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BIPHASIC EFFECT OF ORTHOPHOSPHATE ON THE (Na, K)-PUMP OF HUMAN RED CELLS

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Orthophosphate (P_i) can both stimulate and inhibit the (Na, K)-pump in red cells. At concentrations below 0.5 mmol/l cells, P_i stimulated the pump, but at higher concentrations P_i was inhibitory. The stimulation was demonstrated in intact cells by preincubation with inosine (which leads to a reduction in cellular P_i concentration), and then by incubating cells in media with various P_i concentrations (which relieved the inhibition caused by inosine). In inosine-treated cells there was an inverse relationship between the hematocrit during measurement of the fluxes and inhibition of the (Na, K)-pump; this was also a reflection of cellular P_i, which was lower in inosine-treated cels at low hematocrit. The stimulation of the (Na, K)-pump by P_i below 0.5 mmol/l cells was an indirect effect, due to synthesis of ATP by membrane-bound glycolytic enzymes, which required the appropriate substrates (in addition to P_i). This was shown by studies on inside-out vesicles made from red cell membranes. In the absence of the other substrates, P_i was inhibitory to Na transport in the vesicles. Above 0.5 mmol/l cells P_i was inhibitory to Na transport, both in inside-out vesicles and in intact cells. The mechanism of inhibition, probably a direct effect on the (Na, K)-pump, was not determined, though product inhibition seemed likely. The dependence on P_i of abnormal modes of Na transport by the pump (uncoupled Na efflux and Na/Na exchange) at low P_i concentrations was less than the dependence of normal Na/K exchange. This was attributed to a requirement by the abnormal modes of a lower rate of synthesis of ATP or a lower ATP concentration.

Introduction

Orthophosphate (P_i) may both inhibit and stimulate the (Na, K)-pump, by direct and indirect effects, respectively. Elevated P_i concentrations inhibit the pump in human red cells [1] and inhibit ($Na^+ + K^+$)-ATPase activity in broken membrane preparations [2]. In neither case were the kinetics simple, but direct product inhibition seemed possible.

There have also been suggestions of a mechanism by which P_i should stimulate the (Na, K)-pump indirectly in red cells. Glycolytic enzymes bound to the cytoplasmic surface of the membrane may synthesize ATP and deposit it in a membrane-associated compartment from which it is utilized by the (Na, K)-pump preferentially over ATP free in the cytoplasm [3–7]. Through this mechanism stimulation of the pump by P_i might be expected at low concentrations of P_i.

We recently confirmed this possibility: we demonstrated promotion of active Na transport by P_i in inside-out vesicles made from red cell membranes [8]. The stimulatory effect was observed only in the presence of NAD, glyceraldehyde 3-phosphate, and ADP, which are, along with P_i, the substrates necessary for the synthesis of ATP by glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase. The promotion of the pump's activity by these substrates did not require the addition of ATP. We were also able to show that the ATP synthesized by the membrane-bound enzymes was situated in a compartment associated with the membrane from which is was used by the pump, and where it is inac-

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cessible to degradation by agents added to the extravesicular medium of the inside-out vesicles.

Orthophosphate concentrations in red cells can be reduced by incubating the cells with inosine, which is split phosphorolytically to hypoxanthine and ribose phosphate [9]. However, in two studies no inhibition of the pump, in its normal Na/K exchange mode, was observed after incubation with inosine. Glynn et al. [9], measuring Na efflux activated by external K, and Dunham and Senyk [10], measuring K influx promoted by intracellular Na, found no effect of prior incubation with inosine, though in both studies K/K exchange was inhibited.

We have sought again a dependence on orthophosphate of the (Na, K)-pump in intact cells. In this study we show the conditions under which it is possible to demonstrate such a dependence, and attempt to explain it in terms of stimulation and inhibition of the pump by P_i in inside-out vesicles.

Materials and Methods

Blood was drawn into heparin from adult human donors. The red cells were washed by centrifugation and resuspension in an isotonic medium containing 150 mM NaCl/10 mM Tris-HCl/5 mM glucose, pH 7.4.

Intracellular cation concentrations were altered by the method of Sachs [11] using *p*-chloromercuribenzene sulfonate and dithiothreitol. Choline was used to replace Na and K when appropriate.

Intracellular concentrations of orthophosphate were reduced by incubation of the cells in media containing inosine [9]. In other experiments P_i was controlled by incubation with guanosine, an alternate substrate for nucleoside phosphorylase, or by incubation with hypoxanthine, which reverses the nucleoside phosphorylase reaction.

Fluxes of Na and K in intact cells were measured by methods described before (unidirectional K influx, Sachs et al. [12]; unidirectional Na efflux, Dunham and Hoffman [13]; net fluxes of Na and K, Rodland and Dunham [14]) except that the hematocrits of the suspension during the flux were varied as specified in the presentation of the results.

ATP concentrations were measured on neutralized trichloroacetic acid-extracts of cells using the 3-phosphoglycerate kinase reaction as described by Bergmeyer [15]. Orthophosphate concentrations were measured as phosphomolybdate by the method of Taussky and Shorr [16].

Inside-out vesicles were prepared by the method of Steck [17] with some modifications [8]. We also used Steck's method [17] to measure the fraction of vesicles which were inside-out (our preparations were always close to 80% inside-out). The method for measuring Na influxes into inside-out vesicles was as we described it elsewhere [8].

Results and Discussion

Inhibition of transport by inosine

As suggested above, it ought to be possible to demonstrate a dependence on orthophosphate of the (Na, K)-pump, operating in its normal Na/K exchange mode, if the pump preferentially uses membrane-bound ATP synthesized by membrane-bound phosphoglycerate kinase. Table I shows the results of six separate experiments in which ouabain-inhibitable net fluxes were measured in cells preincubated with glucose or inosine. In the low phosphate (inosine) cells there was a substantial (mean, 66%) inhibition of both active Na efflux and active K influx. Net fluxes rather than unidirectional fluxes were measured because of K/K exchange, which constitutes a substantial fraction of ouabain-inhibitable K influx in cells under physiological conditions, and which is inhibited by reduction of P_i concentration. An inhbition of unidirectional K influx after incubation with inosine could represent K/K exchange, whereas an inhibition of net K influx could not.

To rule out the possibility that the inhibition in Table I was due to reduced ATP, concentrations of ATP were analyzed in cells incubated for 1 h with either 10 mM glucose or 10 mM inosine. In this experiment (not included in Table I) the concentration of ATP in the inosine cells was actually a little higher than in the glucose (control) cells. The ATP concentrations were, in inosine cells: 2.3 mmol/l cells; and in glucose cells: 2.0 mmol/l cells ($n = 3$, results representative of two other experiments). Active Na efflux (measured as unidirectional efflux) was inhibited 63% in these inosine-cells, demonstrating that the inhibition could not be attributed to a decrease in ATP concentration.

TABLE I

OUABAIN-INHIBITABLE Na EFFLUX AND K INFLUX MEASURED AS NET FLUXES IN CELLS PREINCUBATED WITH 10 mM GLUCOSE OR INOSINE

Cells were treated to alter their cation concentrations using *p*-chloromercuribenzene sulfonate and dithiothreitol [11]. They contained $[Na]_c \simeq 30$ and $[K]_c \simeq 10$ mmol/l cells in all experiments. (The low [K] in the cells facilitated measuring net K influx). The balance of intracellular cation was choline. The cells were then incubated for 1 h at 37°C in isotonic media containing 150 mM choline chloride, 10 mM Tris-HCl, pH 7.4, and glucose or inosine at 10 mM. Cells were then centrifuged, resuspended in similar media (2% hematocrit) with or without ouabain (0.1 mM), and incubated at 37°C. After 5 min 150 mM KCl was added to a final concentration of 3 mM, thereby initiating the active fluxes. The net influx of K was calculated from chemical analysis of the cells, and the net efflux of Na from analysis of the medium [14]. (At 2% hematocrit the Na concentrations in the media, after incubation for 1 h, were 30 μM or greater.) The ouabain-insensitive unidirectional fluxes were nearly the same in cells in glucose or inosine. In each experiment the fluxes were determined in quadruplicate. Similar results were obtained using fresh cells (i.e. with unaltered cation concentrations) determined by measuring unidirectional fluxes.

Expt.	Ouabain-inhibitable Na efflux (mmol/l cells per h)			Ouabain-inhibitable K influx (mmol/l cells per h)		
	Preincubation		% Inhibition in inosine cells	Preincubation		% Inhibition in inosine cells
	Glucose	Inosine		Glucose	Inosine	
1	5.73	2.29	60	3.40	1.14	66
2	5.57	1.13	80	3.33	0.77	77
3	3.68	1.41	62	2.47	1.02	59
4	5.02	1.92	62	2.81	0.81	71
5	3.00	0.80	73	2.34	1.21	48
Mean	4.60	1.51	67	2.87	0.99	64

Orthophosphate relieves the inhibition by inosine

If our interpretation of the results in Table I is correct, it should be possible to relieve the inhibition of the pump in the inosine cells by adding P_i to the medium (the cells are permeable to P_i). The results of an experiment testing this possibility are shown in Table II. The cells were prepared in the same way as those in Table I except that some aliquots of cells

TABLE II

THE EFFLUX OF ORTHOPHOSPHATE ON INHIBITION OF UNIDIRECTIONAL Na EFFLUX IN INOSINE-TREATED CELLS

The treatment of the cells and procedure for the experiments were the same as in Table I, except that 10 mM P_i was added to the preincubation media (as Tris-phosphate) as indicated. The cells were preincubated for 60 min. For cells preincubated in media containing P_i, the flux media also contained P_i. The fluxes were determined in quadruplicate.

Expt.	Ouabain-inhibitable Na efflux (mmol/l cells per h)			
	No phosphate added		Phosphate present throughout incubation	
	Glucose	Inosine	Glucose	Inosine
1	3.92	1.94	2.97	4.46
2	4.36	1.51	3.72	4.03
3	2.67	0.44	2.62	3.85
Mean	3.65	1.30	3.10	4.11
Ratio of fluxes: inosine/glucose	0.36		1.33	

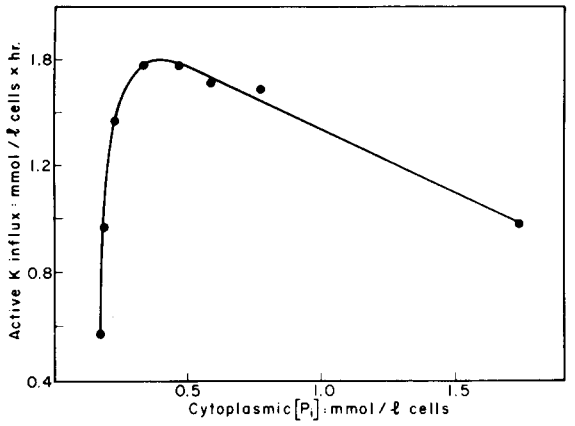

Fig. 1. The effect of varying intracellular orthophosphate (P_i) concentration on unidirectional ouabain-sensitive K influx in intact red cells. The cells were prepared the same as those described in Table II. Unidirectional K influx was measured in cells that had been incubated in media containing 10 mM inosine or 10 mM glucose, and various concentrations of phosphate as indicated. Each point represents the mean of three determinations. Similar results were obtained in two other experiments in which different methods were employed for varying P_i concentration (see text).

were incubated in a medium with P_i at 10 mM in addition to glucose or inosine. The inhibition of Na efflux in the low phosphate (inosine) cells was reversed by the addition of P_i, indicating that the inhibition in inosine is indeed due to the low concentration of P_i. In cells with both inosine and P_i, Na efflux was stimulated somewhat as compared to cells in glucose and P_i. This effect may have been a consequence of a reduction of the high cellular $[P_i]$: the medium contained 10 mM P_i; we will show below that high $[P_i]$ is inhibitory to the pump (Fig. 1). An inhibitory effect is also apparent in Table II (compare the flux in glucose with that in glucose plus P_i). If, in the experiment shown in Table II, inosine had caused a reduction in cellular $[P_i]$ (but not below 0.5 mmol/l), the inhibition would have been relieved, accounting for the stimulation by inosine + P_i as compared with glucose + P_i.

Concentration-dependence of transport on orthophosphate

Fig. 1 shows the dependence of active K influx on the concentration of intracellular P_i, varied from 0.2 to 1.8 mmol/l cells. To achieve this range of intracellular P_i concentrations, cells were incubated in media containing 10 mM inosine or 10 mM glucose and various concentrations of orthophosphate. As cytoplasmic $[P_i]$ was raised from 0.2 to 0.5 mmol/l cells, active K influx was markedly stimulated. As internal $[P_i]$ was increased further the flux was inhibited. Active Na efflux showed a similar dependence on intracellular P_i (results not shown). Two other methods to modify intracellular $[P_i]$ were also used: incubation with guanosine (10 mM) as an alternate substrate for nucleoside phosphorylase [18], and reversal of the nucleoside phosphorylase reaction by incubation with hypoxanthine (0.5–5 mM). Both of these methods gave results similar to those shown in Fig. 2, with the maximum pump activity at approximately 0.5 mmol/l cells of P_i.

Garay and Garrahan [1] had shown that high intracellular $[P_i]$ (2–30 mmol/l cell water) inhibited Na efflux through the pump. They could not have seen the stimulation by P_i since they did not study the effects of concentrations lower than 2 mmol/l.

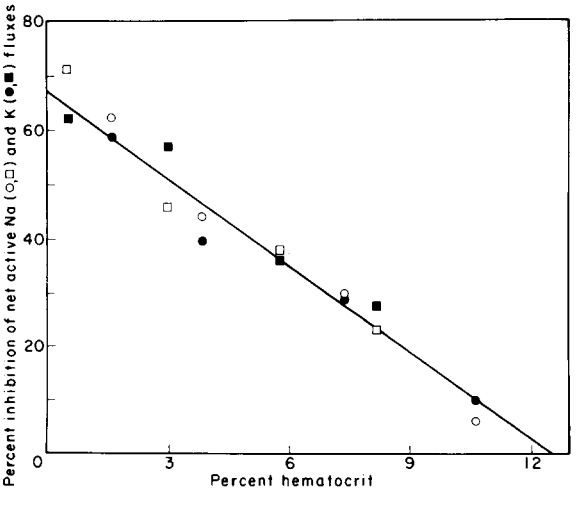

Fig. 2. The effect of hematocrit on net active Na and K fluxes in intact red cells. Cells and conditions were essentially identical to those described in Table I, except that the fluxes were measured in cells at various hematocrit as indicated. The results of two separate experiments are shown. Square symbols, experiment 1; round symbols, experiment 2. Open symbols, Na efflux; solid symbols, K influx. The line was fitted by linear regression ($r = 0.973$). Similar results were obtained in three other experiments.

Relationship of hematocrit to inhibition and P_i concentration

As mentioned above there are two reports in which inhibition of the pump was not observed after preincubation with inosine [9,10]. In the experiments of Glynn et al. [9] inhibition of K/K exchange was observed, but not of Na/K exchange (see their Table IV). However, the medium contained 2.5 mM P_i, and cellular [P_i] was probably not low enough to inhibit Na/K exchange. Therefore, the issue is not why Na/K exchange was not inhibited, but rather why K/K exchange was. Apparently, and not unreasonably, the sensitivity of K/K exchange to [P_i] is greater than that of Na/K exchange.

During the course of experiments undertaken to explain the discrepancy between the present results and our earlier ones [10], it became apparent that the hematocrit of the suspension of cells during measurement of the fluxes influenced active Na and K movements in cells with low [P_i]. Fig. 2 shows the results of two experiments in which percent inhibition of active net fluxes of Na and K was measured following preincubation with inosine. As the hematocrit was increased the inhibition was reduced. The relationship between percent inhibition and hematocrit seemed to be linear, with no inhibition at 12% hematocrit. The active fluxes in glucose cells were not influenced by varying the hematocrit (results not shown).

The influence of hematocrit on the inhibition of transport by inosine may explain in part the difference between our present and earlier results. Dunham and Senyk [10] didn't specify with care the hematocrits of their inosine-treated cells during measurement of fluxes (there was no apparent reason to worry about hematocrit). Apparently the hematocrits were 7–10%, for which we predict an inhibition of about 20% (Fig. 2). While a complete explanation cannot be given for the differences in the results and the apparent lack of inhibition of transport in inosine-treated cells in the earlier study, it is certainly clear from Fig. 2 that there is substantial inhibition of both Na and K transport in inosine-treated cells at low hematocrits. Furthermore, Table II indicates that this inhibition is relieved by the addition of phosphate.

A likely explanation for the difference in the inhibition of active transport caused by inosine in cells at high and low hematocrits is that the concentration of intracellular P_i is lower in cells at low hematocrit as a result of equilibration with a larger extracellular volume. To test this, the intracellular P_i concentrations of cells at high and low hematocrits were determined after the cells has been incubated in flux media with glucose or inosine for 1 h. In five experiments the inosine-treated cells at low hematocrit had a lower concentration of P_i. For example, in one experiment the cells in inosine at a hematocrit of 1.7% had an intracellular [P_i] of 0.22 mmol/l cells, while the cells at a hematocrit of 9.3% had a P_i concentration of 0.34 mmol/l. The [P_i] of the cells in glucose was considerably higher than in inosine, and was the same at high and low hematocrits: at low hematocrit (1.7%), [P_i] = 0.98 mmol/l cells; high hematocrit (9.3%), [P_i] = 0.99 mmol/l. While active unidirectional efflux of Na was nearly the same in the glucose-treated cells at high and low hematocrit (high, 5.57 mmol/l cells per h; low, 5.68 mmol/l cells per h), active Na efflux was inhibited in inosine-cells to a greater extent in cells at low hematocrit as compared to cells at high hematocrit (low hematocrit, 1.13 mmol/l cells per h; high hematocrit, 4.07 mmol/l cells per h). As shown in Fig. 1, the small difference in P_i concentration of the inosine-treated cells at high and low hematocrit can account for the difference in active transport.

Stimulation of transport by P_i in inside-out vesicles

As stated above we have shown that P_i stimulated Na transport in inside-out vesicles made from red cell membranes [8]. Inside-out vesicles are an advantageous system for distinguishing between the stimulatory and inhibitory effects of cytoplasmic P_i. The concentrations of ligands at the cytoplasmic membrane surface can be controlled with certainty, and without the modifications that occur in intact cells and resealed ghosts due to the action of cytoplasmic and membrane-bound enzymes. The explanation for the stimulation of the pump in the vesicles is the synthesis of ATP by membrane-bound phosphoglycerate kinase. Stimulation was observed only when all of the substrates for glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase (glyceraldehyde 3-phosphate, P_i, NAD and ADP) were present, and was abolished by elution of glyceraldehyde-3-phosphate dehydrogenase (band 6) from the vesicles. (With glyceraldehyde 3-phosphate, NAD and

TABLE III

INFLUENCE OF THE SUBSTRATES FOR GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AND PHOSPHOGLYCERATE KINASE ON Na INFLUX INTO INSIDE-OUT VESICLES

The contents of the vesicles and the measurement of Na fluxes were the same as described for Fig. 3. The flux medium contained, in addition to the constituents listed there, the substrates ADP (1 mM) glyceraldehyde 3-phosphate (2 mM), and NAD (4 mM). The control preparation had no further constituents. To the other preparations were added P_i (1 mM) or ATP (1 mM) as indicated. (Unlike the experiment in Fig. 1, there was no ATP in the preparations designated 'control' and 'P_i'.) The stimulation by these additions is shown: Δ. Similar results were obtained in ten other experiments. Values are means ± S.E. or standard errors of differences (for stimulation) (n = 3).

	Na influx (nmol Na/mg protein per min)		
	Control	+P_i	+ATP
Δ (stimulation)	9.5 ± 0.4	12.9 ± 0.5	14.6 ± 0.3
	–	3.4 ± 0.6	5.1 ± 0.5

P_i as substrates, glyceraldehyde-3-phosphate dehydrogenase supplies 1,3-diphosphoglycerate to phosphoglycerate kinase for synthesis of ATP from ADP.) Table III illustrates this stimulation, and compares it with stimulation of Na transport by ATP added to the extravesicular medium.

In intact cells with high intracellular ATP, we have shown here that P_i markedly stimulated the (Na, K)-pump. Apparently in intact cells ATP associated with the membrane is more important to transport than is ATP free in the cytoplasm. However, in the inside-out vesicles P_i, in the presence of the other substrates for glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase, did not stimulate transport as much as 0.5 mM ATP alone. During the preparation of the vesicles, glycolytic enzymes may be eluted from the cytoplasmic membrane surface, accounting for the reduced dependence on P_i of the pumps in vesicles as compared with intact cells.

Inhibition of transport by P_i in inside-out vesicles

The biphasic curve in Fig. 1 shows that there are two modes of action of P_i on the pump. The stimulatory effect of P_i (up to 0.5 mmol/l) can be explained as an indirect effect due to synthesis of ATP by the glycolytic enzymes (Ref. 8; Table III). The inhibition at higher P_i, seen in Fig. 1 and in the results of Garay and Garrahan [1], may be a direct effect on the pump. A similar type ot inhibition by P_i has been seen in studies on $(Na^+ + K^+)$-ATPase [2]. From these considerations one can predict a monophasic inhibitory effect of P_i on Na transport in inside-out vesicles in the absence of the other substrates for glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase. Fig. 3 shows an experiment designed to test this prediction. Inside-out vesicles were suspended in media with ATP (1 mM) and P_i concentrations of up to 4.0 mM. As predicted, P_i inhibited transport with no indication of stimulation at low concentrations, concentrations which stimulated transport in intact cells (Fig. 1) and in inside-out vesicles with all of the other appropriate substrates (Table III).

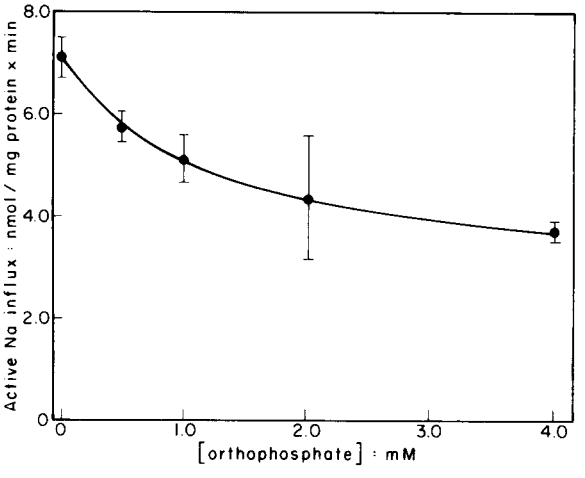

Fig. 3. The effect of varying orthophosphate (P_i) concentration on Na influx in inside-out vesicles. Vesicles had been loaded in a solution containing 5 mM KCl/1.0 mM $MgCl_2$/ 20 mM choline chloride/2.5 mM Tris-glycylglycine, pH 7.4. To measure Na influx, the vesicles were suspended in a medium containing 20 mM NaCl, 5 mM KCl, 1.0 mM $MgCl_2$, 2.5 mM Tris-glycylglycine, 1 mM ATP, 1 μM valinomycin, 1 μCi ^{22}Na/ml, and P_i as indicated. After 15 min 0.2 ml aliquots of the suspension were removed and placed directly onto a 1.2 μm Millipore, type RA 25 mm, filter. The filters were washed with 15 ml of ice-cold non-radioactive medium and the intravesicular ^{22}Na associated with the filter measured with an autogamma counter. Each point represents the mean of three determinations ± S.E.

There are discrepancies in the literature on sensitivity of the (Na, K)-pump to inhibition by P_i, discrepancies which should be pointed out though they are difficult to resolve. Garrahan and Glynn [19] observed inhibition of Na/K exchange at a high P_i concentration (approx. 30 mmol/l) in resealed ghosts; kinetic studies were not carried out. However, they saw no inhibition of $(Na^+ + K^+)$-ATPase activity in broken red cell membranes at 4.5 mM P_i (ATP was 0.5 mM). The inhibition of transport observed by Garay and Garrahan [1] also required high P_i concentrations (half-maximal inhibition at 17 mmol/l). Hexum et al. [2] observed inhibition of $(Na^+ + K^+)$-ATPase from rat brain, but again high concentrations of P_i were required (K_i, 23 mM). In the present study we observed inhibition of Na transport in both intact cells and in inside-out vesicles at rather lower concentrations of P_i. If one assumes that complete inhibition could be achieved (though this is certainly not obviously so) at high concentrations, then $[P_i]$ at half maximal inhibition would be about 3.5 mM for the vesicles and 1.8 mmol/l cells (approx. 2.6 mM) in intact cells. Apparently the sensitivites in the vesicles and the cells are about the same.

It is not easy to account for the higher sensitivity to inhibition in our hands than in the earlier studies [1,2]. A number of conditions differed in the various studies, but, at the same time, the conditions of our vesicles and intact cells also differed. Thus, the treatment with *p*-chloromercuribenzene sulfonate cannot be responsible for the sensitivity in intact cells since the vesicles were not exposed to it; likewise, the low ionic strength of the medium for the vesicles cannot be responsible since it was normal in the cells. Apparently the discrepancy cannot be resolved at the moment.

It is worth considering if the inhibitory effect of P_i is due to a reduction in Mg activity, but several considerations make it unlikely. In the experiment on inside-out vesicles shown in Table III, 0.5 mM P_i, in the presence of the other appropriate substrates, stimulated transport. In Fig. 3 the same concentration of P_i, but without the other substrates, was inhibitory. Therefore the inhibition is unlikely to be due to reduced Mg activity. (This same type of effect was shown in our other study on vesicles (Mercer and Dunham [8]).) Furthermore, P_i is unlikely to affect Mg activity in the presence of nucleotides present at similar concentrations: the binding constant of ATP for Mg is nearly three orders of magnitude higher than that of P_i for Mg, and for ADP nearly two orders of magnitude greater [20]. This consideration is particularly pertinent in reference to Fig. 3, where there was 1 mM ATP. (The measurements in Ref. 20 were made under somewhat different conditions than those of our experiments, but are probably relevant nevertheless.) Finally, in a preliminary experiment we tested the effect of high external [Mg]. $MgCl_2$ at 10 mM in addition to glucose (5 mM) or inosine (5 mM) had little effect on the inhibition by inosine of the active efflux of Na. Ouabain-inhibitable Na efflux was inhibited 85% in cells with inosine + Mg and 81% with inosine alone. This experiment is suggestive, but not conclusive since cellular [Mg] was not measured.

Effect of inosine on abnormal modes of transport

Table IV shows the results of experiments on the effects of inosine-preincubation on Na efflux through the (Na, K)-pump operating in three different modes: normal Na/K exchange, uncoupled Na efflux, and Na/Na exchange. Na/K exchange was promoted (to the exclusion of the other two modes) in Na-free medium containing K. Uncoupled Na efflux, which is inhibited by both external Na and external K, was obtained in medium free of both Na and K. Na/Na exchange was obtained in K-free medium containing Na (see Glynn and Karlish [21] for a review of the modes of operation of the pump). Table IV confirms the inhibition of Na/K exchange by preincubation with inosine. In contrast, there was little inhibition of uncoupled Na efflux and none of Na/Na exchange. The probable explanation for these differences in sensitivity to inosine treatment, and presumably therefore to reduction in P_i concentration, lies with the different interactions of the pump with ATP when operating in the different modes. ATP is required for Na/Na exchange [22], but there is no net hydrolysis [23]. The affinity of the pump for ATP when catalyzing uncoupled Na efflux is much higher than when catalyzing Na/K exchange [24], apparently because the pump does not assume a conformation during uncoupled Na efflux which has a low affinity for ATP, a conformation which does exist during Na/K exchange. Therefore, a lower rate of ATP synthesis can support Na/Na exchange and uncoupled Na efflux than Na/K exchange, and this

TABLE IV

THE EFFECT OF INOSINE INCUBATION ON Na/K EXCHANGE, UNCOUPLED Na EFFLUX, AND Na/Na EXCHANGE, MEASURED AS UNIDIRECTIONAL Na EFFLUX.

Cells and preincubations were identical to those described in Table I. After preincubation with inosine or glucose cells were suspended in one of three kinds of media: Na-free medium with 3 mM K (to measure the normal Na/K exchange); medium free of both Na and K (to measure uncoupled Na efflux); K-free medium with 50 mM Na(to measure Na/Na exchange). Isotonicity was maintained with choline. Shown are the results of two separate experiments, each carried out in quadruplicate, and the means of the two. Total Na efflux, flux with ouabain, and ouabain-inhibitable flux are indicated.

Treatment	Na efflux (mmol/l cells per h)								
Mode of transport:	Na/K exchange			Uncoupled Na flux			Na/Na exchange		
Expt.:	1	2	Mean	1	2	Mean	1	2	Mean
Glucose preincubation	8.80	8.13	8.46	2.24	2.03	2.14	1.66	2.03	1.84
+ Ouabain	1.24	1.42	1.33	1.48	1.45	1.46	1.09	1.32	1.20
Ouabain-inhibitable	7.56	6.71	7.13	0.76	0.58	0.68	0.57	0.71	0.64
Inosine preincubation	4.66	3.14	3.90	2.03	1.57	1.80	1.48	2.13	1.80
+ Ouabain	1.15	1.08	1.12	1.33	1.08	1.20	0.97	1.29	1.13
Ouabain inhibitable	3.51	2.06	2.78	0.70	0.49	0.60	0.51	0.84	0.67
Ratio of fluxes: inosine/glucose	0.46	0.3	0.39	0.92	0.85	0.88	0.90	1.18	1.05

difference in dependence on ATP is reflected in the dependence on P_i as a substrate for ATP synthesis.

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References

1 Garay, R.P., and Garrahan, P.J. (1975) J. Physiol. 249, 51–67
2 Hexum, T., Samson, F.E. and Himes, R.H. (1970) Biochim. Biophys. Acta 212, 322–331
3 Okonkwo, P.O., Longenecker, G. and Askari, A. (1975) J. Pharmacol. Exp. Ther. 194, 244–254
4 Parker, J.C. and Hoffman, J.F. (1967) J. Gen. Physiol. 50, 893–916
5 Proverbio, F. and Hoffman, J.F. (1977) J. Gen. Physiol. 69, 605–632
6 Schrier, S.L. (1966) Am. J. Physiol. 210, 139–145
7 Segel, G.B., Feig, S.A., Glader, B.E., Muller, A., Dutcher, P. and Nathan, D.G. (1975) Blood 46, 271–278
8 Mercer, R.W. and Dunham, P.B. (1981) J. Gen. Physiol., 78, in the press
9 Glynn, I.M., Lew, V.L. and Lüthi, U. (1970) J. Physiol. 207, 371–391
10 Dunham, P.B. and Senyk, O. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 3 099–3 103
11 Sachs, J.R. (1974) J. Gen. Physiol. 63, 123–143
12 Sachs, J.R., Ellory, J.C., Kropp, D.L., Dunham, P.B. and Hoffman, J.F. (1974) J. Gen. Physiol. 63, 389–414
13 Dunham, P.B. and Hoffman, J.F. (1971) J. Gen. Physiol. 58, 94–116
14 Rodland, K.D. and Dunham, P.B. (1980) Biochim. Biophys. Acta 602, 376–388
15 Bergmeyer, H.U. (1963) Methods of Enzymatic Analysis, Academic Press, Inc., New York
16 Taussky, H.H. and Shorr, E. (1953) J. Biol. Chem. 202, 675–685
17 Steck, T.L. (1974) in Methods in Membrane Research. (Korn, E.D., ed.), Vol. 2. pp. 245–281. Plenum Publ. Corp., New York
18 Krenitsky, T.A. (1967) Mol. Pharm. 3, 526–536
19 Garrahan, P.J. and Glynn, I.M. (1967) J. Physiol. 192, 189–216
20 Watanabe, S., Trosper, T., Lynn, M. and Evenson, L. (1963) J. Biochem. 54, 17–24
21 Glynn, I.M. and Karlish, S.J.D. (1975) Annu. Rev. Physiol. 37, 13–55
22 Glynn, I.M. and Hoffman, J.F. (1971) J. Phyiol. 218, 239–256
23 Garrahan, P.J. and Glynn, I.M. (1967) J. Physiol. 192, 217–235
24 Glynn, I.M. and Karlish, S.J.D. (1976) J. Physiol. 256, 465–496