity is considered; (iii) the whole route of the K^+-K^+ exchange may become part of the phosphatase reaction in the presence of ATP (or ADP); (iv) Mg-enzyme complexes as well as the small fraction of K^+-K^+ exchange in the absence of phosphorylation have been omitted (see Discussion); (v) in the phosphatase cycle A is always released before P_i [37].

Any attempt to explain the results of this work must necessarily account for the lack of stimulation of net K^+ extrusion associated with the phosphatase reaction. Fig. 1 describes the reactions required for net K^+ extrusion without of ADP (or ATP). These are:

$$E_1 + K_i^+ \rightarrow E_1K \rightarrow E_2(K)$$

(left vertical axis of the upper square)

$$E_2(K) + AP \rightarrow AE_2PK \rightarrow E_2PK \rightarrow E_2P + K_e^+ \text{ or}$$

$$E_2(K) + AP \rightarrow APE_2K \rightarrow AE_2PK \rightarrow AE_2P + K_e^+$$

(lower loops)
and the returning

$$E_2P \rightarrow E_2 + P_i \rightarrow E_1$$

(upper square).

Some experimental observation support this path: (i) only intracellular K^+ stimulates phosphatase activity [25,26] and a concomitant E-P formation [7,8], and (ii) the prevailing enzyme state during that activity is E_2 [6,7,31]. However, the expected stimulation of net K^+ extrusion was not observed, even when 1 mM ADP was added (Table VI). One way to reconcile these findings is that the $E_1K \rightarrow E_2(K)$ shift acts just as a trigger for the reaction whereas during turnover the enzyme remains most of the time in a K^+ binding E_2 form (lower left loop in Fig. 1). This resembles reversible phosphorylation from phosphate at high [Mg^{2+}] where, without [28] or with (this work) ADP, no effects on net K^+ extrusion are seen. The explanation advanced by Karlish and Stein [28] for the case of phosphate may also apply to phosphatase substrates: in the absence of K_e^+, the E-P formed is actually the K^+-insensitive phosphoenzyme reported by Post et al. [32]. Accordingly, the release of K^+ from E_2PK to the external medium occurs only if there is K_e^+ i.e. K_e^+ prevents the formation of a K_e^+-insensitive phosphoenzyme. This hypothesis is strongly supported by experiments on phosphate and acetyl phosphate phosphorylation of Na^+,K^+-ATPase incorporated into liposomes: with both-phosphate compounds the levels of E_2P are halved when there is K^+ on both sides as compared with K_e^+-free media [8].

If this is so, how does the stimulation of K^+-K^+ exchange come about? A complete overlapping of transport and phosphatase steps is not feasible because the stimulation of K^+-K^+ exchange needs ADP (or ATP). A minimum interaction requires a single common intermediate, E_2PK; in this case, in the presence of intracellular ADP (ATP) and extracellular K^+, the E_2PK formed during phosphatase activity is taken away from its natural path. The reduction in the rate of hydrolysis observed in the presence of ADP is consistent with a removal of the enzyme from its hydrolytic pathway. It is important to stress that this argument does not consider acetyl phosphate and *p*-nitrophenyl phosphate as phosphate substitutes; the idea is that, on their way to being hydrolyzed, they produce a phosphoenzyme eventually leading to an E-P similar to that obtained from inorganic phosphate.

An overlapping of more than one step is also possible. One alternative is given by the solid line in Fig. 1 where part of the K^+-K^+ exchange route [28,33] is shared with the phosphatase reaction which is carried out via the lower left loop. The order of ligand release in this case is: product of hydrolysis A, K^+ (in exchange with K_e^+) and then phosphate, returning via the solid line path. It should be recalled that we found a lack of stoichiometry between K^+-K^+ exchange and phosphatase reaction rates; interestingly, a lack of stoichiometry between the rates of phosphate-dependent K^+ fluxes and water-phosphate oxygen exchange also

exists [34]. In this scheme, E_2PK is the same for the phosphate and phosphatase stimulated fluxes. Unfortunately, this leaves without explanation the 50 percent K^+ deocclusion seen with phosphate [35,36] against the 100 percent observed at maximal phosphatase activity [7]. One way out from this problem is that the exchange of K^+ occurs from different intermediates which differ also in the K^+ releasing rate: E_2PK in the phosphate-dependent, and AE_2PK in the phosphatase-dependent, transport. In the latter case the order of release would be K^+ and then the product of hydrolysis A followed by phosphate; the returning path goes via the upper square in Fig. 1. Here there are two possibilities for the phosphatase cycle: (i) a mixture where the major path is still the lower left loop as before, but with the eventual occurrence of an AE_2PK–$AE_2P + K_c^+$ transition, or (ii) going always via the AE2P intermediate (external lower loop). As stated before, in the absence of external K^+ the phosphatase reaction is assumed to follow the left lower path.

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