THE INTERACTION OF POTASSIUM IONS AND ATP ON THE SODIUM PUMP OF RESEALED RED CELL GHOSTS

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SUMMARY

1. Ouabain-sensitive K or Rb influx was measured into ghosts resealed to contain ATP concentrations of 1 μM-3 mM and no K.

2. Increasing ATP from 1 to 100 μM, at saturating external K, increased K influx about twentyfold while having no effect on the ratio of ouabain-sensitive K influx to ouabain-sensitive ATPase activity.

3. Increasing external K decreased the apparent affinity for ATP. Similarly increasing ATP decreased the apparent affinity for external K.

4. The K influx can be empirically described as: influx $= V_{max} K^2/(K + K_{app})^2$. Increasing ATP increased V_{max} and $(K_{app})^2$ by the same amount.

5. These results are consistent with a consecutive model for the Na pump in which an ATP-dependent reaction follows a K-activated dephosphorylation.

INTRODUCTION

Current models of the sodium pump suggest that the efflux of Na ions from the cell is accompanied by the formation of a phosphoenzyme (EP) which is then hydrolysed by a reaction accelerated by extracellular K ions. Post, Hegyvary & Kume (1972) suggested that the K ions which promote dephosphorylation become occluded within the enzyme as a form which they called E_2K. Release of occluded K to the intracellular surface requires a conformational change (Karlish, Yates & Glynn, 1978) which is accelerated by ATP at a low affinity, non-phosphorylating site. This occlusion of K ions and its reversal by ATP has been shown directly (Beaugé & Glynn, 1979).

$$K_o + EP \rightarrow E_2K \rightarrow E + K_i.$$

In such a scheme where an ATP-dependent reaction follows the K-dependent dephosphorylation, the apparent affinity of the over-all reaction for K should depend on the ATP concentration (Glynn & Lew, 1969). This can be seen intuitively as follows. If ATP is low the conformational change will be slow and very little K will be required to make dephosphorylation much faster than this conformational change, giving an apparent high affinity. As ATP is increased greater rates of dephosphory-

lation are required to saturate the reaction with respect to K. Similarly the apparent affinity for ATP should depend on the external K concentration.

Experimental tests of this prediction have, however, given mixed results. Robinson (1967) working with brain Na^+–K^+ ATPase found that increasing K lowered the ATP affinity, although in this study it was impossible to tell on which side of the membrane K acted to lower the ATP affinity. This result has been confirmed by Hexum, Samson & Himes (1970) and Skou (1974). On the other hand, Peter & Wolf (1972) reported that increasing K raised the ATP affinity with no effect on the rate at saturating ATP concentrations. Work on the intact red cell has, however, been negative. Glynn (1956) and Beaugé & Del Campillo (1976) found that moderate reductions of ATP by starvation had no effect on the apparent affinity for external K. Garay & Garrahan (1975) similarly found no effect of ATP on the K affinity and therefore suggested that the above model was an inadequate description of the Na–K pump. To explain their results Garay & Garrahan postulated that ATP must bind at a low affinity site throughout the pump cycle.

Recent work in the squid axon, however, has shown that lowering ATP increases the affinity for external K as an activator of the Na efflux (Beaugé & DiPolo, 1979, 1981).

In the light of this disagreement the effects of ATP on external K affinity are worth investigating. In order to be able to control ATP over a wide range, the present experiments were performed on erythrocyte ghosts. Most experiments designed to measure Na pump rate in ghosts have measured either ATPase activity or Na efflux. Since the uncoupled Na efflux contributes a large fraction of the Na efflux and ATPase activity at low ATP concentrations, these methods are unsatisfactory for determining the external K affinity. We have therefore measured the K influx. As very few influx studies have been reported in ghosts we first demonstrate the feasibility of measuring the K influx and then report work on the effects of ATP. The experiments show that ATP has marked effects on the affinity for external K in the expected direction.

A preliminary communication of part of this work has already appeared (Eisner & Richards, 1980).

METHODS

The experiments required measuring K inffflux from solutions with very low K concentrations. It was therefore important to be sure that K ions escaping from the ghosts did not affect the specific activity of external K. This was done by replacing intracellular K with Na using the nystatin technique (Cass & Dalmark, 1973). Most of the rest of the steps involved in preparing the ghosts relate to controlling ATP at a constant level by the regenerating system method (Glynn & Karlish, 1976). The steps are dealt with in turn.

1. Starvation and depletion of cells. Blood was obtained from the blood bank and washed three times with depletion solution (120 mM-choline Cl; 10 mM-NaCl; 10 mM-KCl; 10 mM-Tris Cl (pH 7·5 at 20 °C); 1 mM-$MgCl_2$; 0·1 mM-(Tris) EGTA. The cells were then incubated in depletion solution at 37 °C for 24–36 hr at about 10 % haematocrit in the presence of chloramphenicol (25 μg/ml.). Following this incubation, to reduce ATP further, the cells were incubated in depletion solution at 37 °C for 3 hr in the presence of 5 mM-inosine and 5 mM-iodoacetamide. This procedure has been shown to reduce ATP to less than 1 μM (Lew, 1971; Glynn & Karlish, 1976).

2. Removal of intracellular K with nystatin. The cells were then washed three times with nystatin loading solution (155 mM-NaCl; 10 mM-(Tris) Hepes; 1 mM-$MgCl_2$; 0·1 mM-(Tris) EGTA; 29 mM-sucrose). Nystatin was made up as a solution of 2·5 mg/ml. in methanol. This was then added to a suspension of cells (4 % haematocrit) to give a final nystatin concentration of 50 μg/ml. The cells

were left for 1 hr in the dark at room temperature. Following this they were washed six times with nystatin loading solution. They were then washed three times with choline wash medium (155 mM-choline Cl; 5 mM-(Tris) Hepes (pH 7·5, 20 °C); 1 mM-MgCl$_2$; 0·1 mM-(Tris) EGTA). The treated cells were then kept overnight. The K content of the cells was reduced to less than 30 μM, so the K in the ghosts was less than 1 μM.

3. *Lysis.* The lysing solutions generally contained (for influx experiments): 2·5–10 mM-creatine phosphate (di Na salt); 30 μM-creatine; 5 units creatine kinase/ml.; 1·5 mM-MgCl$_2$; 5 mM-(Tris) Hepes (pH 7·5 at 20 °C); 0·2 mM-(Tris) EGTA; 20 mM-(choline Cl + NaCl). In experiments in which ATPase was also measured, the lysing solution also contained 5 mM-inosine and 5 units hexokinase/ml. ATP was also added to the desired concentration with [γ^{32}P]ATP added as required. One ml. of cells was squirted into the 30 ml. of lysing solution at 0 °C. After 5 min sufficient 2·5 M-choline Cl was added to restore tonicity. After another 3 min the ghost suspension was warmed to 37 °C to allow resealing to occur for 15 min. The ghosts were then washed three times at 0 °C by centrifugation (the first spin for 5 min at 18,000 g and the others for 2 min at 15,000 g). With ghosts containing radioactive ATP, four washes (the first at 37 °C to allow the inosine to escape) were performed. The ghosts were then resuspended (generally at haematocrits of < 1 %) at 0 °C in media containing appropriate salts with or without ouabain (10^{-3} M) and tracer Rb or K where required.
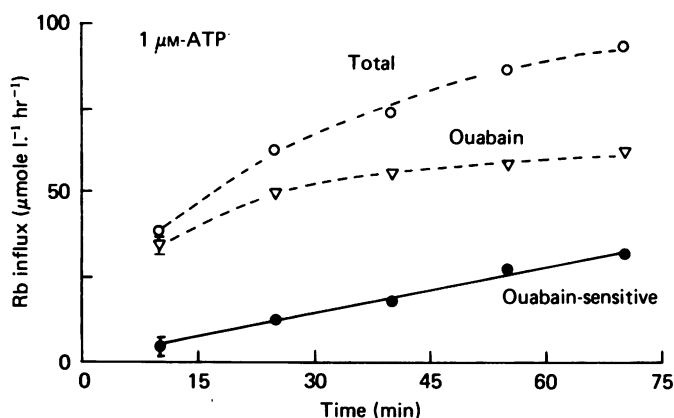

Fig. 1. Linearity of ouabain-sensitive Rb influx into ghosts resealed to contain 1 μM-ATP. The uptake of Rb is shown as a function of time after warming the ghosts from 0 to 37 °C. The incubating medium consisted of choline wash solution (see Methods) containing 150 μM-^{86}RbCl and 5 mM-NaCl. The symbols show: ○, total influx; ▽, influx in the presence of 10^{-3} M-ouabain; ●, ouabain-sensitive influx. All points are the means of three determinations. Error bars represent S.E. of means and have been omitted when they would be smaller than the symbol.

Detailed procedure. The cells were starved and then depleted of nucleotides with inosine and iodoacetamide as described in the Methods. Since the external Rb concentration was comparatively high in this experiment, the nystatin treatment was omitted. The cells were lysed 1 volume in 30 of a solution containing 2·5 mM-creatine phosphate (disodium salt); 5 units creatine kinase/ml.; 1·5 mM-MgCl$_2$; 5 mM-Tris Hepes; 0·2 mM-EGTA; 20 mM-choline chloride. After washing the resealed ghosts were resuspended at a final haematocrit of 0·8 % in ice-cold choline wash solution containing 5 mM-NaCl and 150 μM-^{86}RbCl with or without ouabain (10^{-3} M). 1 ml. aliquots were added to Eppendorf tubes which were then transferred to 37 °C. At the indicated times sample tubes were cooled and processed as described in the Methods.

The measurement of K influx

The incubation period was started by warming tubes containing 1 ml. ghost suspension with appropriate isotopes to 37 °C. Following the end of the incubation period (generally 1 hr) the tubes

were cooled to 0 °C. The ghosts were then washed three times with ice-cold choline wash medium. After washing, the pellet was lysed with 2 ml. distilled water. The radioactivity of the lysate was then measured. Samples of the suspension were then used as standards.

The experiment illustrated in Fig. 1 was designed to investigate whether one can measure a linear ouabain-sensitive influx into ghosts. Rb influx from a solution of 150 μM-Rb was measured into ghosts containing 1 μM-ATP. The open circles show the total Rb uptake as a function of time after warming to 37 °C. The *total* influx was non-linear with time. There is an initial component of uptake which levels off to a slower influx. The triangles show the influx in the presence of 10^{-3} M-ouabain. Again, a large initial component is apparent. This initial uptake is reminiscent of the initial, rapid component of efflux produced by warming ghosts up to 37 °C (Hoffman, 1962) and may represent a population of ghosts which are leaky at 37 °C. They must, however, be tight to Rb at 0 °C since their contents are not lost in the washes. The ouabain-sensitive component may be obtained by subtraction. The filled circles show that there is a significant ouabain sensitive component and that this is linear with time. Thus, although a leaky population of ghosts is present, an ouabain-sensitive flux can still be measured. Under the conditions shown the ouabain-sensitive flux is about one third of the total Rb uptake.

Measurements of ATP concentration in ghosts. ATP was estimated by a firefly method. The technique was similar to that of Glynn & Hoffman (1971) with the exception that trichloroacetic acid (TCA) was used rather than perchloric acid. TCA was then removed by extraction into ether. All ATP concentrations quoted in the paper are those in the lysing solution. Measurements of ATP suggest that the actual concentration of ATP in the ghosts did not differ by more than 10 % from this value.

ATPase experiments. The method used was similar to that of Glynn & Karlish (1976) and details are given in Table 1.

Isotopes. Most influx experiments were performed with ^{86}Rb which was counted by measuring the Cerenkov radiation. This method was, however, unsuitable for experiments in which ATPase and influx were measured simultaneously since ^{32}P and ^{86}Rb are similar β emitters. For those experiments ^{42}K was generally used and measured with a γ counter. ^{42}K was unsuitable for experiments at low K concentrations, since our supply had a low specific activity. We therefore used ^{43}K which can be obtained at much higher specific activity. Our ^{43}K samples contained about 5 % ^{42}K. However, the counter discriminator could be set to exclude most of the ^{42}K counts.

Calculation of results. In order to express the results with respect to the volume of ghosts, we have measured the Na concentration in both the resealing solution and in the final suspension (before adding Na). This allowed us to calculate the original volume of the ghosts resealed to Na. All points represent the mean of triplicates and are given as $\pm$S.E. of mean.

Sources of materials. Blood was obtained from the Regional Transfusion Centre, Cambridge and was a few days old. [γ^{32}P]ATP, ^{42}K and ^{86}Rb were from the Radiochemical Centre, Amersham. ^{43}K was from the M.R.C. cyclotron, Hammersmith. Creatine kinase, hexokinase, creatine phosphate, Tris, ouabain and inosine were from Sigma. NaCl and KCl were Specpure grade (Johnson Matthey Ltd., London). Creatine (A grade) was from Calbiochem. Choline chloride was recrystallized from hot absolute ethanol.

<div align="center">RESULTS</div>

One of the aims of this work was to use the K influx through the Na pump as a measure of pump activity. It was therefore important to check that this influx is a satisfactory measure of the turnover of the pump. If, for example, the number of K ions transported per pump cycle depended on the ATP concentration, the K influx would not be an adequate measure of the turnover. We therefore measured the effects of changing ATP on both ATPase activity and K influx. Table 1 shows a summary of results from three such experiments. In each experiment ATPase and K influx were measured at 1 and 100 μM-ATP at an external K concentration of either 150 or 300 μM. This value of external K was chosen to be sufficiently high for all of the ouabain-sensitive ATPase to reflect Na/K exchange rather than uncoupled Na efflux. It is obvious that increasing ATP increases both the ATPase and K influx. The ratio

of K influx to ATPase is also given. At both low and high ATP the value for this ratio is generally less than the expected value of 2·0. However, as well as the errors indicated, problems of systematic errors due to different numbers of ghosts being sealed to ATP compared to K must also be allowed for. It is not therefore certain that the difference from 2·0 is significant. This problem should not, however, interfere with comparing the stoichiometry in 1 and 100 μM-ATP. Such a comparison shows no significant difference in this ratio on increasing ATP from 1 to 100 μM. This

TABLE 1. The effects of ATP concentration on both ouabain-sensitive ATPase activity and K influx

	1 μM-ATP			100 μM-ATP		
	ATPase (m-mole.l.$^{-1}$ hr^{-1})	K influx (m-mole.l.$^{-1}$ hr^{-1}	$\dfrac{\text{K influx}}{\text{ATPase}}$	ATPase (m-mole.l.$^{-1}$ hr^{-1})	K influx (m-mole.l.$^{-1}$ hr^{-1})	$\dfrac{\text{K influx}}{\text{ATPase}}$
(i)	0·095 ± 0·005	0·134 ± 0·002	1·41 ± 0·05	0·514 ± 0·047	0·756 ± 0·003	1·47 ± 0·09
(ii)	0·077 ± 0·007	0·090 ± 0·002	1·17 ± 0·11	0·593 ± 0·015	0·720 ± 0·007	1·21 ± 0·03
(iii)	0·071 ± 0·019	0·142 ± 0·025	2·00 ± 0·64	1·382 ± 0·036	1·959 ± 0·024	1·42 ± 0·03

In each experiment the three left-hand columns refer to 1 μM-ATP and the three right hand ones to 100 μM-ATP. At each ATP concentration the columns denote (from left to right); ATPase activity; K influx; ratio of K influx/ATPase activity. Errors are s.e. of means.

Detailed procedure. After starvation and depletion with iodoacetamide and inosine the cells were lysed 1 volume in 30 in a solution of: 1 or 100 μM-[γ^{32}P]ATP (disodium salt) in isotopic equilibrium with 5 mM-[^{32}P]creatine phosphate, 30 μM-creatine, 5 units creatine kinase/ml., 5 units hexokinase/ml., 1·5 mM-MgCl$_2$, 5 mM-(Tris) Hepes (pH 7·5 at 20 °C), 0·2 mM-(Tris) EGTA, 20 mM-choline chloride, 5 mM-inosine. The subsequent washing of the ghosts was as described by Glynn & Karlish (1976). The ghosts were resuspended in ice-cold choline wash solution containing 5 mM-NaCl and either radioactive KCl (flux measurements) or cold KCl (ATPase measurements). 10^{-3} M-ouabain was added to half the suspension. One ml. aliquots were distributed to either Eppendorf tubes (flux experiments) or glass tubes (ATPase). The measurement of influx is described in the Methods and that of ATPase by Glynn & Karlish (1976). Both influx and ATPase were measured after 10 and 70 min and the difference gave the activity per hr. The ouabain-sensitive activity was obtained by subtracting the ouabain activity from the control. KCl was 150 μM in experiments (i) and (ii) and 300 μM in (iii). ^{43}K was used in experiments (i) and (ii) and ^{42}K in (iii).

suggests that the stoichiometry of K transport to ATPase is fixed over a range of ATP in which the V_{max} for K influx changes by a factor of about 20 (see Table 2). This result extends the work of Mullins & Brinley (1969) who showed that the ratio of Na–K transport in squid axons did not change significantly on varying ATP over the range 10 μM–10 mM. The present work shows that ATP concentration has no great effect on the amount of K ions pumped per ATP split.

The effects of external K on the ATP affinity of the Na pump

Fig. 2 shows an experiment designed to measure the ATP affinity of the pump. In this and the majority of subsequent experiments, Rb was used as a K cogener because of its convenient half-life. Ghosts were prepared to contain various ATP concentrations from 1 μM to 3 mM. Rb influx was measured from an external Rb concentration of either 10 or 200 μM. Fig. 2 shows that increasing ATP increases the Rb influx and, not surprisingly, the Rb influx is much greater at 200 μM than at 10 μM-Rb. It is, however, obvious that the apparent affinity for ATP is much higher at 10 than at 200 μM-Rb. This is emphasized in the inset which shows the two curves normalized to the same maximum value. Whereas the 200 μM curve is half-saturated

at about 100 μM-ATP, the influx at 10 μM-Rb is half-saturated by 10 μM-ATP. These results are therefore in agreement with those of Robinson (1967) on the isolated Na$^+$–K$^+$ ATPase in showing that the apparent affinity for ATP depends on the concentration of K. Furthermore, they show that at least part of the effect of K is at the external site.

The fact that external K affects the ATP affinity implies that ATP will affect the affinity for external K. Experiments designed to test the effects of ATP on the K
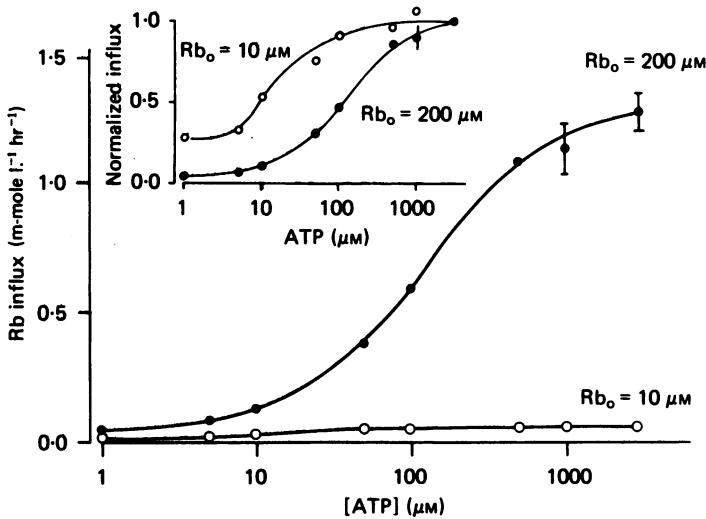
Fig. 2. Effects of external Rb concentration on the apparent affinity for ATP. Rb influx as a function of ATP concentration. Ouabain-sensitive Rb uptake is plotted as a function of ATP concentration from solutions containing either 200 μM (●) or 10 μM (○) RbCl (NaCl = 1 mM). The ouabain-insensitive Rb influx did not vary significantly with ATP concentration and amounted to 3 μmole l.$^{-1}$ hr^{-1} at 10 μM-Rb and 57 μmole l.$^{-1}$ hr^{-1} at 200 μM-Rb. The ATP concentrations given are those in the lysing solutions. Control experiments suggest that the actual intracellular ATP was, at most, about 10% different. The inset shows normalized Rb influx as a function of ATP concentration. The two curves have been normalized to the same level in 3 mM-ATP to facilitate comparison of the affinities.

Detailed procedure. The cells were starved and depleted and then treated with nystatin to remove intracellular K. 2·6 ml. of cells was then lysed in 80 ml. ice-cold solution containing 10 mM-creatine phosphate (di Na salt); 5 units creatine kinase/ml.; 1 mM-MgCl$_2$; 5 mM-(Tris) Hepes; 0·2 mM-EGTA; 5 mM-inosine. Aliquots of 8·6 ml. were then added to each of eight vials containing a small volume of: enough ATP to achieve the final desired concentrations; MgCl$_2$ such that the final free Mg^{2+} was 1 mM (after allowing for the chelating properties of ATP and creatine phosphate; see Dawson, Elliott, Elliott & Jones (1969) for values of the affinity constants); and sufficient choline chloride to restore tonicity. After washing, the ghosts were resuspended in a choline wash solution containing 1 mM-NaCl, 10 or 200 μM-^{86}RbCl with or without ouabain (10^{-3} M). Rb uptake was measured as described in the Methods. The final resealed haematocrit in the incubating media was 0·6%.

affinity have certain advantages over the sort of experiment shown in Fig. 2. It is impossible to control reliably ATP concentration at levels below 1 μM and at low external K a large component of influx is present even at 1 μM-ATP (Fig. 2). It is therefore difficult to get an accurate measure of the ATP affinity. No such problem exists with respect to external K which can be varied over any range. Therefore in

the rest of this paper we will investigate the interactions of ATP and K by studying the effects of ATP on the external K affinity.

The dependence of K influx on external K

In the experiment of Fig. 3 we examined the K influx into ghosts as a function of the external K concentration at two different ATP concentrations: 1 and 100 μM. At 100 μM-ATP the influx is still increasing with external K at the highest K
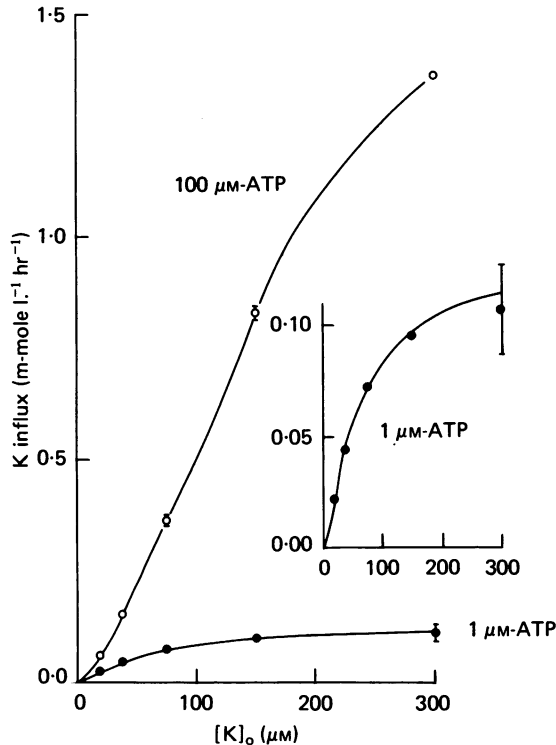

Fig. 3. The dependence on external K of the ouabain-sensitive K influx into ghosts containing 1 or 100 μM-ATP. The main Figure shows a comparison of K uptake at 1 μM-ATP (●) and 100 μM-ATP (○). An enlargement of K uptake at 1 μM-ATP is given in the inset. The incubating media consisted of choline wash solution containing 5 mM-NaCl and a variable concentration of ^{43}KCl with or without ouabain (10^{-3} M).

  Detailed procedure. After starvation, depletion and nystatin treatment the cells were lysed in the solution described in the Methods with the difference that creatine phosphate was 2·5 mM at 1 μM-ATP and 5 mM at 100 μM-ATP. The difference in Na concentration was compensated for by adding NaCl. After washing, the ghosts were resuspended in a choline wash solution containing 5 mM-NaCl and a variable concentration of ^{43}KCl.

concentration examined (300 μM). This is in agreement with previous work at high ATP where the K influx (in an external Na concentration of 5 mM) was half-saturated at about 200 μM (Garrahan & Glynn, 1967b). However, at 1 μM-ATP, the K influx is much less than at 100 μM. It is also obvious that the affinity for external K is much higher at 1 μM- than at 100 μM-ATP. This is emphasized in the inset which shows that the influx is more or less saturated by 300 μM-K. Fig. 4 shows the same data in the form of a Lineweaver–Burk graph. It is well known that, in the presence of

external Na, the K influx curve is sigmoid (Sachs & Welt, 1967); K influx seems to require the occupation of two sites for external K. Such sigmoid behaviour is obvious for the 100 μM curve in Fig. 3. We have therefore plotted $v^{-\frac{1}{2}}$, rather than v^{-1}, as a function of K_0^{-1} in Fig. 4. This plot is justified in the Discussion. The sigmoid behaviour is less obvious for the lower ATP concentration. However, this is probably
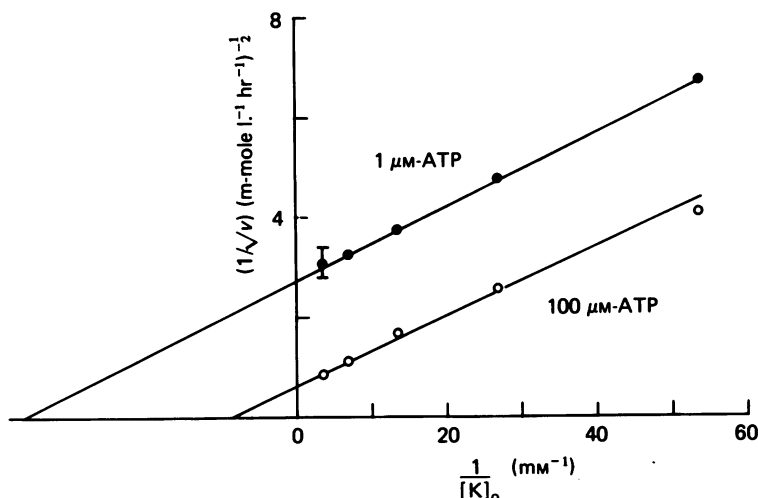

Fig. 4. Double reciprocal plots of K activation curve. Data from Fig. 3. Plot of (K influx)$^{-\frac{1}{2}}$ as a function of $[K]_o^{-1}$. The straight lines were obtained by a least squares, unweighted linear regression on an Eadie plot ($[K]_o v^{-\frac{1}{2}}$ as a function of $[K]_o$). The following parameters were obtained: 1 μM-ATP: $K_m = 24 \cdot 8\ \mu$M, $V_{max} = 0 \cdot 13$ m-mole l.$^{-1}$ hr^{-1}. 100 μM-ATP: $K_m = 113\ \mu$M; $V_{max} = 2 \cdot 53$ m-mole l.$^{-1}$ hr^{-1}. Symbols show: 1 μM-ATP ($\bullet$); 100 μM-ATP (O).

because the affinity is so much higher that none of the points are at low enough concentrations to show an inflexion. Even at 1 μM-ATP the influx curve is better fitted by assuming that two K ions rather than one must bind for transport to occur (not shown). The line at 1 μM-ATP is parallel to that at 100 μM-ATP. In other words, increasing ATP increases the apparent K_m by exactly the same fraction that it increases the $\sqrt{V_{max}}$.

In Fig. 5 data from a similar experiment (using Rb rather than K) are presented. The graph shows the value of the ratio (influx at 100 μM-ATP divided by influx at 1 μM-ATP) as a function of the external Rb concentration. It is clear that the value of this ratio, in other words the fractional increase of Rb influx produced by increasing ATP, depends markedly on the Rb concentration. ATP has very little effect on the Rb influx at low external Rb concentration but a large effect at higher Rb concentration. This result differs markedly from that of Garay & Garrahan (1975) who demonstrated that the fractional reduction of K influx produced by lowering ATP was independent of external K concentration.

A more complete range of ATP concentrations is examined in the experiment of Fig. 6. It is again clear that both the V_{max} and apparent K_m for external Rb increase with increasing ATP. Both K_m and $\sqrt{V_{max}}$ have the same dependence on ATP. The magnitude of the change of K_m is very large: it varies from about 14 μM at 1 μM-ATP
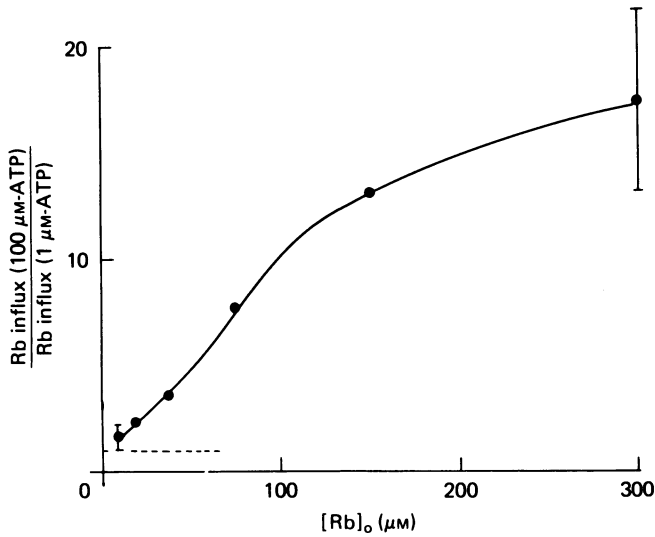
Fig. 5. The dependence of the increase of ouabain-sensitive Rb influx with increasing ATP on the external Rb concentration. Ghosts were prepared to contain either 1 or 100 μM-ATP. The symbols show the value of the ratio: (Rb influx in 100 μM-ATP)/(Rb influx in 1 μM-ATP) as a function of external Rb. The detailed procedure was similar to that of Fig. 3. The ghosts were suspended during the incubation in a choline wash medium containing 5 mM-Na and a variable concentration of [86]RbCl at a resealed haematocrit of 0·5%. The horizontal dashed line corresponds to a ratio of unity.
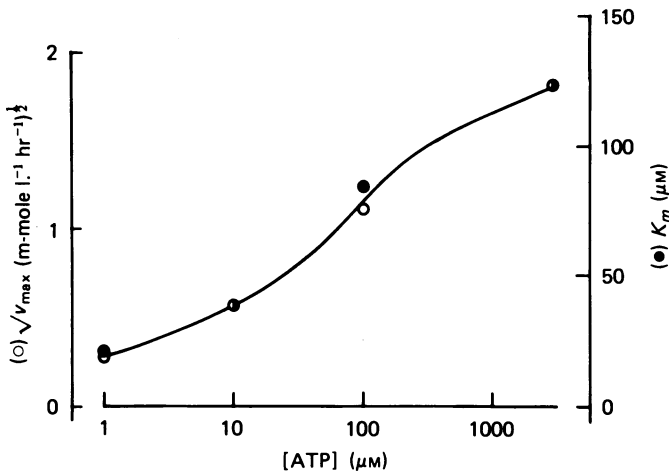

Fig. 6. The dependence on ATP of K_m and $\sqrt{V_{max}}$ for the ouabain-sensitive Rb uptake. The ghosts were prepared to contain various ATP concentrations. The detailed procedure was similar to that of Fig. 3. In particular the lysing solution contained 10 mM-creatine phosphate (di Na salt). The ghosts were finally incubated in a choline wash solution containing 5 mM-NaCl and various amounts of [86]RbCl. The final resealed haematocrit was 0·9% at 1 and 10 μM-ATP but was reduced to 0·4% at 100 μM and 3 mM-ATP so that the larger fluxes would not significantly deplete external Rb. The values of K_m and V_{max} were obtained from Eadie plots.

to about 100 μM at 3 mM-ATP. Data from more experiments are summarized in Table 2. This shows the values of K_m and V_{max} at both 1 and 100 μM-ATP. On average, increasing ATP from 1 to 100 μM increases both V_{max} and $K_m{}^2$ about twentyfold whereas the ratio $\sqrt{V_{max}}/K_m$ does not change significantly.

TABLE 2. Mean values for K_m and V_{max} of Rb influx at 1 and 100 μM-ATP. Values of K_m and V_{max} were obtained as described for Fig. 6. The number of experiments is indicated in column 2

ATP (μM)	n	K_m (μM)	V_{max} (μmole l.$^{-1}$ hr^{-1})	$\sqrt{V_{max}}/K_m$ (hr μM)$^{-\frac{1}{2}}$
1	6	$15 \cdot 2 \pm 1 \cdot 3$	66 ± 8	$0 \cdot 534 \pm 0 \cdot 056$
100	3	$60 \cdot 0 \pm 5 \cdot 0$	1210 ± 230	$0 \cdot 577 \pm 0 \cdot 074$

The K affinity at low ATP in the absence of external Na

All the experiments on the affinity of the Na pump for external K were performed in an external Na concentration of 5 mM. This was so that Na leaking out of the ghosts
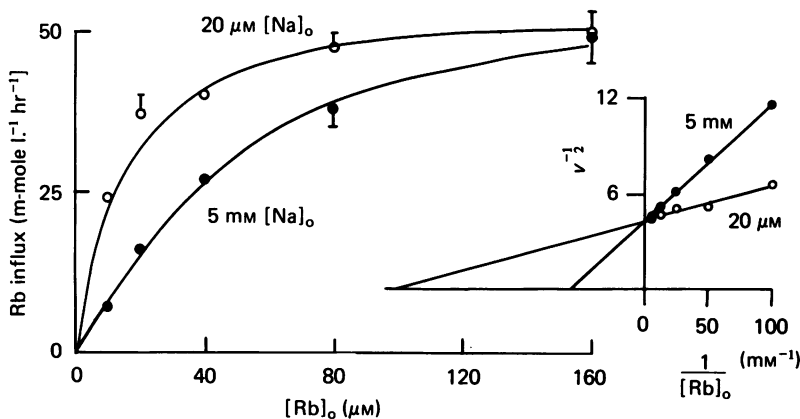

Fig. 7. The effects of external Na on the affinity for external Rb of the ouabain-sensitive Rb uptake at 1 μM-ATP. The main Figure shows the dependence of ouabain-sensitive Rb influx on external Rb at either 5 mM ($\bullet$) or 20 μM ($\bigcirc$) external NaCl. The inset is a double reciprocal plot of (Rb influx)$^{-1}$ as a function of [Rb]$_o{}^{-1}$. The detailed procedure was similar to that of Fig. 3 except that the ghosts were finally resuspended in choline wash solutions containing either 5 mM or 20 μM-NaCl and a variable concentration of ^{86}RbCl. Final resealed haemotocrit 0·14 %.

would change external Na by a negligible amount. It further decreases the magnitude of the uncoupled Na efflux (Garrahan & Glynn, 1967a; Lew, Hardy & Ellory, 1973; Glynn & Karlish, 1976). External Na, however, also lowers the affinity for external K (Garrahan & Glynn, 1967b) and it is therefore possible that ATP exerts its effect on the K affinity by affecting the inhibition by external Na. This possibility is, however, excluded by the experiment of Fig. 7. In this experiment external Na was either 20 μM or 5 mM. Twenty μM was chosen to be close to zero whilst still reducing the effects of Na escaping. To further reduce problems of variations in external Na due to Na escape, the experiment was performed at a very low haematocrit (0·14 %). It should be noted that 20 μM-Na produces a negligible effect on the K influx, even at low external K (Cavieres & Ellory, 1975). At this Na

concentration the Rb influx is half-saturated at an external Rb concentration of 12 μM whereas at 5 mM-Na it only reaches half-saturation at 46 μM-Rb. A similar value for the half-saturation point is seen for the K influx in 20 μM-Na (not shown). The inset shows a Lineweaver-Burk plot emphasizing the very high affinity for external Rb in the low Na solution. Lew *et al.* (1973) examined the affinity for external K in Na-free solutions at high (2 mM) ATP. They obtained a value for the $K_{0.5}$ for external K of about 100 μM. The ionic conditions used in the experiments of Lew *et al.* (1973) were the same as those of the present work: $[\text{Na}]_i$, 10 mM; $[\text{K}]_i < 1$ mM. It is therefore likely that it is the reduction of ATP which is responsible for reducing the value of $K_{0.5}$ to 12 μM at low ATP. Therefore the effects of ATP on the K affinity are not merely due to a change of the effectiveness of external Na as a competitor. From a practical point of view this result emphasizes the need to avoid contaminating escape of K when studying the uncoupled Na efflux from low ATP ghosts.

DISCUSSION

Analysis of the results of this paper requires a model for the reactions of the Na pump. A very simple scheme suffices for the present work since the experiments were done under conditions in which most of the steps were irreversible. There was no ADP in the cells, thus removing Na–Na exchange; the cells were K-free and contained very little inorganic phosphate, thus making dephosphorylation irreversible and abolishing K–K exchange (Glynn, Lew & Lüthi, 1970; Simons, 1974). Nevertheless, for ease of subsequent discussion the model allows for the presence of inorganic phosphate. The schemes of Post *et al.* (1972) and Karlish *et al.* (1978) have been reduced to the following.
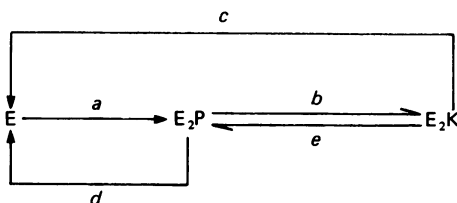
E represents all the unphosphorylated E_1 forms; several intermediates such as E_1P are missing; their presence does not affect the conclusions of this discussion. *a* is a lumped rate constant for phosphorylation which will depend on the internal Na concentration and the saturation of the high affinity (phosphorylating) ATP site. *b* is the rate constant of dephosphorylation by external K and therefore depends on $[\text{K}]_o$. *c* is the rate constant for the conformational change and release of occluded K and depends on the binding of ATP to a low affinity site. *d* is the rate of spontaneous breakdown of the phosphoenzyme which accompanies the uncoupled Na efflux (Lew *et al.* 1973). *e* is the rate constant of phosphorylation of E_2K from inorganic phosphate and therefore depends on the inorganic phosphate concentration. The total concentration of enzyme is E_T; $E_T = E + E_2P + E_2K$. A steady-state analysis shows that the rate of K influx is given by:

$$\text{K influx} = cE_2K = \frac{abc}{ae+ca+de+cd+bc+ba}E_T. \tag{1}$$

The small size of the uncoupled Na efflux compared to Na–K exchange demonstrates that, at least in the presence of saturating internal Na, d must be considerably less than a. Therefore d can be ignored. With this assumption:

$$\text{K influx} = \frac{abc}{ae + ca + bc + ba}\,\mathbf{E_T}. \tag{2}$$

Under the conditions of the present experiments, inorganic phosphate must have been very low, allowing e to be ignored:

$$\text{K influx} = \frac{abc}{ca + bc + ba}\,\mathbf{E_T}. \tag{3}$$

The apparent affinity for ATP. It is easy to show from eqn. (3) that if the rate constant for the conformational reaction is given by $c = c_{\max}x/(\beta + x)$ where β is the real affinity for ATP (x) at the low affinity site, the apparent affinity is given by $\beta_{\text{app}} = \beta ba\,c_{\max}/((b + a)\,c_{\max} + ba)$ (cf. Glynn & Lew, 1969). This is only equal to β in the trivial case when $c_{\max}$ is small compared to both b and a and therefore the conformational change is rate-limiting. The curves relating K influx to ATP concentration are, unfortunately, not simply described by hyperbolae. The influx at $1\ \mu\text{M}$-ATP is much greater than would be expected from a rectangular hyperbola drawn to fit the higher ATP concentrations and passing through the origin. A similar phenomenon was noticed for the $\text{Na}^+\text{–K}^+$ ATPase activity of red cell ghosts (Glynn & Karlish, 1976). It may represent the fact that, even in the absence of ATP, the conformational reaction has a finite rate.

It is nevertheless obvious from Fig. 2 that changing external K affects the affinity for ATP in the expected manner. This suggests that part of the effect of K on the affinity for ATP of the isolated $\text{Na}^+\text{–K}^+$ ATPase is likely to be due to an action at external sites. However, in the isolated ATPase, K acting at internal sites may also lower the ATP affinity. This is because E_2K can be produced from E_1 when K binds to the internal sites (Karlish *et al.* 1978). The greater the K concentration, the greater the concentration of ATP required to displace this K and the lower the apparent affinity for ATP. This reduction of the ATP affinity by *internal* K has been demonstrated in squid axons by Beaugé & DiPolo (1979).

The affinity for external K

In order to analyse quantitatively the effects of ATP on the K affinity one needs to know the explicit form of the dependence of b, the rate of dephosphorylation on the external K concentration. If the rate of dephosphorylation depended on external K in a hyperbolic manner the problem would be trivial. The apparent affinity for K would be distorted in a manner exactly analogous to that of the ATP affinity discussed above. However the K influx depends in a sigmoid manner on external K. It is easy to show that, in this case, not only is the apparent affinity for external K distorted but the qualitative shape of the dependence of K influx on external K will bear no simple relation to the shape of the activation curve for K on the rate of dephosphorylation. The point is easily demonstrated if one arbitrarily assumes that dephosphorylation has a very simple dependence on external K, being proportional

to the degree of occupation of two sites to which K ions can bind randomly with equal affinity.

Thus dephosphorylation rate $= b = b_{\max}\dfrac{K^2}{(K+\alpha)^2}$.

By substituting this into eqn. (3) it can be shown that

$$K\ \text{influx} = \frac{\delta K^2}{K^2(b_{\max}+\gamma)+2\alpha\gamma K+\gamma\alpha^2},$$

where δ and γ are constants which are independent of external K concentration (K) and depend on the other rate constants. Since the denominator has no real roots, this expression does not appear to describe random binding to any two sites. The equation appears to represent the ordered binding to two different sites and has been used to fit K activation curves (Sachs & Welt, 1967; Cavieres & Ellory, 1974). The above analysis shows, however, that even a model describing the K influx as the ordered binding to two sites is consistent with dephosphorylation depending on the random occupation of two identical sites. The only case in which the K influx will appear to result from two identical sites is the trivial one when $b_{\max}$ is very small. In this case dephosphorylation is rate limiting and the over-all rate reflects that of dephosphorylation. As is discussed later this is unlikely to be the case, even at physiological ATP concentrations.

One is therefore forced to adopt a more empirical approach and consider the case when external K tends towards zero and b becomes very small. Then from eqn. (3) K influx $= bE_T$ and is a faithful representation of the rate constant of dephosphorylation. In particular the model predicts that the K influx should become independent of ATP at low external K. This result has been observed (Fig. 5). It should be noted that the prediction is totally general and is independent of the precise shape of the K activation curve. As an experimental convenience we have fitted these activation curves to an equation of the form

$$\text{Influx} = V_{\max}K^2/(K+K_{\text{app}})^2 \qquad (4)$$

where K_{app} is the apparent affinity for each of the two sites. As discussed above this has no physical significance. When K is very small eqn. (4) states that influx $= V_{\max}K^2/K_{\text{app}}^2$ and, since (from eqn. (3)) the influx is independent of ATP under these conditions, this implies that $V_{\max}/K_{\text{app}}^2$ should also be independent of ATP. This has been experimentally verified by showing (Fig. 4) that graphs of $v^{-\frac{1}{2}}$ against $1/[K]_0$ are parallel. Thus the kinetic data are consistent with the predictions of a model in which the ATP-dependent conformational change follows dephosphorylation. The results are also in agreement with those obtained in the squid axon by Beaugé & Di Polo (1979, 1981) where ATP was shown to affect the affinity for external K to activate the Na efflux. In this case, however, more quantitative analysis was impossible since the Na efflux contains a large uncoupled Na efflux and the above analysis breaks down.

This analysis assumes that ATP only affects the Na pump by changing the rate constant for the conformational change (c). A sufficient decrease of ATP will, however, decrease the rate constant for phosphorylation (a). However, so long as a is large compared to c, changes of a will have no effect on the over-all rate of K influx.

That a is always much greater than c, even at 1 μM-ATP (the lowest concentration used in the present experiments) is suggested by the following argument. Mårdh & Zetterqvist (1972) found that in brain enzyme at room temperature, the rate of phosphorylation produced by adding 5 μM-ATP was 45 sec^{-1}. This implies that, even if phosphorylation rate was a linear function of ATP, the rate at 1 μM would be about 10 sec^{-1}. Since the rate is probably a saturating function of ATP (Kanazawa, Saito & Tonomura, 1970), the rate at 1 μM-ATP will be greater than this. This rate is much faster than that for the conformational reaction at 1 μM-ATP of 0·4 sec^{-1} in the kidney enzyme (Karlish & Yates, 1978). It is therefore likely that in the present experiments, the rate of phosphorylation was always greater than that of the conformational reaction and therefore that changes of phosphorylation rate can be ignored.

Comparison with previous work in the red cell. Although the results of this paper provide clear support for a model in which the ATP-dependent and K-dependent reactions are in series, previous work on the red cell has failed to show any effect of ATP on the affinity for external K (Glynn, 1956; Garay & Garrahan, 1975; Beaugé & Del Campillo, 1976). There are probably at least two explanations of this discrepancy. First, since these previous experiments were performed in intact cells, with the methods used it was probably not possible to lower ATP below about 100 μM. In fact, since the cells contained K, if ATP had been lowered significantly more, internal K would probably have inhibited Na–K exchange by forming the occluded species E_2K from internal K (Karlish *et al.* 1978). Due to the comparatively small reduction of ATP the maximum reductions of V_{max} were never very large (V_{max} was decreased to about 50 % in Fig. 6 of Garay & Garrahan, 1975). Since we have shown that $\sqrt{V_{max}}/K_m$ remains constant one would only expect K_m to fall to 70 % of its control value. Therefore a contributory factor to the failure to observe changes of affinity may well have been the modest size of the expected change.

There are, however, other differences with respect to the previous studies. In the present experiments internal K was zero and the intracellular inorganic phosphate concentration must have been very low. In the experiments of Garay & Garrahan (1975), however, P_i was almost 2 mM and the cells had a normal K concentration. In the presence of P_i dephosphorylation becomes reversible (Post, Toda & Rogers, 1975). Non-parallel Lineweaver-Burk plots are expected in the presence of reaction products (Cleland, 1970). Equation (2) shows that, in the presence of P_i (when $e > 0$), the influx is no longer independent of ATP (c) even at low external K. It is obvious that the influx at low external K increases with ATP when phosphate is present. From eqn. (4) this implies that increasing ATP will increase $\sqrt{V_{max}}/K_{app}$. Therefore the apparent affinity will change by even less than $\sqrt{V_{max}}$. It is therefore not surprising that studies in intact cells have failed to show changes of K affinity produced by changing ATP. It is noteworthy that P_i was zero in the dialysed squid axons used to demonstrate the effects of ATP on the K affinity (Beaugé & DiPolo, 1979, 1981). We have found (D. A. Eisner & D. E. Richards, in preparation) that when ghosts are prepared to contain inorganic phosphate, changing ATP produces non-parallel Lineweaver-Burk lines. This supports the idea that failure to see changes of affinity in previous experiments was indeed due to the presence of inorganic phosphate.

The flux experiments presented in this paper are consistent with biochemical work on the isolated ATPase showing an effect of K on the affinity for ATP. Furthermore, they show that this effect is at least partly due to external K ions.

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