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ACTIVATION BY LITHIUM IONS OF THE INSIDE SODIUM SITES IN ($Na^+ + K^+$)-ATPase

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Summary

1. Purified pig kidney ATPase was incubated in 30—160 mM Tris-HCl with various monovalent cations. 130 mM LiCl stimulated a ouabain-sensitive ATP hydrolysis (about 5% of the maximal ($Na^+ + K^+$) activity), whereas 160 mM Tris-HCl did not stimulate hydrolysis. Similar results were obtained with human red blood cell broken membranes.

2. In the absence of Na^+ and with 130 mM LiCl, the ATPase activity as a function of KCl concentration showed an initial slight inhibition (50 μM KCl) followed by an activation (maximal at 0.2 mM KCl) and a further inhibition, which was total at 25 mM KCl. In the absence of LiCl, the rate of hydrolysis was not affected by any of the KCl concentrations investigated.

3. The lithium-activation curve for ATPase activity in the absence of both Na^+ and K^+ had sigmoid characteristics. It also showed a marked dependence on the total LiCl + Tris-HCl concentration, being inhibited at high concentrations. This inhibition was more noticeable at low LiCl concentrations.

4. In the absence of Na^+, 130 mM Li^+ showed promoted phosphorylation of ATPase from 1 to 3 mM ATP in the presence of Mg^{2+}. In enzyme treated with *N*-ethylmaleimide, the levels of phosphorylation in Li^+-containing solutions, amounted to 40% of those in Na^+- and up to 7 times of those in K^+-containing solutions.

5. The total ($Na^+ + K^+$)-ATPase activity was markedly inhibited at high buffer concentrations (Tris-HCl, Imidazole-HCl and tetramethylammonium-HEPES gave similar results) in cases when either the concentration of Na^+ or K^+ (or both) was below saturation. On the other hand, the maximal ($Na^+ + K^+$)-ATPase activity was not affected (or very slightly) by the buffer concentration.

6. Under standard conditions (Tris-HCl + NaCl = 160 mM) the Na^+-activation curve of Na^+-ATPase had a steep rise between 0 and 2.5 mM, a fall between 2.5 and 20 mM and a further increase between 20 and 130 mM. With 30 mM Tris-

HCl, the curve rose more steeply, inhibition was noticeable at 2.5 mM Na^+ and was completed at 5 mM Na^+. With Tris-HCl + NaCl = 280 mM, the amount of activation decreased and inhibition at intermediate Na^+ concentrations was not detected.

Introduction

It is generally accepted that the interaction of the intracellular Na^+ with the (Na^+ + K^+)-ATPase pump is related to the phosphorylation of the enzyme by ATP, in the presence of Mg^{2+} and Na^+, whereas K^+ uptake, in exchange for the release of Na^+ into the external medium, is associated with dephosphorylation of the enzyme. In an incomplete pumping cycle, it is believed that external Na^+ exchanges with internal Na^+ by means of another incomplete biochemical cycle, the phosphoryl group exchange between ATP and ADP [1,2]. In order to accept the notion that a given cation can be inwardly translocated by the Na^+ pump, in a K^+-like action, it must activate Na^+ efflux and it must also stimulate the dephosphorylation of the phosphoenzyme. Following the same criteria, any cation which can act as internal Na^+ in the pump cycle must be able to promote an active K^+ influx and also stimulate the phosphorylation of the enzyme from ATP in the presence of Mg^{2+}.

Several monovalent cations (Rb^+, Cs^+, NH_4^+, Li^+) are able, to a greater or lesser degree, to replace external K^+ in the activation of the Na pump [1—3]. However, the maximal velocities obtained with Li^+ (a cation which shows many chemical and physiological similarities with Na^+), both for Na^+ transport and ATP hydrolysis, never reached the velocity obtained with the other monovalent cations [3—5]. These similarities between Na^+ and Li^+ have prompted several investigators to study the possible Na^+-like effect from the inside of the cell. Until a short time ago, all efforts were either negative [6—8] or, where positive, accompanied by the fact that the complete absence of Na^+ could not be asserted [5,9]. Recently, however, Dunham and Senyk [10] have provided convincing evidence that Li^+, acting from the inside of human red cells, can promote a ouabain-sensitive K^+ influx and can be transported outwards by a process which is stimulated by external K^+ and inhibited by ouabain. These results very strongly suggest that Li^+ can replace Na^+ for the activation of the internal sites of the Na^+ pump. One difficulty with the Dunham and Senyk experiments is that the internal Na^+ concentration could not be reduced below 1 mM. Although no K_0^+-stimulated Na^+ efflux was seen under this condition [10], there is still the possibility of some Na^+-Li^+ interaction. On the other hand, no proof of simultaneous ATP hydrolysis was provided.

As discussed above, if Li^+ indeed can replace internal Na^+, then it would have two simultaneous and contrasting properties: (1) K^+-like actions (promoting dephosphorylation and competing with Na^+), and (2) Na^+-like actions. This means that it should be possible to observe, in the presence of Li^+ only, a ouabain-sensitive hydrolysis of ATP and a phosphorylation of the enzyme from ATP in the presence of Mg^{2+}. The purpose of this work was to investigate these possibilities. These results have been briefly reported elsewhere [11].

Materials and Methods

Purified ATPase and red cell membrane fragments. The purification of pig kidney ATPase was performed according to the method of Jorgensen [12] and stored at 0°C. The human red cell membrane fragments were prepared as described by Dunham and Glynn [13] with modifications. The cells were lysed in 1 mM Tris-EDTA (pH 7.4) and washed in 0.1 mM Tris-EDTA. After freezing and thawing twice, they were stored at −80°C in the same washing solutions. Before being used they were thawed and suspended in 50 mM Tris-HCl (pH 7.4) at 37°C.

ATPase activity. The ATPase activity was estimated by a modification of the method of Fiske and SubbaRow [14] using the Amidol reagent. For 4 ml total volume, 1 ml 5% ammonium molybdate in 1 M H_2SO_4 plus 1 ml 0.15 g/100 ml Amidol/1.5 g/100 ml Na_2SO_3 were used. Protein was determined by the method of Lowry et al. [15]. Unless otherwise stated, the concentration of the different ligands was: ATP (Na^+-free); 3 mM $MgCl_2$; 0.1 mM EGTA-Tris; Tris-HCl (pH 7.4, 37°C) 160 mM (or was matched to the monovalent cation concentration). Under all conditions studied the total hydrolysis of ATP was 10% or less. All assays were carried on in triplicate, at 37°C.

Phosphorylation by ATP. Phosphorylation levels were obtained after incubation for 10 s at 37°C. The reaction was started by adding 0.1 or 0.2 mg enzyme into 0.5 ml reaction mixture. The reaction was stopped by transferring 0.4 ml into 0.6 ml ice-cold solution containing: 50 mM ATP; 10 mM P_i; 20% $HClO_4$ and 10% polyphosphoric acid (Sigma) (w/v). After 15 min, the mixtures were centrifuged at 12 000 × *g* for 3 min and the pellet washed 5 times with the same solution containing 5% $HClO_4$. The final pellet was dissolved in 0.5 ml 1 M NaOH and incubated at 55°C for 30 min. After mixing, 0.4-ml aliquots were taken and dried on planchettes and the remaining 0.1 ml was used for protein determination by the method of Lowry et al. [15]. The ^{32}P activity was assayed in a gas-flow Beckman counter (background 1 cpm). When possible, the counting time was long enough to permit standard errors of 1% on Triplicate samples. In the *N*-ethylmaleimide treatment, the enzyme was incubated at 37°C in 25 mM imidazole (pH 7.5) at 20°C and 5 mM *N*-ethylmaleimide until the maximal ($Na^+ + K^+$)-ATPase activity was reduced by 95% (usually 45 min). Enough 2-mercaptoethanol was then added to obtain a final molar ratio of 2-mercaptoethanol : *N*-ethylmaleimide = 6 : 1.

Solutions. The sodium and potassium salts used were Ultrex grade from Baker. All other reagents were analytical grade. ATP was obtained from Boehringer and made Na^+-free by passing it through a Amberlite IR-120 column; after the pH was adjusted to 7.0 with Tris base, the solution was stored at 0°C. The sodium contamination of the solutions was checked by atomic absorption spectrophotometry and, in the ATP, was usually less than 0.01%. In Na^+-free Li^+ solutions, the Na^+ concentration in the complete reaction mixture, including the enzyme, was 30 μM or less. The [γ-^{32}P]ATP was obtained from New England Nuclear as a tetra(triethylammonium) salt with a specific activity of about 3000 Ci/mmol.

Results

ATPase activities in the presence of different monovalent cations. If Li^+ can occupy and activate the external K^+ sites and the internal Na^+ sites of the Na^+-pump, then it should be possible to detect a ouabain-sensitive hydrolysis of ATP in solutions where Li^+ is the only monovalent cation. This was done using Tris-HCl as a buffer, which has shown no Na^+-like effect in studies on ATP-ADP exchange reaction [16]. As a comparison, the hydrolytic activities using Na^+-containing solutions and in optimal ($Na^+ + K^+$) concentrations were investigated (Table I). In both human red cell broken membranes and purified pig kidney enzyme, ouabain-sensitive ATPase activity was detected in Na^+ alone (this was not investigated in red cells), ($Na^+ + K^+$) and Li^+ alone in the media. On the other hand, with the concentrations used (Table I), no ouabain-sensitive activity was observed when Tris-HCl was the only monovalent cation. The Li^+-stimulated hydrolysis was higher in broken red cell membranes than in purified pig kidney enzyme. However, the larger fraction of a ouabain-resistant hydrolysis in the former makes the purified enzyme a much better system to study its properties in detail. It must be emphasized that when the Na^+ concentration was checked by atomic absorption photometry the values found were 30 μM or less with both preparations.

Effects of K^+ on the Li^+-activated ATPase activity in the absence of Na^+. The experiments of the previous section suggest that Li^+ can simultaneously occupy both the Na^+ and K^+ activating sites of the ATPase. K^+ will compete with Li^+ for both sites. The competition would be expected to result in a mixture of inhibition of the ATPase activity (displacement of Li^+ from the internal Na^+ sites) or further activation (displacement of Li^+ from the external K^+ sites), the final result depending on the K^+ and Li^+ concentrations, as well as on the

TABLE I

MONOVALENT CATION ACTIVATED ATPase ACTIVITIES IN DIFFERENT PREPARATIONS

Human red blood cell (HRBC) broken membranes and purified pig kidney enzyme were prepared. The composition of the incubation (at 37°C) solutions was as follows: Na + K, 130 mM NaCl and 20 mM KCl; Na, 130 mM NaCl; Li, 130 mM LiCl. In all cases using 3 mM $MgCl_2$; 3 mM ATP (Na^+-free); 0.1 mM EGTA-Tris and Tris-HCl (pH 7.4 at 37°C). The rates of ATP hydrolysis were determined from phosphate production. The Na^+ concentration in solutions nominally Na^+-free was 30 μM or less. The results are expressed as the mean ± S.E.M. of triplicate determinations.

	Preparation		$Na^+ + K^+$	Na^+	Li^+	$Tris^+$
1	Broken HRBC	Total	3.67 ± 0.04	—	1.59 ± 0.01	1.45 ± 0.01
	(mmol $P_i \cdot h^{-1}$ per l cells)	Ouabain	1.81 ± 0.05	—	1.34 ± 0.01	1.42 ± 0.02
		Ouabain-sensitive	1.86 ± 0.06	—	0.25 ± 0.02	0.03 ± 0.03
2	Broken HRBC	Total	—	—	1.42 ± 0.01	1.16 ± 0.02
	(mmol $P_i \cdot h^{-1}$ per l cells)	Ouabain	—	—	1.19 ± 0.02	1.19 ± 0.01
		Ouabain-sensitive	—	—	0.23 ± 0.02	−0.03 ± 0.02
3	Pig kidney	Total	13.73 ± 0.02	0.53 ± 0.02	0.81 ± 0.02	0.13 ± 0.01
	(μmol $P_i \cdot mg^{-1} \cdot min^{-1}$)	Ouabain	0.12 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	0.13 ± 0.01
		Ouabain-sensitive	13.61 ± 0.02	0.40 ± 0.02	0.69 ± 0.02	0.01 ± 0.01

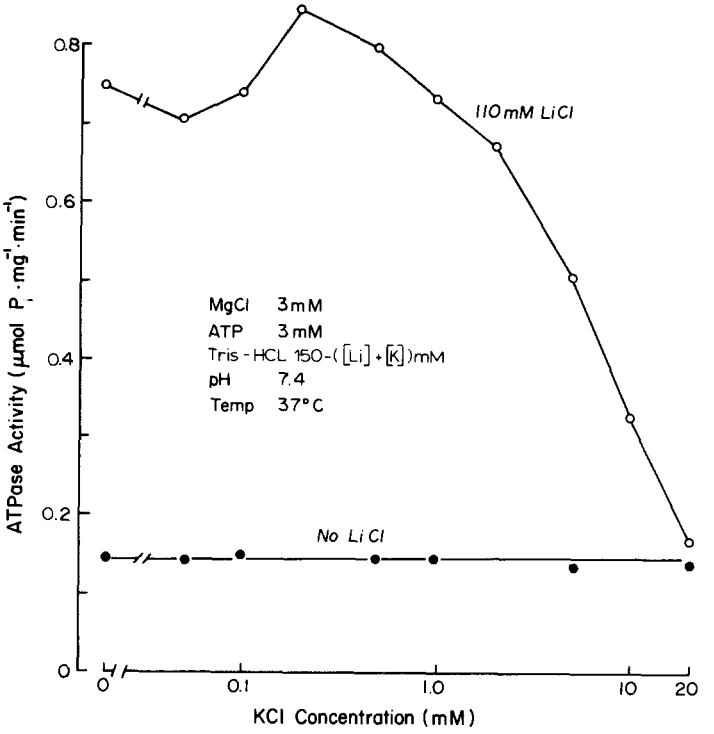

Fig. 1. Effect of varying KCl concentration on the rate of ATP hydrolysis by purified pig kidney enzyme in Na^+-free solutions in the presence and absence of 110 mM LiCl. Purified kidney enzyme was incubated in the presence of the ligands specified and the rate of ATP hydrolysis determined from phosphate production. All points represent the mean of three determinations. The Na^+ contamination in the whole incubations mixture was always 30 μM or less.

affinity of the sites for both cations. Fig. 1 shows the rate of ATP hydrolysis as a function of K^+ concentration, determined in the presence and absence of 110 mM Li^+. In the presence of Li^+ and, after a slight inhibition of 50 μM K^+ (which was statistically significant), the addition of K^+ produced an increase in the rate of hydrolysis above the Li^+ levels (which was maximal at 0.2 mM). At more than 0.2 mM, a further increase in K^+ concentration brought about an inhibition of the rate of hydrolysis, which was 50% at 5 mM K^+. At 25 mM K^+ the activity was similar to that observed in Tris-HCl. In the absence of Li^+, there was no detectable effect of K^+ on the ATPase activity.

Effect of Na^+ on the ATPase activity in the presence of Li^+. Atomic absorption photometry revealed that the maximal concentration of Na^+ in the Li^+-containing incubation mixtures (including the enzyme) was about 30 μM. Although this is a very low concentration, it could be enough to sustain some degree of phosphorylation in the absence of K^+. The activating effect of Li^+, which is a poor replacement for K^+, could be then purely a consequence of its well-known effect on the external enzyme sites. If this were the case, an increase in the Na^+ concentration to 100 or 150 μM should produce a further increase in the rate of ATP hydrolysis. However, in the presence of 130 mM lithium, an increase in Na^+ concentration up to 1 mM failed to produce a significant increment in the rate of ATP hydrolysis. On the other hand (Fig. 2), this

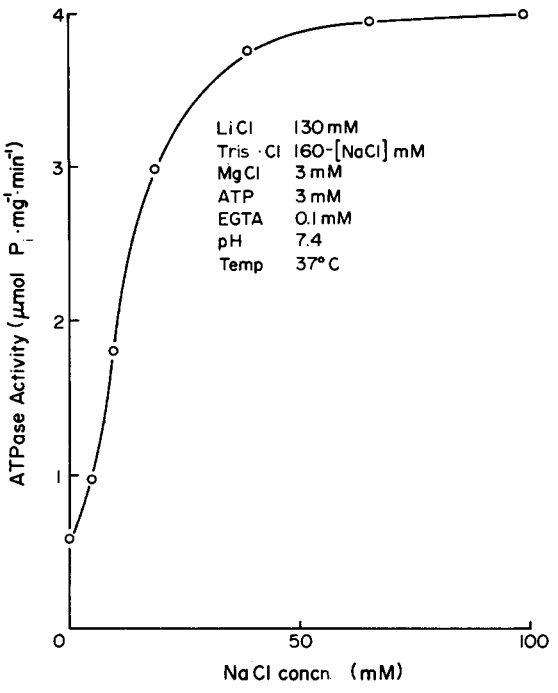

Fig. 2. The effect of NaCl concentrations on ATPase activity of purified pig kidney enzyme in the presence of 130 mM LiCl. All points are the mean of three determinations.

system still responds in the usual way to Na^+ concentrations in the millimolar range. Obviously in Fig. 2, at saturating Na^+ concentrations, the Li^+ effect must be almost all exerted through the external K^+ sites. In agreement with previous reports on ATPase activity and Na^+ transport [3—5], the maximal velocity in Na^+ plus Li^+ is lower than with Na^+ and other monovalent cations.

Activation curves for Na^+-ATPase and Li^+-ATPase as a function of Na^+ and Li^+ concentration. In Fig. 3 the activation curves for Na^+- and Li^+-activated ATP hydrolysis are plotted together. In both cases the total concentration of Tris-HCl plus that of NaCl (or LiCl) was maintained at 160 mM. The Na^+-activation of Na^+-ATPase showed a steep rise between 0 and 2.5 mM, an actual statistically significant decrease between 2.5 and 20 mM and a further increase between 20 and 130 mM. A complex Na^+-activation curve of Na^+-ATPase, with a plateau at intermediate Na^+ concentrations has already been described [16,17] and it has also been reported for ATP-ADP exchange in native enzyme [16,18], where a similar fall between 2.5 and 10 mM Na^+ was observed. The Li^+-activation curve in the absence of Na^+ showed a continuous sigmoid rise in the rate of hydrolysis. At low concentrations the activity in Li^+-containing solutions was lower than in those containing Na^+, but it became higher at concentrations above 50 mM. With both cations, the addition of 10^{-4} M ouabain reduced the rate of hydrolysis to the Tris levels.

The ability of Li^+ to promote enzyme phosphorylation by ATP. If Li^+ can indeed stimulate the inner Na^+ sites of ATPase, it must be able to promote its phosphorylation from ATP in the presence of Mg^{2+}. In some experiments the

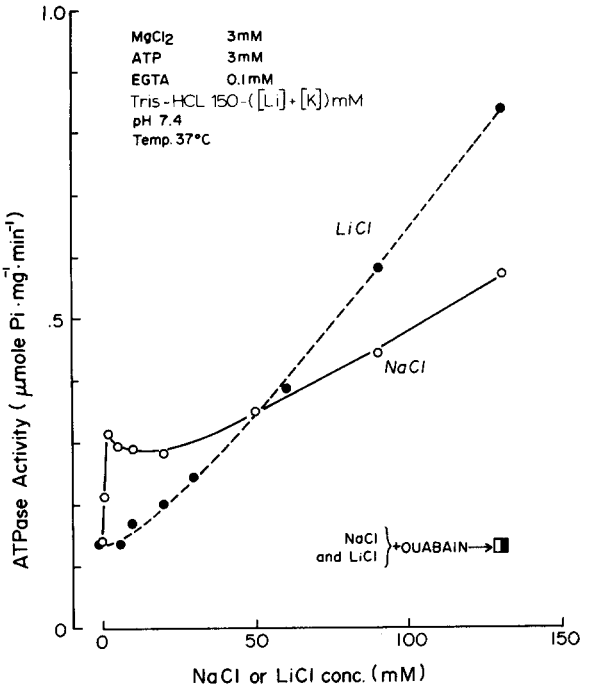

Fig. 3. A comparison between the rates of ATP hydrolysis by purified pig kidney enzyme at different concentrations of NaCl (○) and LiCl (●) in the absence of K^+. All points are the mean of 3 determinations. The squares on the lower right side are the rates observed with 10^{-4} M ouabain.

K^+-like effect of Li^+ was minimized by the use of *N*-ethylmaleimide, which is thought to prevent formation of K^+-sensitive phosphoenzyme [19,20]. The levels of enzyme phosphorylation after 10 s in media containing Tris-HCl, NaCl, LiCl or KCl were determined. Several attempts where these experiments were done with 4 μM ATP, either at 0°C of 37°C, failed to show any difference between the Li^+, Tris and K^+ levels, although a large Na^+-dependent phosphorylation was observed. This was true both in native and *N*-ethylmaleimide treated enzymes.

Because the experiments of ATP hydrolysis were all performed at 37°C and with 3 mM ATP, it was decided to reexamine this problem but with ATP at 1 and 3 mM (Table II). Both in native, but more conspicuously in *N*-ethylmaleimide-treated, enzyme, LiCl was able to promote phosphorylation by ATP above the levels found in Tris-HCl or KCl. In all cases however, the levels of phosphorylation in LiCl were lower than in NaCl; this Na^+-Li^+ difference was more marked in the native enzyme. Another interesting observation regarding the possible mechanism of action of *N*-ethylmaleimide was that both the Na^+- and Li^+-dependent phosphorylations in *N*-ethylmaleimide-treated enzyme were reduced by the addition of 10 mM K^+. The amount of that inhibition was 25% for Na^+ and 70% for Li^+.

Effect of ionic strength on the rate of ATP hydrolysis. A common observation was that when concentrations of LiCl higher than 130 mM were used, the rate of ATP hydrolysis either did not increase any further or was even reduced.

TABLE II

EFFECT OF DIFFERENT MONOVALENT CATIONS ON PHOSPHORYLATION BY ATP OF PURIFIED PIG KIDNEY ENZYME

Purified pig kidney enzyme (0.1 mg for 1 mM ATP and 0.2 mg for 3 mM ATP) was mixed with 0.5 ml reaction mixture containing the ligands as specified for 10 s at 37°C. The Tris-HCl concentration was as in Table I and the $MgCl_2$ concentration was matched to that of ATP. In all cases 0.1 mM EGTA was used. Denatured enzyme refers to the same procedure but where the enzyme was first added to the $HClO_4$ stopping solution and then mixed with the incubation mixture. The results are expressed as the mean ± S.E.M. of triplicate determinations.

ATP (mM)		Enzyme treatment	
		Native (nmol P_i/mg protein)	*N*-ethylmaleimide (nmol P_i/mg protein)
1	Denatured enzyme	0.011 ± 0.001	0.007 ± 0.001
	160 mM Tris-HCl	0.041 ± 0.001	0.037 ± 0.002
	150 mM Tris-HCl/10 mM KCl	0.023 ± 0.001	0.029 ± 0.002
	130 mM NaCl	0.770 ± 0.015	0.367 ± 0.006
	130 mM LiCl	0.074 ± 0.003	0.140 ± 0.005
3	Denatured enzyme	—	0.045 ± 0.005
	160 mM Tris-HCL	—	0.092 ± 0.009
	150 mM Tris-HCl/10 mM KCl	—	0.081 ± 0.002
	130 mM NaCl	—	0.493 ± 0.007
	130 mM NaCl/10 mM KCl	—	0.373 ± 0.015
	130 mM LiCl	—	0.222 ± 0.003
	130 mM LiCl/10 mM KCl	—	0.099 ± 0.003
	130 mM KCl	—	0.067 ± 0.002

This could be an inhibitory effect of Li^+ per se or a consequence of changes in the ionic strength. In order to investigate this, activation curves for ATP hydrolysis as a function of LiCl concentration were determined at different concentrations of the Tris-HCl buffer. These results (Fig. 4) indicate that there was an inhibitory effect of Tris-HCl. The inhibition was more noticeable at low Li^+ concentrations, suggesting that part of it was a consequence of reduction in the apparent affinity of the enzyme towards Li^+.

The fact that (1) with 30 mM Tris-HCl, inhibition also developed at high Li^+ and (2) all curves seem to approach the same value at high LiCl + Tris-HCl concentrations, could suggest that these results are more related to an unspecific ionic strength effect rather than to inhibition by Tris-HCl. On the other hand, if part of this inhibition is due to reduction in the apparent affinity of the enzyme for Li^+, this could have happened on the internal sites, external sites or both. The next group of experiments (Table III) was designed to test these possibilities. The results with $(Na^+ + K^+)$-ATPase activity showed a marked inhibition at high buffer concentrations when the internal Na^+ sites, external K^+ sites, or both, were unsaturated. On the other hand, little inhibition was detected when both sites were at or near saturation. Also the rates of hydrolysis in NaCl, LiCl and Tris-HCl alone were largely reduced at high buffer concentrations. The changes in the rates of hydrolysis were rather similar when Tris-HCl or imizadole-HCl were used. In another experiment for the $(Na^+ + K^+)$-ATPase activity at low Na^+ and K^+, similar results were obtained using tetramethylammonium-HEPES buffer combination. This suggests that the inhibi-

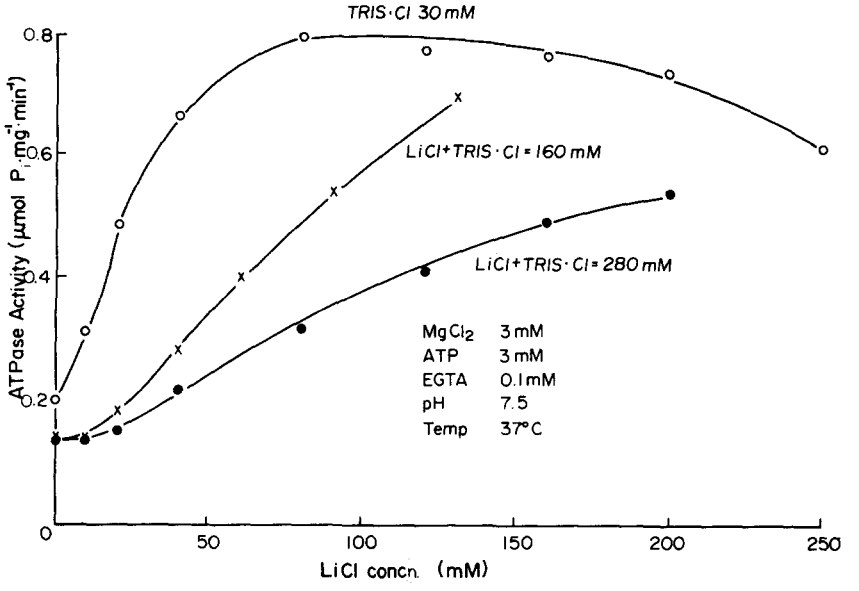

Fig. 4. The effect of increasing Tris-HCl concentration on the rate of ATP hydrolysis by purified pi kidney enzyme as a function of the LiCl concentration in solutions free of both Na^+ and K^+. ○, 30 mM Tris-HCl; X, Tris-HCl + LiCl = 160 mM; ●, Tris-HCl + LiCl = 280 mM. All points represent the mean o three determinations. The experiments at 30 mM Tris-HCl and 280 mM Tris-HCl + LiCl were performe simultaneously. As a comparison, a previous experiment with Tris-HCl + LiCl = 160 mM was alsc included.

TABLE III

EFFECTS OF BUFFER CONCENTRATION ON THE ATPase ACTIVITY OF PIG KIDNEY ENZYME INCUBATED WITH DIFFERENT MONOVALENT CATIONS

Purified pig kidney enzyme was prepared and ATPase activities were determined from phosphate produc tion at 37°C. The results are expressed as the mean ± S.E.M. of three determinations, in $\mu mol\ P_i \cdot mg^{-1}\ min^{-1}$.

Buffer	Monovalent cations		
	None	80 mM Na^+	80 mM Li^+
30 mM Tris-HCl	0.21 ± 0.01	0.44 ± 0.01	0.80 ± 0.02
200 mM Tris-HCl	0.15 ± 0.01	0.31 ± 0.01	0.30 ± 0.01
30 mM Imidazole-HCl	0.24 ± 0.01	0.39 ± 0.01	0.90 ± 0.03
200 mM Imidazole-HCl	0.17 ± 0.01	0.27 ± 0.01	0.40 ± 0.01

Buffer	Monovalent cations		
	72 mM K^+ 8 mM Na^+	0.15 mM K^+ 80 mM Na^+	10 mM K^+ 70 mM Na^+
30 mM Tris-HCl	1.16 ± 0.05	1.22 ± 0.03	9.00 ± 0.03
200 mM Tris-HCl	0.53 ± 0.03	0.55 ± 0.03	8.70 ± 0.05
30 mM Imidazole-HCl	1.29 ± 0.07	1.10 ± 0.03	8.98 ± 0.03
200 mM Imidazole-HCl	0.70 ± 0.04	0.43 ± 0.02	8.90 ± 0.06

tion seen at high buffer concentration is likely to be an ionic strength effect.

The results in this section indicate that ionic strength strongly influences the interactions between monovalent cations and their activating sites in the (Na^+ + K^+)-ATPase. It would be interesting to know if similar effects can also be found for other sites where these cations are believed to have a regulatory function. One site easily accessible to study is the external site at which Na^+ inhibits with high affinity [21]. This is manifested by a reduction in the rate of ATP-ADP exchange or ATP hydrolysis when Na^+ concentration is increased from 2.5 to 10—20 mM [16,18,21] (Fig. 3). Na^+-activation curves for Na^+-ATPase activity were then determined at 30 mM Tris-HCl and at a total Tris-HCl + NaCl concentration of 280 mM (Fig. 5). At 30 mM Tris-HCl, there was a very steep rise reaching its maximum at 1 mM Na^+ followed by a marked inhibition already noticeable at 2.5 mM Na^+ and maximal at 5 mM Na^+. At higher concentrations, the rate of hydrolysis increased with tendency towards saturation. The steepness of the early activation and both the steepness and magnitude of the inhibition are much more marked than those seen in Fig. 3, where the total Tris-HCl + NaCl was 160 mM. In contrast with the above, when the total Tris-HCl +

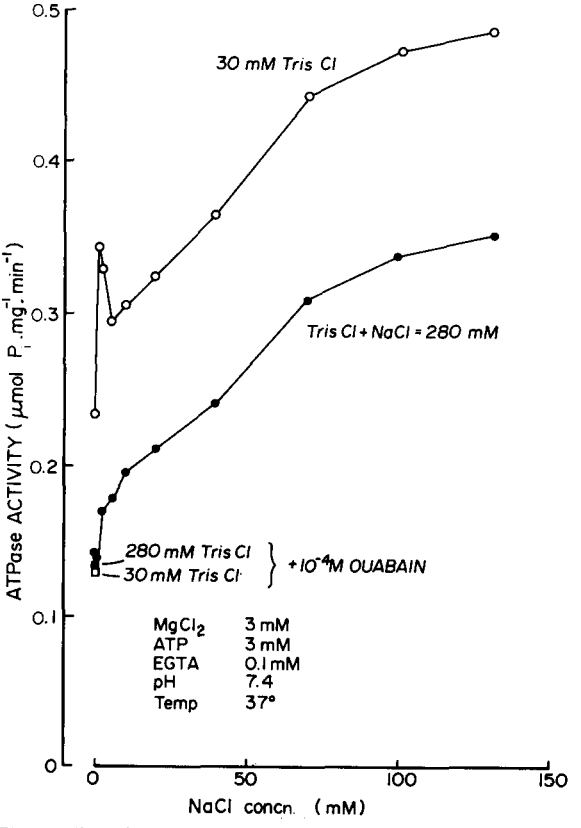

Fig. 5. The effect of increasing Tris-HCl concentration on the rate of ATP hydrolysis by purified pig kidney enzyme as a function of NaCl concentration in solutions free of K^+ and Li^+. ○, 30 mM Tris-HCl; ●, Tris-HCl + NaCl = 280 mM. □, 30 mM Tris-HCl; ■, 280 mM Tris-HCl in the presence of 10^{-4} M ouabain. All symbols are the mean of three determinations.

NaCl was 280 mM, the activation curve showed a much slower rise (slower than in Fig. 3) and no actual decrease in the rate of hydrolysis at intermediate concentration of Na^+. Perhaps the only sign of possible inhibition is a downward bend in the curve at about 40 mM Na^+. At all Na^+ concentrations, the rates of hydrolysis were inhibited by increasing the Tris-HCl concentration and, above 5 mM, both curves rose in a similar way. In agreement with the results of Table III, in the absence of Na^+, the rates of ATP hydrolysis were also sensitive to the Tris-HCl concentration. All the increase observed when Tris-HCl was reduced from 280 mM to 30 mM was abolished by 10^{-4} M ouabain. With 280 mM Tris-HCl (as was seen for 160 mM Tris-HCl, Fig. 3), there was no ouabain-sensitive ATPase activity.

Discussion

All the data presented in this paper are consistent with Li^+ stimulating the inside Na^+ sites of the ATPase enzyme. This Na^+-like effect of Li^+ is superimposed on its widely-accepted K^+-like effect on the external membrane side.

The existence of ATP hydrolysis in the presence of Li^+ and $MgCl_2$ has been reported [5,9]. However, in one of these cases [5], the experimental procedure was not explained in detail and one cannot be sure if some Na^+ was present as a contaminant; in addition, no data on the effect of ouabain were provided. In the other work [9], the red cell ghosts contained 4 mM disodium-ATP, which would give enough Na^+ to activate the Na^+ pump. In the present experiments, careful Na^+ analysis by atomic absorption spectrophotometry revealed an Na^+ contamination of no more than 30 μM. The additional observation that, in Li^+ solutions, increasing the Na^+ concentration up to 1 mM failed to promote any further stimulation of hydrolysis seems to rule out any effect due to contamination of Na^+ in the micromolar range. ATP hydrolysis stimulated by Li^+ acting on the Na^+ sites has also been observed recently in inside-out membrane vesicles of red cells (Blostein, R., personal communication).

The K^+ effects on the ATP hydrolysis in the presence of Li^+ are also in agreement with a Na^+-like effect of the latter. The inhibition between 0.2 mM and 25 mM KCl could be accounted for by K^+-Li^+ competition at the inside Na^+ sites of the enzyme, and the small activation between 0.05 mM and 0.2 mM KCl would agree with a binding of potassium to the external K^+ sites. However, a quantitative analysis, based on the estimated apparent affinity constants for these cations, would be rather difficult due to the overlapping of activation and inhibition. In addition the picture is further complicated by the small inhibition at 0.05 mM KCl for which we have no explanation. Furthermore, it would seem that the way Li activates the ATPase is not exactly the same way Na and K do. This view is supported by the fact that the maximal rates of (Na^+ + Li^+) ATPase activity are always lower than those of the (Na^+ + K^+) activity (ref. 5 and this paper). Similar results were also obtained for the rates of Na^+ efflux activated by external Li^+ and K^+ [3,4].

The most convincing evidence indicating a Na^+-like effect of Li^+ on the ATPase enzyme comes from its ability to phosphorylate the enzyme at 37°C in the presence of millimolar concentrations of ATP and $MgCl_2$. Although this was more striking in enzyme treated with *N*-ethylmaleimide, it was also

statistically significant in the native enzyme. It is interesting that we consistently failed to see any Li^+-promoted phosphorylation when the ATP was kept in the micromolar range. This happened in native as well as in enzyme treated with *N*-ethylmaleimide, both at 0 and 37°C. Earlier attempts by other laboratories to phosphorylate ATPase from ATP in the presence of Li^+ and Mg^{2+} have failed [19,20,22]. Perhaps the reason for the failure is that, in those cases, either the temperature was subnormal for the species or the ATP was in the micromolar range.

As shown in Table II, *N*-ethylmaleimide reduced the Na^+-dependent phosphorylation by about 50% under conditions, where the $(Na^+ + K^+)$-ATPase activity was inhibited by more than 95%. This agrees with experiments with the eel enzyme [23], where the ATPase activity was always more sensitive to *N*-ethylmaleimide than phosphorylation by ATP. On the other hand, using enzyme from ox brain, this treatment reduced in the same proportion both ATPase activity and ATP binding [24]. It is believed that *N*-ethylmaleimide blocks the conversion of the enzyme into a form which can be dephosphorylated by K^+ [19,20]. However, the addition of 10 mM K^+ partially inhibited both the Na^+- and Li^+-dependent phosphorylations (Table II). This inhibition by K^+ could have been accomplished by a reduction in the rate of incorporation, an increase in the rate of dephosphorylation, or both. In the Na^+ experiments, the ratio of the K^+ to Na^+ was 1/13. This ratio seems too small to shift the enzyme from the Na^+-form into the K^+-form, both in the absence and presence of *N*-ethylmaleimide [25]. This makes it unlikely that inhibition of phosphorylation was due to K^+ acting on the inside Na^+ sites of the enzyme. So if inhibition has occurred on the inside it must have been on different sites. The alternative is that K^+ was actually acting on the outside, either increasing the dephosphorylation rate or, on other types of sites, reducing the rate of incorporation. The existence of external sites other than the catalytic ones has already been proposed [26]. These results concur with a similar K^+ inhibition reported for the Na-dependent ATP-ADP exchange reaction in the presence of 4 mM ATP in *N*-ethylmaleimide-treated guinea pig enzyme [27].

The experiments with different buffers and buffer concentrations indicate that the ionic strength markedly influences the interactions between monovalent cations and the $(Na^+ + K^+)$-ATPase. The fact that the inhibition of the $(Na^+ + K^+)$ activity was readily seen when the sites were unsaturated suggests that a decrease in the apparent affinities for Na^+ and K^+ was involved. This agrees with a decrease in the apparent affinity for K^+ in the activation of the *p*-nitrophenylphosphatase activity reported in ox brain ATPase when the ionic strength was increased [28]. An increase in Tris-HCl concentration was also reported to reduce the affinity of the ox enzyme for ATP [24] and for strophanthidin [29]. Interestingly enough, the hydrolysis rates in 80 mM Na^+ and, especially, in 80 mM Li^+ were very sensitive to the buffer concentration. With 80 mM Na^+ the internal Na^+ sites must have been completely saturated; that means the ionic strength affects either a spontaneous dephosphorylation in the absence of K^+ or a Na^+-dependent dephosphorylation due to a K^+-like effect of Na^+. With 80 mM Li^+, in the absence of both Na and K, we would expect the external and internal sites to be almost completely saturated. However, the ATPase activity under these conditions was as much sensitive to buffer con-

centration as was the ($Na^+ + K^+$) activity with unsaturated sites; this may be related to the inhibition of the Li^+-ATPase at high Li^+ concentration, which is very unlikely to involve reduction of the affinity towards Li^+. Perhaps this is also a consequence of an imperfect (or incomplete) interaction between Li^+ and the enzyme. The ouabain-sensitive ATPase activity seen in the presence of 30 mM Tris-HCl (plus ATP and $MgCl_2$) could be a consequence of some Na^+ contamination, but it could also be a spontaneous rate of hydrolysis which in the absence of any Na^+ and K^+ is triggered by a low ionic strength.

Finally, at normal and low ionic strengths, a high affinity inhibitory site for Na^+ could be readily demonstrated. The fact that in some cases it appears just as a plateau in the Na^+ activation curve of the Na^+-ATPase [16,17], whereas in others an actual fall in the activity was observed, could be a consequence of different ionic strengths and different sensitivity to ionic strength of the preparations used.

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