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EFFECTS OF ATP ON THE INTERMEDIARY STEPS OF THE REACTION OF THE ($Na^+ + K^+$)-ATPase

IV. EFFECT OF ATP ON $K_{0.5}$ FOR Na^+ AND ON HYDROLYSIS AT DIFFERENT pH AND TEMPERATURE

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Summary

The pH optimum for ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) depends on the combination of monovalent cations, on the ATP concentration and on temperature.

ATP decreases the Na^+ concentration necessary for half maximum activation, $K_{0.5}$ for Na^+ ($Na^+ + K^+ = 150$ mM), and the effect is pH and temperature dependent.

At a low ATP concentration a decrease in pH leads to an increase in $K_{0.5}$ for Na^+, while at the high ATP concentration it leads to a decrease.

$K_{0.5}$ for ATP for hydrolysis decreases with an increase in pH.

The fractional stimulation by K^+ in the presence of Na^+ decreases with the ATP concentration, and at a low ATP concentration K^+ becomes inhibitory, this being most pronounced at 0°C.

The results suggest that (a) ATP at a given pH has two different effects: it increases the Na^+ relative to K^+ affinity on the internal site ($K_{0.5}$ for ATP at pH 7.4, 37°C, is less than 10 μM); it increases the molar activity in the presence of $Na^+ + K^+$ ($K_{0.5}$ for ATP at pH 7.4, 37°C, is 127 μM), (b) binding of the cations to the external as well as the internal sites leads to pK changes (Bohr effect) which are different for Na^+ and for K^+, i.e. the selectivity for Na^+ relative to K^+ depends both on ATP and on the degree of protonation of certain groups on the system, (c) ATP involves an extra dissociable group in the determination of the selectivity of the internal site, and thereby changes the effect of an increase in protonation of the system from a decrease to an increase in selectivity for Na^+ relative to K^+.

Introduction

In the presence of Na^+ the apparent affinity of the ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) for ATP as substrate is decreased by the low activating concentrations of K^+ [1]. When the enzyme is prephosphorylated in the presence of Mg^{2+}, Na^+ and ATP, the addition of K^+ leads to a dephosphorylation and to a binding of K^+ to the dephospho enzyme; ATP increases the rate of release of K^+ when ATP is added together with Na^+ in a following step [2]. From this it has been proposed that it is the ATP induced release of K^+ from the dephospho enzyme which limits the rate of the hydrolysis at least at the low ATP concentrations when the system is turning over in the presence of $Na^+ + K^+$, and this explains that K^+ in the low activating concentrations decreases the apparent affinity for ATP for hydrolysis [2]. This finds support from the observations that the enzyme can exist in two different forms depending on the presence of K^+ or Na^+ [3,4,5] and that the conversion between the two forms is slow and is accelerated by FTP, a fluorescent ATP analogue [6].

When the system is turning over in the presence of $Na^+ + K^+$, ATP decreases $K_{0.5}$ for Na^+ for activation [7]. According to the above proposed scheme the ATP induced release of K^+ is followed by the binding of Na^+, and this is followed by the hydrolysis, i.e. the ATP which decreases the $K_{0.5}$ for Na^+ must have released K^+. The effect of ATP on $K_{0.5}$ for Na^+ may follow from the 'opening' of the dephospho enzyme and the relase of K^+, or there may be two different effects of ATP, one on the 'opening' of the dephospho form, and another on a competition between K^+ and Na^+ for Na^+ entering the 'opened' dephospho form. In order to test this, the effect of ATP on $K_{0.5}$ for Na^+ has been compared to the effect of ATP on the hydrolysis. The experiments have been performed at three different pH values in order to see how a change in protonation influences the ATP effect. In relation to this it has been tested how ATP, Na^+, Li^+, and K^+ influence the pH optimum of the ATPase reaction. It has finally been tested how a decrease in temperature to 0°C influences the effect of ATP on activation by K^+ compared to the effect on $K_{0.5}$ for Na^+.

Methods

The enzyme was prepared from ox-brain as described in a previous paper [8]; the specific ($Na^+ + K^+$) activity was 250—300 μmol $P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$ (4 mM Mg^{2+}, 3 mM ATP, 130 mM Na^+, 20 mM K^+, pH 7.4, 37°C). The Mg^{2+} activity (equal to the ouabain-insensitive activity) was less than 0.5% of the total activity.

The activity was tested by measuring the amount of ^{32}P released from ATP labelled with ^{32}P in the γ position. The test volume was 1 ml, which besides 30 mM buffer, histidine-HCl or Tris-HCl adjusted to required pH at 37°C or 0°C, contained [^{32}P]ATP, Na^+, K^+ and Mg^{2+} in the concentrations given on the figures. The reaction time was in all the experiments adjusted in such way that the maximum hydrolysis of the added ATP was less than 20%. Under these conditions, the hydrolysis was a linear function of time. The reaction was stopped by the addition of 0.1 ml 50% trichloroacetic acid; blank was with

10^{-4} M ouabain in the test medium. The hydrolysis of [^{32}P]ATP was determined according to the method of Lindberg and Ernster [9]. The ^{32}P was counted in a liquid scintillation counter. [^{32}P]ATP was from The Radiochemical Centre, Amersham, England. ATP was from Boehringer. [^{32}P]ATP and ATP were purified and converted to the Tris salt by chromatography on a DEAE-Sephadex A-25 (Pharmacia) column [3].

With ATP concentrations higher than 500 μM, the activity was tested by measuring the P_i released by the method of Fiske and SubbaRow [10].

The figures show typical results, which have all been repeated several times; for variability in the experimental results, see legend to Fig. 5.

Results

Ionic strength

With 3 mM ATP and with $Na^+ + K^+$ equal to 150 mM the maximum rate of hydrolysis is obtained with 20 mM K^+ + 130 mM Na^+, i.e. with a $K^+ : Na^+$ ratio of 1 : 6.5. This ratio is also optimum when the sum of $Na^+ + K^+$ is varied. The activity at a fixed 1 : 6.5 ratio gives a bell shaped curve with maximum when $Na^+ + K^+$ equals 150 mM; at a higher concentration the activity decreases, Fig. 1. This suggests that an increase in ionic strength leads to a decrease of activity. In agreement with this the activity is lower when the experiment is repeated but with Tris included to keep the ionic strength constant ($Na^+ + K^+ +$

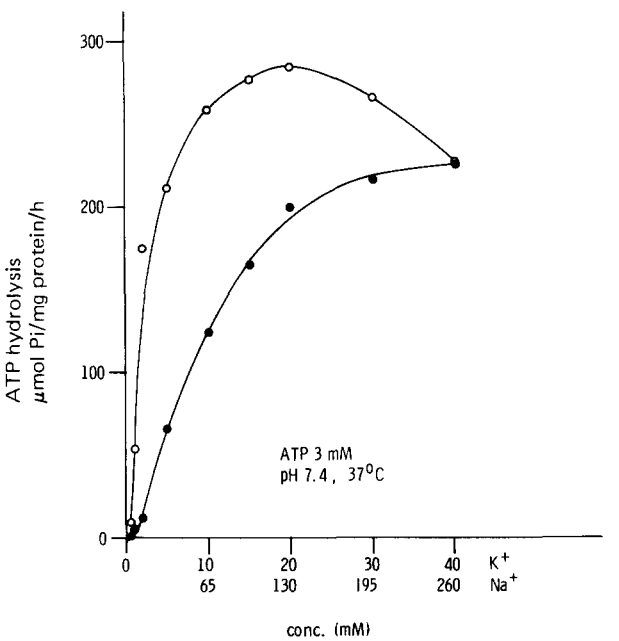

Fig. 1. The effect of a variation in the $Na^+ + K^+$ concentration on the catalytic activity of ($Na^+ + K^+$) ATPase. The $K^+ : Na^+$ ratio was kept constant at 1 : 6.5 which is the optimum for activation. In the one set of experiments the ionic strength varied with the $Na^+ + K^+$ concentration (○), in the other tris was included and $Na^+ + K^+$ + ionized Tris was kept constant at 300 mM (●). 3 mM ATP, 4 mM Mg^{2+}, pH 7.4.

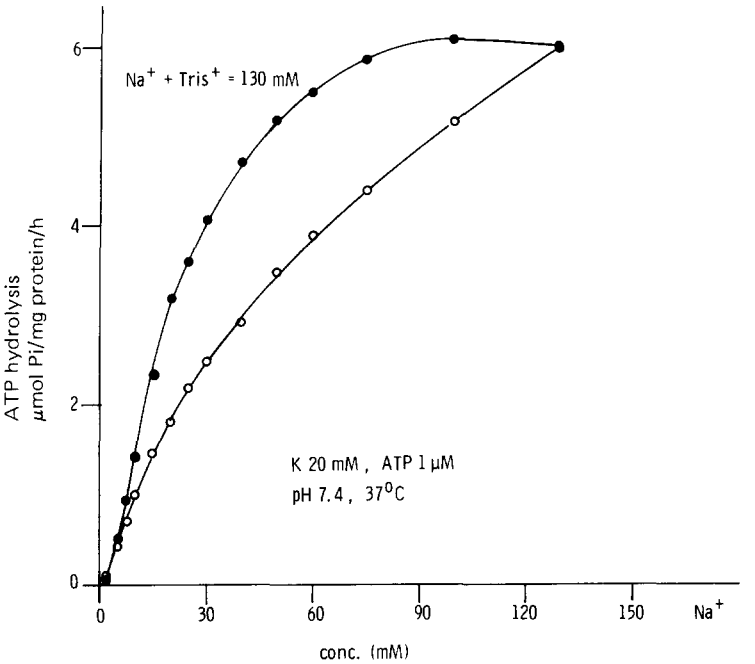

Fig. 2. The effect of a variation of the Na^+ concentration on the catalytic activity of ($Na^+ + K^+$)-ATPase in the presence of 20 mM K^+. In the one set of experiments, the ionic strengths varied with the Na^+ concentration, in the other, the sum of Na^+ and ionized Tris was kept constant at 130 mM while the Na^+ concentration was varied. 1 μM ATP, 100 mM Mg^{2+}, pH 7.4, 37°C.

ionized Tris = 300 mM, chloride as anion) (Fig. 1).

With a lower ATP concentration (1 μM) inclusion of Tris to keep the ionic strength constant has the opposite effect; it increases the activity. This is seen in Fig. 2 where K^+ has been kept constant at 20 mM and Na^+ has been varied without and with Tris (Na^+ + ionized Tris = 130 mM).

Tris has no activating effect on the system with Na^+ but no K^+ or with K^+ but no Na^+. It seems therefore most likely that the stimulating effect of Tris with the low ATP concentration is due to an ionic strength effect.

It is difficult to exclude that Tris or any other monovalent cation used to keep the ionic strength constant competes for Na^+ and/or K^+ on the specific sites on the system. To avoid this problem the ionic strength in the following experiments was kept constant by varying Na^+ and K^+ inversely; Na^+ plus K^+ was kept constant at 150 mM.

pH optimum

There is an optimum for the magnesium concentration for activation and it varies with the ATP concentration; in concentrations higher than the optimum, magnesium inhibits [7].

The ratio between free ATP, MgATP and free magnesium depends on pH. In agreement with this it is found that the optimum magnesium concentration for activity at a given ATP concentration depends on pH. The optimum magnesium concentration has therefore been determined for each pH and ATP concentra-

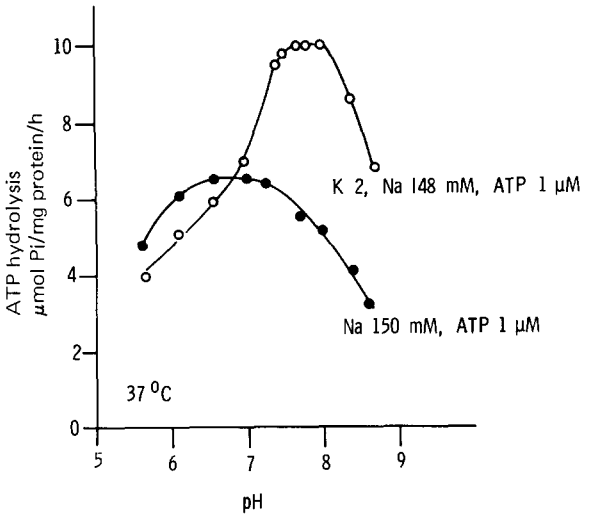

Fig. 3. The effect of pH on the catalytic activity of $(Na^+ + K^+)$-ATPase with 150 mM Na^+ and with 148 mM Na^+ + 2 mM K^+, respectively, and with 1 μM ATP. The Mg^{2+} concentration was the optimum for activity at each pH value. Temp. 37°C.

tion and the values used in the experiments.

The pH optimum for hydrolysis of ATP depends on the ligands used for activation and on the ATP concentration.

With 150 mM Na^+ and no K^+ and with nonsaturating concentrations of ATP (1 μM) the pH optimum is 6.6—7.0, Fig. 3. With saturating concentrations of ATP, 25 μM or higher, the pH optimum is the same (not shown).

With 2 mM K^+ in the presence of 148 mM Na^+ the pH optimum with 1 μM ATP is shifted towards a higher value, 7.6—8.0, Fig. 3. This effect of K^+ is

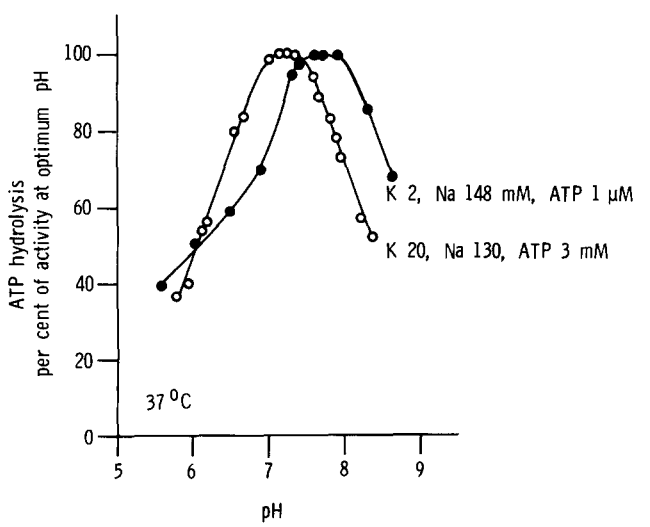

Fig. 4. The effect of pH on the catalytic activity of $(Na^+ + K^+)$-ATPase in the presence of 1 μM ATP, 2 mM K^+ 148 mM Na^+ and with 3 mM ATP, 20 mM K^+ 130 mM Na^+. The Mg^{2+} concentration was the optimum for activity for each pH avalue and ATP concentration. Temp. 37°C. The ordinate shows the activity as a percentage of the activity at the optimum pH.

partly prevented by an increase in the ATP concentration; with saturating concentrations of ATP (3 mM) the pH optimum is shifted downwards to pH 7.1–7.4, Fig. 4.

20 mM is the optimum K^+ concentration with 3 mM ATP and at all the tested pH values. 2 mM K^+ is optimum with 1 μM ATP and with a pH equal to or higher than 6.85; at a lower pH, 2 mM K^+ becomes inhibitory compared to the activity with Na^+ alone (see Fig. 3). The decrease in pH optimum when the ATP concentration is increased from 1 μM to 3 mM is not due to a difference in the K^+ concentration used in the two experiments.

With 150 mM Li^+, which is the only other cation besides Na^+ which can activate the system alone, the pH optimum is 6.2–6.4 which is lower than with Na^+ alone. Li^+ has a K^+-like effect on the activity in the presence of Na^+ [11]. With 1 μM ATP and with 50 mM Li^+ plus 100 mM Na^+ (optimum for activation) the pH optimum is shifted towards a higher pH values, 7.0–7.3; this is higher than seen with Na^+ alone, but lower than with Na^+ plus K^+ with 1 μM ATP. With 3 mM ATP the pH optimum with $Li^+ + Na^+$ is 6.3–6.9.

ATP and pH on $K_{0.5}$ for Na for activation

Fig. 5 shows the effect of an increase in Na^+ concentration (and a decrease in K^+ concentration, Na^+ plus K^+ = 150 mM) on the activity with 0.1 μM and with 3 mM ATP at three different pH values, 8.4, 7.4 and 5.7. The activity is expressed in percent of maximum obtainable at the given ATP and pH, i.e with optimum concentrations of Na^+ plus K^+.

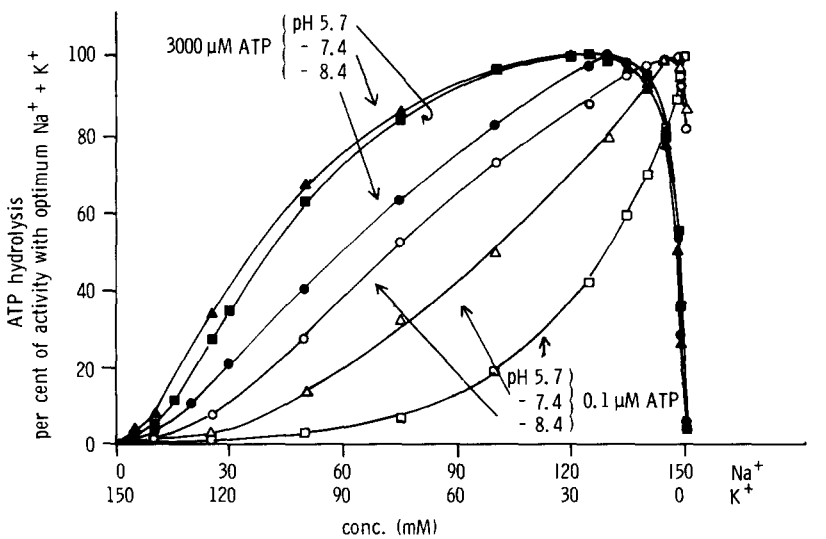

Fig. 5. The effect of $Na^+ + K^+$ ($Na^+ + K^+$ = 150 mM) on the catalytic activity of ($Na^+ + K^+$)-ATPase with 3 mM and 0.1 μM ATP, respectively, and for each ATP concentration at three different pH values: 5.7, 7.4 and 8.4, 37°C. The Mg^{2+} concentration was the optimum for activity at each pH and ATP concentration. The ordinate shows the percentage of the activity with optimum concentrations of $Na^+ + K^+$. The mean of Na^+ ± S.E. for half maximum activation (the left part of the curves) was at 3 mM ATP, pH 5.7, 40.5 ± 0.3 mM (n = 3), pH 7.4, 37.3 ± 0.9 mM (n = 3), pH 8.4; 61.2 ± 1 mM and at ATP 0.1 μM, pH 5.7, 130.7 ± 0.9 mM (n = 3), pH 7.4, 92 ± 0.7 (n = 9) and pH 8.4, 75.4 ± 0.2 mM (n = 4).

The left part of the curves show the activation by Na^+ in the presence of K^+.

In agreement with previous observations an increase in the ATP concentration at a given pH shifts the curves towards a lower $K_{0.5}$ for Na^+ [7]. The effect is seen at all the three pH values tested, but the change in $K_{0.5}$ for Na^+ is more pronounced the lower the pH.

The effect of a change in pH depends on the ATP concentration. With 0.1 μM ATP a decrease in pH shifts the curve to the right, to a higher $K_{0.5}$ for Na^+, and the effect is seen in the pH interval from 8.4 to 5.7. With saturating concentrations of ATP (3 mM) the effect is the opposite; a decrease in pH shifts the curve towards a lower $K_{0.5}$ for Na^+. The effect is seen by a decrease from pH 8.4 to 7.4, while a further decrease in pH has little or no effect.

ATP on K^+ activation

In agreement with observations made by others, Fig. 5 also shows that $K_{0.5}$ for K^+ activation [1,2] (the right part of the curves) as well as the optimum concentration of K^+ for activation [2] decreases with a decrease in the ATP concentration. The concentration of K^+, which is optimum for activation at 3 mM ATP, becomes inhibitory at the lower ATP concentration [2,12–14]. However, with a lower K^+ concentration there is still a stimulation when the pH is higher than 6.85; at a lower pH and with 1 μM ATP or lower, K^+ has no stimulating effect, it only inhibits.

ATP on $K_{0.5}$ for Na^+ and on rate of hydrolysis

Fig. 6 shows $K_{0.5}$ for Na^+ as a function of the ATP concentration at pH 5.7, 7.4 and 8.4. The $K_{0.5}$ is read from a family of curves like the ones shown in Fig. 5.

Fig. 6 also shows the hydrolytic activity with 130 mM Na^+ and 20 mM K^+ as a function of the ATP concentration (plus the optimum magnesium concentration for each ATP concentration) at the three pH values.

At pH 8.4 an increase in the ATP concentration up to about $K_{0.5}$ for ATP for hydrolysis gives a decrease in $K_{0.5}$ for Na^+. A further increase in the ATP concentration gives an increase in $K_{0.5}$ for Na^+. This double effect for ATP is not, or to a much less extent, seen at pH 7.4 and 5.7. Due to this shift in effect of ATP the curve at pH 8.4 crosses the two other curves and this explains why a change in pH has a different effect on $K_{0.5}$ for Na^+ at a low and at a high ATP concentration. At a low ATP concentration an increase in pH gives a decrease in $K_{0.5}$ for Na^+; at the high ATP concentration it is a decrease in pH which gives a decrease in $K_{0.5}$ for Na^+ and the pH interval in which the effect is seen is shifted towards a higher pH value (cf. Fig. 5).

Fig. 6 furthermore shows that the $K_{0.5}$ for ATP (plus magnesium) necessary for hydrolysis decreases with an increase in pH. At pH 5.7 it is 195 ± 24 μM ATP (n = 3), at pH 7.4 127 ± 11 μM ATP (n = 3) and at pH 8.4 45 ± 1 μM ATP (n = 3).

Fig. 6 also shows that at the low ATP concentrations, ATP has a much more pronounced effect on $K_{0.5}$ for Na^+ than on the rate of hydrolysis. At pH 7.4 the ATP concentration necessary to give a half maximum effect on $K_{0.5}$ for Na^+ is less than 10 μM while $K_{0.5}$ for ATP for hydrolysis is 127 μM. Saturation of the effect on $K_{0.5}$ for Na^+ is obtained with 500 μM ATP while 3000 μM ATP is necessary for saturation of the hydrolytic activity.

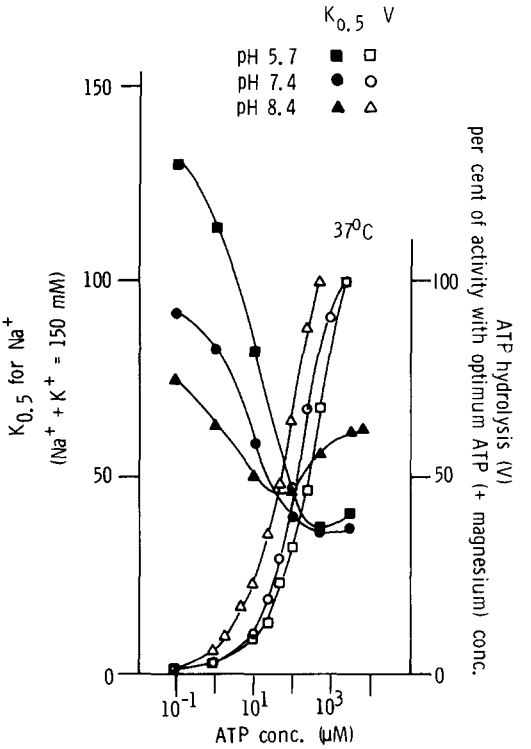

Fig. 6. The $K_{0.5}$ for Na^+ for activation in the presence of K^+ ($Na^+ + K^+$ = 150 mM) at different ATP concentrations and for each ATP concentration at three different pH values: 5.7, 7.4 and 8.4, 37° C. $K_{0.5}$ is read from curves like the ones shown in Fig. 5; they are given on the left abscissa. The figure furthermore shows the hydrolytic activity as a function of the ATP concentration at the same three pH values. The activity was measured with 130 mM Na^+ and 20 mM K^+ and for each ATP concentration with the optimum Mg^{2+} concentration. Temp. 37° C. The activity is expressed as a percentage of the activity obtained with optimum ATP (+magnesium) and is given on the right abscissa.

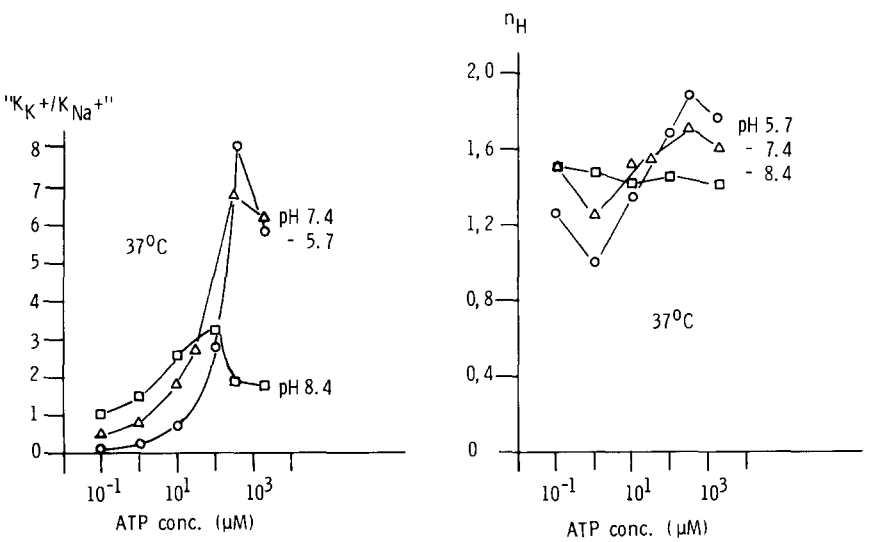

Fig. 7. 'K_K/K_{Na}' values and slopes n_H (n_{Na^+/K^+}) read from a Hill plot from the left part of the curves like the ones shown in Fig. 5 and at different ATP concentrations and for each ATP concentration at three different pH values: 5.7, 7.4 and 8.4. Temp. 37° C.

A $\log(v/V-v)$ versus $\log(Na^+/K^+)$ of the left part of the curves at a given ATP concentration gives a straight line which at the extremes becomes S-shaped. From this can be read a 'K_{Na}/K_K' and a slope n_{Na^+/K^+}. In Fig. 7 is shown $1/'K_{Na}/K_K'$ ('K_K/K_{Na}') and n_{Na^+/K^+} as a function of the ATP concentration.

At pH 7.4 the apparent K^+ relative to Na^+ affinity varies from about a three times higher affinity for K^+ than for Na^+ at 0.1 μM ATP to about a six times higher affinity for Na^+ than for K^+ at saturating concentrations of ATP. The slope varies from 1 to 1.9 around 1.5 and is dependent both on pH and on the ATP concentration.

Temperature effect

pH optimum. The system still has activity at 0°C. The optimum pH (measured at 0°C) for hydrolytic activity with $Na^+ + K^+$ is shifted towards a higher pH value than at 37°C. With 1 μM ATP it is 8.2—8.5, and as it was seen at 37°C, an increase in ATP shifts it towards a lower pH (7.9—8.1) with 3 mM ATP.

ATP on $K_{0.5}$ for Na^+ and on activation by K^+. With 3 mM ATP and optimum pH 8.0 at 0°C the activity as a function of a variation in Na^+ relative to K^+ ($Na^+ + K^+ = 150$ mM) follows a curve like the one at 37°C, pH 7.4, but the $K_{0.5}$ for Na^+ is lower, 25 mM ($K^+ = 125$ mM) compared to 37 mM ($K^+ = 113$ mM) at 37°C. The right part of the curve, which shows the activation by K^+ in the presence of Na^+, is shifted towards a lower $K_{0.5}$ for K^+. The activity with 150 mM Na^+ is 29% of the activity with optimum $Na^+ + K^+$ compared to 3% at 37°C, i.e. the fractional activation by K^+ is lower at 0°C (not shown).

The optimum concentration of K^+ for activation and the fractional stimulation by K^+ decreases with a decrease in the ATP concentration. With 1 μM and 0.1 μM ATP, K^+ has no activating effect, it only inhibits, even at the pH which

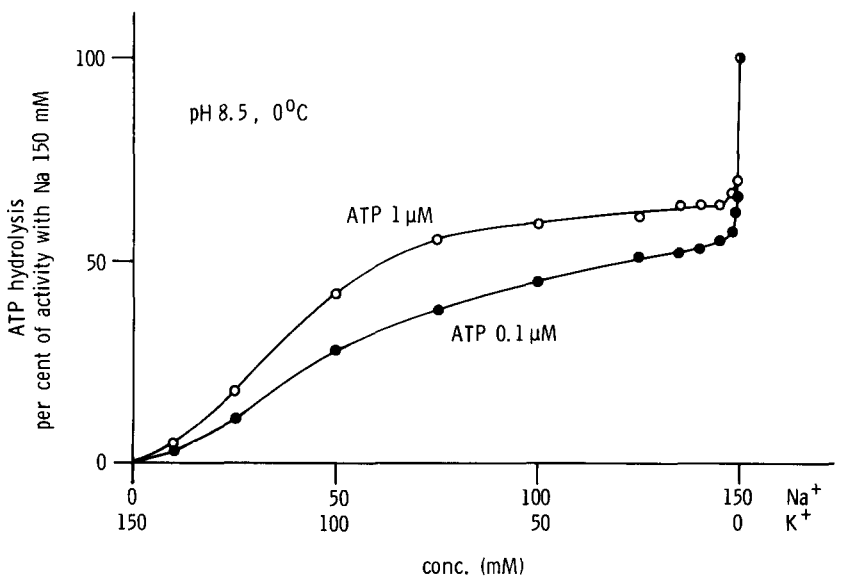

Fig. 8. The effect of $Na^+ + K^+$ ($Na^+ + K^+ = 150$ mM) on the catalytic activity of ($Na^+ + K^+$)-ATPase in the presence of 0.1 μM and 1 μM ATP, respectively. Temp. 0°C, pH 8.5.

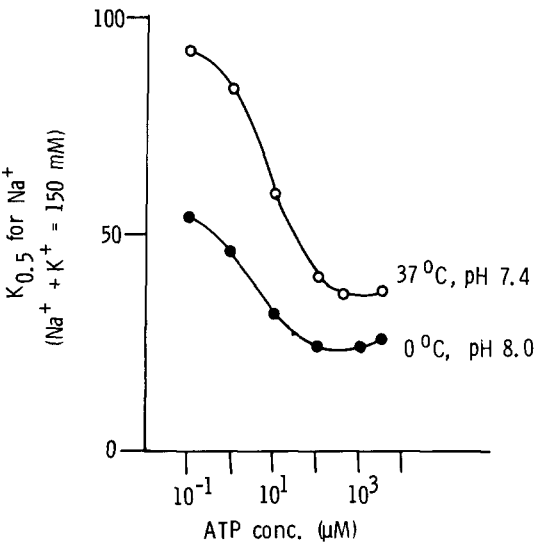

Fig. 9. The $K_{0.5}$ for Na^+ for activation in the presence of K^+ ($Na^+ + K^+ = 150$ mM) at different ATP concentrations at pH 8.0, 0°C. For comparison $K_{0.5}$ is shown for Na^+ at different ATP concentrations at 37°C, pH 7.4.

is the optimum for activation in the presence of K^+ (Fig. 8). The shape of the curves suggests two different inhibitor effects of K^+. At low concentrations, K^+ gives a steep decrease in activity. This occurs in concentrations which at a higher ATP concentration increase the activity by an effect of K^+ on the site which in the intact membrane is facing the external solution (the external site in the following), suggesting that this inhibition is due to a K^+ effect on this site. The inhibition which is seen by a further increase in the K^+ concentration follows a curve which has resemblance to the left part of the curve seen at the higher concentrations of ATP. It seems likely that this part of the curve (read from the left) shows the same as the left part of the curve at the higher ATP concentrations, namely the competition between Na^+ and K^+ for Na^+ activation on the internal site. This part of the curve is shifted to the left, towards a lower $K_{0.5}$ for Na^+, compared to what was seen with the low concentrations of ATP at 37°C, cf. Figs. 5 and 6 with Figs. 8 and 9. On this site the ability of Na^+ to displace K^+ is increased.

Fig. 9 shows the $K_{0.5}$ for Na^+ as a function of the ATP concentration. At the ATP concentrations where K^+ had no activating effect, the $K_{0.5}$ for Na^+ is read from that part of the curve which, as discussed above, is assumed to represent the activation by Na^+ at the internal site. It is seen from a comparison of Figs. 9 and 6 that the $K_{0.5}$ at 0°C is lower than the $K_{0.5}$ at 37°C at the tested pH values. As the difference is seen also with saturating concentrations of ATP, it cannot be due to differences in affinity for ATP at the two temperatures.

At the low ATP concentration, the decrease in temperature thus leads to an increase in the inhibitory effect of the low K^+ concentrations but the requirement for Na^+ for diplacement of K^+ for Na^+ activation is decreased.

In Fig. 10 is shown the 'K_K/K_{Na}' values and the slope n_{Na^+/K^+} read from a

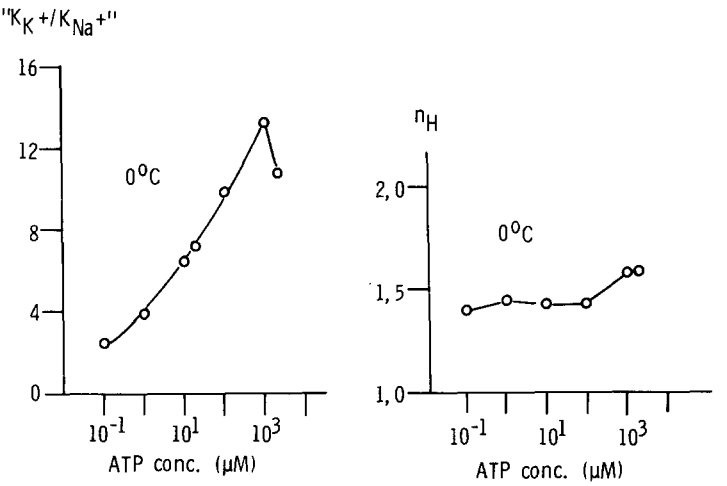

Fig. 10. 'K_K/K_{Na}' values and slopes n_H (n_{Na^+/K^+}) read from a Hill plot of the left ascending part of curves showing the activation by Na^+ in the presence of K^+ ($Na^+ + K^+ = 150$ mM) at different ATP concentrations, pH 8.9, 0°C.

$\log(v/V - v)$ versus $\log(Na^+/K^+)$ of that part of the curves which shows the activation by Na^+. At 0°C with 0.1 μM ATP the apparent affinity for Na^+ is about 2 times higher than for K^+ and with saturating concentrations of ATP it is about 12 times higher. At 0°C the apparent affinity for Na^+ is thus higher than for K^+ at all the tested ATP values in contrast to 37°C where the apparent affinity was higher for K^+ than for Na^+ at the low ATP concentrations. The slope increases from 1.4 to about 1.6 with the ATP concentration.

Discussion

pH optimum

In agreement with observations by Neufeld and Levy [15] it is found that the pH optimum with Na^+ alone is lower than with Na^+ plus K^+. In their experiments it was 6.4, while in the present experiment it is 6.6—7.0.

The effect of a change in the protonation of the system (and substrate) is influenced by the nature and combination of the monovalent cations. It suggests either that it is differently protonated forms of the system which have activity with the different combination of monovalent cations, and/or that the monovalent cations influence the protonation of the system at a given pH, i.e. change the pK values.

With Na^+ in the medium, Li^+ and K^+ has the effect on the pH optimum in concentrations which are optimum for activation, i.e. it is an effect on the external site of the system. This site has a lower affinity for Li^+ than for K^+, and the activating effect of Li^+ is lower [11]. The different effect of K^+ and Li^+ on the pH optimum raises the question whether there is a relationship between differences in selectively and/or activating effect of the cations on the external site and their effect on the protonation of the system.

ATP on $K_{0.5}$ for Na and on turnover rate

ATP at low concentrations has a much stronger effect on $K_{0.5}$ for Na^+ than on the hydrolysis. At pH 7.4, the maximum effect of ATP on $K_{0.5}$ for Na^+ is obtained with less than 10 μM ATP, while the $K_{0.5}$ for ATP on hydrolysis is about 127 μM ATP. Saturation of the effect on $K_{0.5}$ for Na^+ is with 500 μM ATP, while for hydrolysis it is 3000 μM ATP. At pH 8.4 ATP has a dual effect on $K_{0.5}$ for Na^+, but this is not reflected in the substrate requirement. A decrease in temperature at 1 and 0.1 μM ATP leads to an increase in the inhibitory effect of the low K^+ concentrations. This is not accompanied by a decrease in the ability of Na^+ to be exchanged for K^+; on the contrary, the $K_{0.5}$ for Na^+ is decreased.

This seems to suggest two different effects of ATP. One on the K^+—Na^+ exchange on the internal site and with a relatively high affinity for ATP and another on the rate of turnover and with a lower affinity.

It is K^+ in the low activating concentrations [1], i.e. K^+ on the external site, which sets the requirement for ATP for the effect on the turnover rate. K^+ on this site decreases the apparent affinity for ATP [1] and vice versa, suggesting a negative cooperative effect between K^+ and ATP in one of the steps of the reaction; the ATP-induced decrease in the affinity to K^+ seems to be necessary in order to get a K^+ stimulation of the turnover rate.

The different effects of ATP on the K^+ affinity and on $K_{0.5}$ for Na^+ seem to be reflected by the effect of ATP on the *p*-nitrophenolphosphatase activity. Without Na^+, ATP decreases the apparent affinity for K^+ for activation of the *p*-nitrophenolphosphatase activity [16—19] and with 10 mM K^+, 90 mM choline, and saturating concentrations of *p*-nitrophenolphosphate and Mg^{2+}, the $K_{0.5}$ for ATP inhibition of the K^+ activation is about 150 μM [18], of the same order as the $K_{0.5}$ for ATP for the effect on the turnover rate. With high Na^+ and low K^+, ATP stimulates the *p*-nitrophenolphosphatase activity (see Refs. 18, 19). With 10 mM K^+ and 90 mM Na^+, the $K_{0.5}$ for ATP for stimulation is about 13 μM [18], of the same order as for the effect on $K_{0.5}$ for Na^+. On the same enzyme preparation and with the same batches of nucleotides (same degree of purity) the stimulation of the *p*-nitrophenolphosphatase activity [18] and the effect on $K_{0.5}$ for Na^+ [7] follows the same order: ATP > CTP > ITP > GTP. The effect on the K^+ affinity follows another order: ATP > GTP > CTP > ITP. It supports the view that there are two different effects of ATP on the system and suggests that it is at sites with different conformations.

There is a good deal of evidence that the stimulation of the *p*-nitrophenolphosphatase is related to a phosphorylation of the system from ATP (see Ref. 20), i.e. ATP on the site where it stimulates *p*-nitrophenolphosphatase and increases $K_{0.5}$ for Na^+ is hydrolysed. With Na^+ but no K^+ in the medium ATP is bound to the system with a K_D of 0.12—0.29 μM [3,4] and the system is phosphorylated from ATP with an apparent $K_{0.5}$ of the same order [21,22]. Nucleotides are bound to the high affinity site in an order which for ATP, CTP, and GTP is the same as for their effect on $K_{0.5}$ for Na^+ [4,23]. K^+ increases K_D for ATP while Na^+ has no effect [3,4] which means that ATP in the presence of Na^+ and K^+ increased the apparent affinity for Na^+. It suggests that it is the ATP on the site which binds ATP with the high affinity without K^+ in the medium which has the effect on $K_{0.5}$ for Na^+ for the ATPase activation in the presence of Na^+ and K^+.

Thus it seems to be an ATP at a high affinity site which shifts the equilibrium towards the Na^+ form of the internal site in the presence of $Na^+ + K^+$ and that this ATP is a substrate for the reaction. ATP at a low affinity site is necessary in order to get K^+ to increase the turnover rate, i.e. this ATP so to say releases an inhibition due to K^+, and this allows K^+ to act as an activator.

ATP transforms an 'occluded' K^+ dephospho form $E_2(K_m)$ into an 'open' E_1K_m (E_1 = 'Na^+ form') and thereby increases the rate of release of K^+ [2,6]. In the presence of Na^+, E_1K_m exchanges K^+ for Na^+ and becomes E_1Na_n (m and n are numbers); the enzyme has a higher affinity for ATP in the presence of Na^+ than in the presence of K^+ [3,4] meaning that ATP in the presence of $Na^+ + K^+$ shifts the equilibrium towards E_1Na_n. This scheme could explain the two different ATP effects and two different affinities: ATP 'opens' $E_2(K_m)$ with a low affinity [6] and shifts the equilibrium between E_1K and E_1Na towards E_1Na_n with a high affinity. It could be an effect of ATP on the same site but in different conformations in consecutive steps.

This scheme could also explain the two different effects of ATP on *p*-nitrophenolphosphatase activity. It has been suggested that it is $E_2(K_m)$ which has the *p*-NPPase activity [2]. $E_2(K_m)$ is formed when the systems reacts with K^+ but without Na^+ and ATP [6]. ATP 'opens' this form and decreases the apparent affinity for K^+ just as ATP decreases the apparent affinity for K^+ for activation of *p*-nitrophenolphosphatase activity. $E_2(K)$ can also be formed via a K^+ dephosphorylation of a phospho enzyme formed in the presence of Na^+ + ATP. With $K^+ + Na^+$ it is low concentrations of ATP which stimulate *p*-nitrophenolphosphatase activity. The $K_{0.5}$ for ATP is of the same order as for the ATP effect on the K^+—Na^+ exchange (see above). Whith higher concentrations of ATP the activity again decreases, the $K_{0.5}$ is about 1 mM [18,19]. With the low ATP concentration the rate of 'opening' of the $E_2(K_m)$ formed by the K^+ dephosphorylation will be slow, but when 'opened' the low ATP concentration will shift the equilibrium from E_1K_m towards E_1Na_n allowing a new phosphorylation and a following K^+ dephosphorylation with formation of $E_2(K_m)$, i.e. a main part of the system will be on $E_2(K_m)$ which as suggested [2] has *p*-nitrophenolphosphatase activity. With the high ATP concentration the equilibrium will be shifted away from $E_2(K_m)$.

From the effect of Na^+ on the K^+ activation of *p*-nitrophenolphosphatase activity it has been suggested that without ATP, K^+ activates the *p*-nitrophenolphosphatase activity by an effect on the internal sites of the system while with Na^+ and ATP it is K^+ on the external sites which activates [18]. This agrees with the proposal that without Na^+ and ATP, $E_2(K_m)$ is formed by a reaction of K^+ with the internal sites [6], while with Na^+ and ATP it is a dephosphorylation due to an effect of K^+ on the external sites which leads to $E_2(K_m)$.

In the scheme discussed the two ATP effects are consecutive. An alternative is that the ATP effect on the turnover rate is on a step in the reaction prior to the translocation of K^+ from the external site, i.e. prior to hydrolysis of ATP at the high affinity site (prior to a phosphorylation and/or to a dephosphorylation). ATP on the low affinity site changes the interaction between the polypeptides in the system, and in this form the system has a lower apparent affinity for K^+ on the external site. When K^+ activates the hydrolysis (or dephosphorylation) of this 'open' form of the system, K^+ is translocated and not

bound in an 'occluded' form. While without ATP on the low affinity site K^+ is not translocated but bound in an 'occluded' form. This means that ATP at the low affinity site increases the turnover rate by 'opening up' for the K^+ translocation and not by a release of a translocated K^+ from an 'occluded' form. With consecutive addition of the ligands this reaction scheme will also show an 'occluded' $E_2(K_m)$ form which is opened by ATP, and the explanation given above of the effect of ATP on *p*-nitrophenolphosphatase activity will also apply to this scheme. The main difference is, however, that a system following this scheme must have two different sites for ATP, a low and a high affinity site, existing simultaneously.

pH on $K_{0.5}$ for Na^+

At the low ATP concentrations the decrease in $K_{0.5}$ for Na^+ seen when pH is increased could be due to an increase in apparent affinity for ATP, cf. Fig. 6. However, as seen from the figure, about 20 μM ATP is necessary to give the same effect on the $K_{0.5}$ for Na^+ at pH 5.7 as 0.1 μM at pH 8.4, i.e. 200 times more gives a four-fold decrease in $K_{0.5}$ for ATP for hydrolysis by going from pH 5.7 to 8.4. This may suggest that there is a pH effect on $K_{0.5}$ for Na^+ which goes beyond an effect on the affinity for the substrate.

The effect of a pH change is different at the low and the high ATP concentration; this seems to indicate that the pH effect is not, or not only, due to a change in protonation of the substrate. The bell-shaped curve for the effect of ATP at pH 8.4 and the different pH interval, in which the effect is seen at a low and a high ATP concentration, suggests that there are at least two different dissociable groups involved. One group is at the low ATP (or no ATP) and the more this is deprotonated, the higher the selectivity is for Na^+ over K^+. ATP involves another dissociable group, but with a higher pK, and when both groups are involved it is not a deprotonation but a protonation which increases the selectivity for Na^+ relative to K^+.

An effect of a change in pH on the selectivity suggests that the reaction with Na^+ and with K^+ at a given pH leads to pK changes — a Bohr effect. The decrease in $K_{0.5}$ for Na^+ with an increase in pH, a deprotonation, at a low ATP concentration suggests that the release of K^+ and uptake of Na^+ on the internal site leads to relase of a proton, a pK decrease. With saturating concentrations of ATP it is a decrease in pH, a protonation, which leads to a decrease in $K_{0.5}$ for Na^+, suggesting that in the presence of ATP the release of K^+ and uptake of Na^+ on the internal site leads to uptake of a proton, a pK increase.

The dissociable groups may be part of the sites for the cations, and/or they may be involved in an interchain interaction between the polypeptides and thereby indirectly influence the structure of the binding sites, a haemoglobin-like situation.

Hill plot

Considering that the Na^+ : K^+ transport ratio in a number of experiments has been found to be about 3 : 2 (1.5 : 1) (see Ref. 13) it is suggestive that n_{Na^+/K^+} varies around this value. If, however, the slope shows the competition ratio between Na^+ and K^+ for reaction with the 'Na^+-site', the present experiments suggest that this ratio is variable between 1 : 1 to 2 : 1, dependent on pH, ATP

concentration and on temperature. A variable ratio would be in agreement with some of the observations on stoichiometry, cf. Ref. 24.

Functional significance

It seems likely that it is of importance for the exchange of cations across the membrane by the system, that the Na^+ binding site is a flexible structure which can change its selectivity. But is there a functional significance of the effect of ATP, pH and temperature on $K_{0.5}$ for Na^+ which goes beyond this? Is the effect of importance for the regulation of the system in relation to the homeostatis of the cell? And/or does the uptake and release of protons, which seem to be dependent on the reaction with ligands, mean that the system besides Na^+ also pumps out protons, and by this not only regulates the internal K^+/Na^+ concentration, but also the H^+ concentration?

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