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THE EFFECT OF ATP AND Ca^{2+} ON THE CELL VOLUME IN ISOLATED KIDNEY TUBULES*

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SUMMARY

The effect of external ATP on the steady-state levels of water and electrolytes in isolated renal tubules of healthy adult rabbits was studied:

1. In the absence of external Ca^{2+}, ATP produced an increase of tissue Na^+, Cl^-, and Ca^{2+}, and a loss of K^+, at all pH values tested (pH 6.2 to 8.2); only at pH 7.2 was a marked ATP-induced cellular swelling observed. No such effects of ATP were found in the presence of 2.5 mM $[Ca^{2+}]_0$.

2. The swelling effect of ATP was not affected by 0.1 mM and 0.5 mM ouabain or the absence of Na^+ (Li^+ saline) and was in part reversed by raising $[Ca^{2+}]_0$ to 2.5 mM. Increased Mg^{2+} (5.5 mM final concn) could replace Ca^{2+} in preventing the effect of external ATP.

3. ADP and GTP did not produce changes of cell volume under conditions where ATP was effective.

4. Isolated kidney tubules were found to hydrolyse added ATP or other triphosphonucleotides. The rate of hydrolysis was increased by raising the medium pH from 6.2 (0.13 μmole P_i/mg protein per 10 min) to 8.2 (0.24 μmole P_i/mg protein per 10 min) and was to some extent inhibited by Ca^{2+}.

5. A soluble protein with a nucleotidase specificity, activated by Mg^{2+} or Ca^{2+}, was isolated from the tubules. At pH 7.2 this protein was precipitated from solution by the addition of ATP and Ca^{2+}; this phenomenon was found to be specific for ATP.

6. It is suggested that external ATP affects the water and ionic contents of the cells of kidney tubules by an interaction with the cell membrane and Ca^{2+}, thus involving the physical (contractile?) properties of the membrane structure and/or membrane permeability.

INTRODUCTION

Convincing evidence has been repeatedly reported that in addition to the leak-and-pump mechanism, an ouabain-insensitive system is involved in the volume

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regulation of kidney cells (for review, see ref. 1). This system has been shown to be sensitive to Ca^{2+} and pH, and an involvement of cell ATP has been demonstrated[2]. Recently, a contractile protein with an ATPase (ATP hydrolase, EC 3.6.1.3.) activity stimulated by Ca^{2+} has been shown to be present in (or at) the membrane of red blood cells[3], and evidence has been obtained demonstrating that Ca^{2+} and ATP affect the red cell volume by modification of the membrane deformability[4] and permeability[5,6]. External ATP has also been shown to produce changes in the water and ionic contents of ascites tumor cells[7,8]. It was thus of interest to investigate whether the kidney cells showed properties similar to those observed in erythrocytes and ascites tumor cells, *viz.* (a) the interrelationship between cell volume, Ca^{2+} and ATP, and (b) a (contractile) protein with an ATPase activity stimulated by Ca^{2+}, located at or near the cell membrane.

In order to avoid difficulties associated with the complex diffusional pathways in the kidney tissue, isolated tubules[9] instead of kidney cortex slices were used in these experiments.

After conclusion of this investigation, a report has appeared[10] on a Ca^{2+}- and Mg^{2+}-stimulated ATPase from kidney cortex. The properties of this enzyme are rather similar to those described below.

METHODS

The experiments described below were carried out mostly using suspensions of renal tubules isolated from rabbit kidney cortex (see ref. 2 for details of preparation and experimental techniques). The tubules were incubated aerobically (O_2) at 25 °C in salines of the Krebs–Ringer type containing 3 % (v/v) calf serum at pH varying from 6.2 to 8.2, and the effects of ATP and Ca^{2+} on the steady-state levels of tissue water and electrolytes were investigated. As in the previous communication[2], these results are expressed as kg water and mequiv of electrolytes per kg tissue dry wt.

The hydrolysis of added ATP by the tubules was studied as follows: The tissue was first brought to steady-state level of its components (aerobic preincubation of 100 mg tubules at 25 °C in 10 ml saline with or without Ca^{2+} for 30 min). Subsequently, a 50 mM ATP solution was added (final concn 4 mM) and 2-ml samples were removed at time 0 and usually at 60 min for the determination of P_i and protein. The spontaneous hydrolysis of ATP was followed under identical conditions using saline only (without tissue).

The suspension of isolated tubules was also used as the starting material for the preparation of ATPase extracts.

Unless otherwise stated, Ca^{2+}-activated ATPase was prepared by extracting kidney tubules for 1 h at 0 °C with a 10-fold volume of a modified Edsall solution (composition[11]: 0.6 M LiCl, 0.01 M Na_2CO_3, 0.04 M $NaHCO_3$, 1 mM EDTA, final pH 8.5). The suspension was then centrifuged for 30 min at 10000 rev./min at 0 °C using a RC2-B Sorvall refrigerated centrifuge. The pH of the supernatant was adjusted to 7.2 with 0.33 M HCl.

The ATPase activities of the preparation were measured essentially as described by Nakamaru *et al.*[12].

Mg^{2+}-ATPase assay: The tube contained 0.25 ml tissue extract, 0.25 ml 0.4 M Tris–HCl buffer, pH 7.4, 0.25 ml 40 mM $MgCl_2$, 0.25 ml 33 mM ATP (Tris salt), the final volume was adjusted to 2 ml with water.

Ca^{2+}-ATPase: In the above mixture, 0.25 ml 40 mM $CaCl_2$ was substituted for $MgCl_2$.

$(Na^+ + K^+)$-activated ATPase: 0.25 ml tissue extract, 0.25 ml 0.4 M Tris–HCl buffer, pH 7.4, 0.25 ml 0.04 M $MgCl_2$, 0.25 ml 1 M NaCl, 0.25 ml 0.2 M KCl, 0.25 ml 5 mM ouabain was added to a portion of the test tubes. The final volume was adjusted to 2 ml with water.

In each type of assay, the reaction was allowed to proceed at 37 °C for 30 min, then was stopped by transfer of the tubes to ice and addition of 0.4 ml ice-cold 35 % (w/v) trichloroacetic acid. The tubes were then centrifuged for 10 min at 10000 rev./min at 0 °C. In the supernatant P_i was measured by the method of Fiske and SubbaRow[14]. The protein pellet was dissolved in 1 M NaOH, and in the solution protein was determined by either the biuret reaction or the procedure of Lowry *et al.*[13], employing bovine serum albumin as standard. The activity of the ATPase was then expressed as μmoles P_i liberated/mg protein per h. In each case, a correction had to be applied for the spontaneous hydrolysis of ATP, measured as the amount of P_i liberated under identical experimental conditions in the absence of tissue extract. This correction was required in view of the relatively high content of P_i in some samples of ATP. The activity of the Mg^{2+}- or Ca^{2+}-activated ATPase was defined as the difference between P_i liberated in the presence and the absence of the respective divalent cations. As usual, the $(Na^+ + K^+)$-ATPase activity was taken to be the difference between the amount of P_i liberated in the absence and presence of ouabain.

In some of the experiments subcellular fractions were prepared by the procedure of Fitzpatrick *et al.*[15], and identified by microscopic and enzymatic methods[15]. No attempt was made to prepare highly purified preparations. These fractions were then used for an assay of ATPase activity; $(Na^+ + K^+)$-ATPase activity was determined in the preparations directly, while for the assay of the Ca^{2+}-activated ATPase the fractions were first extracted with the Edsall solution (see above); no $(Na^+ + K^+)$-ATPase activity was found in the extract. An extract of the light microsomes was also employed to measure the effect of Ca^{2+} and ATP on the absorbance at 550 nm in a Perkin–Elmer spectrophotometer at 25 °C.

Reagents: All chemical reagents used were of analytical grade. ATP (disodium and Tris salts), ADP (Tris salt), GTP, ITP, and UTP were purchased from Sigma, St. Louis, Mo., U.S.A. The pH of the nucleotide solutions was checked and where required, adjusted to pH 7.4 using 0.3 M Tris.

RESULTS

Effect of external ATP on the water and ionic contents of isolated tubules

Preliminary experiments showed that in the presence of Ca^{2+} (2.5 mM), external ATP (4 mM) did not affect the level of water and bulk electrolytes in isolated tubules; tissue Ca^{2+} was increased (Table I).

In Ca^{2+}-free media ($[Ca^{2+}]_0 < 0.1$ mM), marked effects of ATP on tissue water and electrolytes were obtained (Fig. 1 and Table II). At pH 7.2, ATP at concentrations of 2 mM and higher produced a net increase in tissue water, Na^+, Cl^-, and Ca^{2+}, as well as a considerable loss of K^+; at lower ATP concentrations, no significant effects were found. Concerning the effect of ATP on tissue Ca^{2+}, it has been pointed out previously[2] that Ca^{2+} originating from the calf serum added to the incubation saline

may have served here as a source for the cellular accumulation of this electrolyte, particularly in the presence of increased P_i produced by the hydrolysis of ATP (see below).

It has been shown previously[2] that pH markedly affects the water and electrolyte content of kidney cortex cells incubated in Ca^{2+}-free saline. It thus was of interest whether the observed effect of external ATP was dependent on medium pH. The results of such studies are shown in Fig. 2 and Table II. It will be seen that at pH 6.2 ATP (4 mM) did not produce cellular swelling while increasing the level of tissue Na^+ and decreasing that of K^+. With increasing pH, ATP had a considerable swelling effect, characterized particularly by a net increase in cell Na^+ and Ca^{2+}, and a marked decrease in K^+. Raising the pH still further (pH 7.7 and 8.2), the cells in the controls swelled in the absence of Ca^{2+}, as demonstrated previously[2]; under these conditions, ATP depressed the tissue water and Na^+, whereas the level of K^+ was lower than in the control. At all pH values, the presence of ATP brought about decrease in tissue K^+ and Mg^{2+}, and an increase in Ca^{2+}.

It was noted that the Mg^{2+} content of the tissue significantly increased with increasing pH.

The ATP-induced increase in tissue Na^+ and loss of K^+ recalled effects produced

TABLE I

EFFECT OF ATP ON THE WATER AND THE IONIC CONTENT OF ISOLATED TUBULES

The tissue preparation was incubated aerobically (O_2) for 1 h at 25 °C in saline (pH 7.2) containing 2.5 mM Ca^{2+}. All values are the mean of four determinations.

	Control (no ATP)	*4 mM ATP*
Water (kg/kg dry wt)	3.37	3.25
Na^+ (mequiv/kg dry wt)	253	258
K^+ (mequiv/kg dry wt)	300	287
Ca^{2+} (mequiv/kg dry wt)	21.1	57.4
Cl^- (mequiv/kg dry wt)	260	230

TABLE II

EFFECT OF ATP ON THE WATER AND ELECTROLYTE CONTENT OF ISOLATED TUBULES

Tubules were incubated aerobically for 1 h at 25 °C in Ca^{2+}-free saline in absence (line a) or in presence of 4 mM ATP sodium salt (line b). Values are the mean ± S.E. of eight determinations from two tubule preparations.

		Water (kg/kg dry wt)	*Na^+ (mequiv/kg dry wt)*	*K^+ (mequiv/kg dry wt)*	*Ca^{2+} (mequiv/kg dry wt)*	*Mg^{2+} (mequiv/kg dry wt)*
pH 6.2	(a)	3.40 ± 0.05	322 ± 6	239 ± 5	10.3 ± 0.4	26.3 ± 0.1
	(b)	3.49 ± 0.03	446 ± 17	100 ± 2	28.3 ± 3.9	20.5 ± 0.1
pH 7.2	(a)	3.27 ± 0.02	326 ± 5	241 ± 5	10.8 ± 0.5	28.0 ± 0.4
	(b)	4.78 ± 0.02	675 ± 13	93 ± 3	33.4 ± 0.9	25.4 ± 0.5
pH 8.2	(a)	6.13 ± 0.05	866 ± 21	85 ± 3	30.1 ± 0.6	32.2 ± 0.9
	(b)	5.35 ± 0.06	782 ± 16	73 ± 2	44.0 ± 1.0	26.8 ± 0.7

by an inhibition of the Na^+ pump. It was therefore of interest to compare the effect of ATP in Na^+-free (Li^+) medium and in Na^+ medium in the presence of ouabain.

In Na^+-free saline, isolated kidney tubules incubated at pH 7.2 in the absence of Ca^{2+} markedly swelled, as reported previously; addition of ATP produced a further increase in tissue water (Fig. 3). Similar results were obtained using Na^+ salines in the presence of ouabain (Table III).

The described effect of ATP was found to be specific for this nucleotide; 4 mM GTP or ADP were ineffective, as shown in Table IV.

The external Mg^{2+} concentration was found to be of importance for the effect of ATP on tissue water and electrolytes. An increase of $[Mg^{2+}]_0$ from 1.5 to 5.5 mM completely suppressed the swelling effect of ATP, and also eliminated associated changes of tissue electrolytes (Table V). In fact, the values found in the presence of ATP and 5.5 mM Mg^{2+} were essentially identical with those found when ATP was

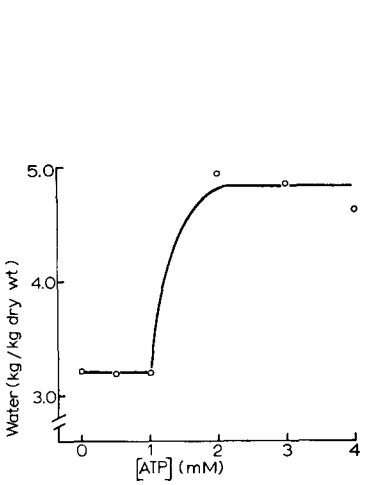

Fig. 1. Effect of external ATP on the water content of isolated kidney tubules. Tubule suspensions were incubated aerobically (O_2) for 1 h at 25 °C in Ca^{2+}-free saline at pH 7.2 in the presence of varying concentrations of ATP. Each value is the mean of four analyses.

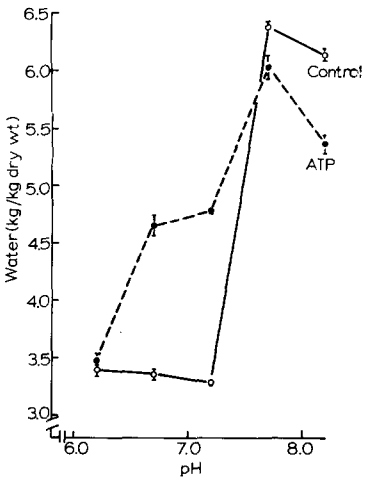

Fig. 2. Effect of pH and external ATP on the water content of isolated kidney tubules. Tubules were incubated aerobically (O_2) at 25 °C in Ca^{2+}-free salines at different pH values for 1 h without (control, ○) and with 4 mM ATP (●). Each value is the mean of eight analyses, ± S.E.

TABLE III

EFFECT OF ATP AND OUABAIN ON THE WATER AND IONIC COMPOSITION OF ISOLATED TUBULES

Preparations were incubated aerobically for 1 h at 25 °C in Ca^{2+}-free saline, pH 7.2, with no additions (control), in the presence of 4 mM ATP (Tris salt), 0.5 mM ouabain, or both compounds. Values are the means ± S.E. of eight determinations.

	Control	*ATP*	*Ouabain*	*ATP + ouabain*
Water (kg/kg dry wt)	3.27 ± 0.02	3.60 ± 0.08	4.81 ± 0.09	5.34 ± 0.09
Na^+ (mequiv/kg dry wt)	326 ± 5	423 ± 10	642 ± 11	690 ± 12
K^+ (mequiv/kg dry wt)	241 ± 5	129 ± 1	67 ± 1	70 ± 5
Cl^- (mequiv/kg dry wt)	257 ± 10	383 ± 8	564 ± 4	615 ± 8
Ca^{2+} (mequiv/kg dry wt)	10.8 ± 0.5	27.2 ± 4.0	10.2 ± 1.5	44.5 ± 0.9

added to standard saline (2.5 mM Ca^{2+}). The results obtained thus suggest that Mg^{2+} can to some extent replace Ca^{2+} at or near the membrane and thus prevent the swelling effect of external ATP.

Fig. 4 shows that the effect of ATP in Ca^{2+}-free medium was at least partly reversible. In these experiments the tissue was first incubated in the presence of 4 mM ATP and thus the swelling process started. After 1 h incubation, Ca^{2+} was added to a portion of the tubule suspension (final $[Ca^{2+}]_0$ 2.5 mM), and the tissue water (and electrolytes) were then followed for further 60 min and compared with controls where no Ca^{2+} was added. It will be seen that the addition of Ca^{2+} produced a significant small extrusion of water in the first 10 min (0.3 kg). Subsequently, a new steady state of cell water was reached, but, as opposed to the behavior of ascites tumor cells[7], did not return to the original value. The net extrusion of water was

TABLE IV

EFFECT OF VARIOUS NUCLEOTIDES ON THE WATER CONTENT OF ISOLATED RENAL TUBULES

Tubules were incubated aerobically for 1 h at 25 °C in Ca^{2+}-free saline at pH 7.2 without (control) and with various nucleotides (final concn 4 mM) present. The values of tissue water (kg/kg dry wt) are the mean ± S.E. ($n = 5$).

Nucleotide present (4 mM)	*Tissue water (kg/kg dry wt)*
None	3.49 ± 0.09
ATP	4.21 ± 0.04
ADP	3.34 ± 0.04
GTP	3.46 ± 0.05

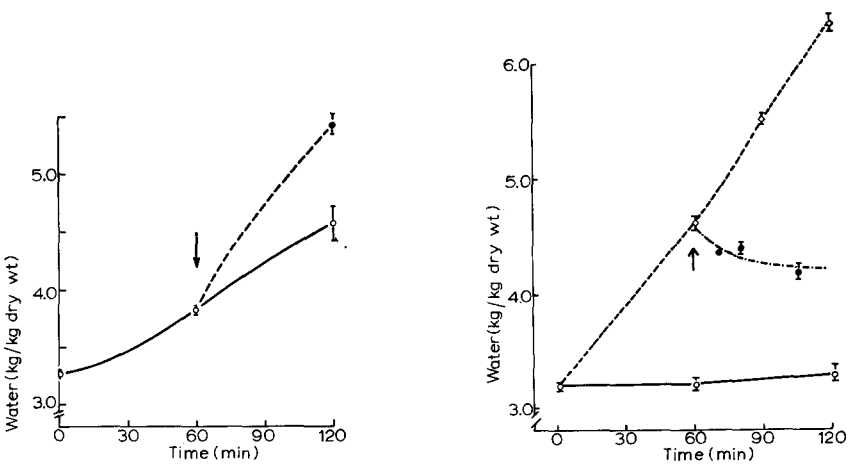

Fig. 3. Effect of ATP on tissue water of kidney tubules incubated in Na^+-free medium. Tubules were incubated aerobically (O_2) at 25 °C in Li^+ saline free of Ca^{2+} (pH 7.2) (control, ○). At 60 min ATP (4 mM, final concn) was added to a portion of the suspension (●). Each value is the mean of eight analyses, ± S.E.

Fig. 4. Effect of Ca^{2+} on the tissue swelling produced by external ATP. Tubules were incubated aerobically (O_2) at 25 °C in Ca^{2+}-free saline in the absence (control, ○) or presence of 4 mM ATP (◇). At 60 min, Ca^{2+} (final concn 2.5 mM) was added to a portion of the suspension containing ATP (●). Each value is the mean ± S.E. ($n = 8$).

TABLE V

EFFECT OF Mg^{2+} ON THE ATP-INDUCED SWELLING OF ISOLATED KIDNEY TUBULES

The tubule preparation was first preincubated aerobically (O_2) for 30 min at 25 °C in Ca^{2+}-free saline ($[Mg^{2+}]_0$ = 1.5 mM), pH 7.2. Subsequently, a portion of the preparation was incubated further for 60 min (control); to other portions of the suspension, ATP (4 mM final concn) or ATP *plus* Mg^{2+} (final $[Mg^{2+}]_0$ 5.5 mM) were added. The results are the means of four analyses.

Experimental conditions		*Tissue water* (*kg/kg dry wt*)	*Tissue electrolytes* (*mequiv/kg dry wt*)			
ATP (*mM, final concn*)	Mg^{2+} (*mM, final concn*)		Na^+	K^+	Cl^-	Ca^{2+}
None	1.5	3.29	343	144	334	20
4	1.5	4.03	464	97	429	36
4	5.5	3.50	302	178	331	24

associated with a small net loss of Na^+ and Cl^- (50 mequiv/kg dry wt); tissue K^+ changed insignificantly (+9 mequiv) whereas tissue Ca^{2+} increased considerably (+47 mmoles/kg dry wt). After 45 min incubation, tissue Ca^{2+} reached values as high as 136.6 ± 5.5 mmoles/kg dry wt. The Mg^{2+} content of the tissue was not affected.

The observed differences between the effect of external ATP on the swelling of the tissue in the presence or absence of Ca^{2+} (or, to a lesser degree, of Mg^{2+}) might be due to either (a) hydrolysis of ATP by a Ca^{2+}-activated nucleotidase localized on the outer face of the cells, or (b) to an increased membrane permeability to ATP in Ca^{2+}-free medium, thus allowing ATP to reach a site of action present at the internal face of the membrane, as demonstrated for the red blood cells[5]; pH might then affect either of these mechanisms. Some experiments were directed to elucidate these points.

Hydrolysis of ATP by renal tubules

As shown in Fig. 5, ATP added to the medium was rapidly split by isolated tubules. The rate of ATP hydrolysis was linear for the first 20 min and subsequently somewhat decreased. The inflection of the hydrolysis curve corresponded to the liberation of one phosphate equivalent from the added ATP. These properties of the enzyme correspond to observations reported for the splitting of ATP by rat myo-

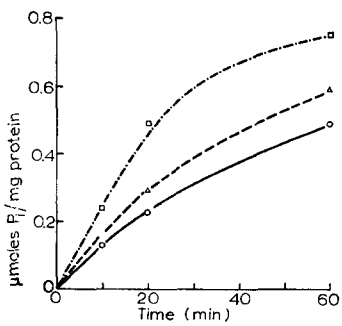

Fig. 5. ATP hydrolysis by isolated kidney tubules. Tubules were incubated aerobically (O_2) in standard saline (2.5 mM Ca^{2+}) containing 4 mM ATP; pH: ○, 6.2; △, 7.2; □, 8.2. Each point is the mean of four analyses.

metrium[15], chick embryo fibroblasts[16], and also the Ca^{2+}-activated ATPase of brain microsomes[12]. The rate of ATP hydrolysis by isolated tubules was increased by 50 % by increasing the medium pH from 6.2 to 8.2 (Fig. 5 and Table VI). The pH of the medium affected particularly the relatively fast rate of ATP hydrolysis.

TABLE VI

EFFECT OF pH AND Ca^{2+} ON THE RATE OF ATP HYDROLYSIS BY ISOLATED TUBULES

Tubules were incubated aerobically (O_2) for 1 h at 25 °C in saline containing 4 mM ATP, with or without Ca^{2+}. Values are the mean of four determinations.

Conditions		*ATP hydrolysis rate* (*μmoles P_i/mg protein per h*)
Saline pH	$[Ca^{2+}]_0$ (*mM*)	
6.2	2.5	0.498
	0	0.580
7.2	2.5	0.590
	0	0.690
8.2	2.5	0.748
	0	0.758

Table VI shows that the absence of Ca^{2+} did not suppress the rate of ATP hydrolysis by isolated tubules; in fact, at pH 6.2 and 7.2, the rate of hydrolysis of ATP was even somewhat higher with tubules incubated in Ca^{2+}-free salines than in the controls (tubules incubated in salines containing 2.5 mM Ca^{2+}).

The above results are compatible with the view that at the outer face of the renal tubular cells a nucleotidase is located which hydrolyses external ATP and this enzyme is to some extent sensitive to Ca^{2+}. Preliminary experiments indicated that the slight entry of labelled ATP into the cells (of the order of 20 μmoles/g dry wt per h) was not greatly affected by variations of external pH; such effect would be expected if the second of the above views were correct. Thus, renal cells share the characteristic of a nucleotidase at the outer face of the membrane with a variety of other mammalian cells[16–19]. As compared with the enzyme activity described for other cell types, the specific ATPase activity of the kidney tubular cells appears to be rather low, possibly due to suboptimal ratios of ATP and divalent cations (Ca^{2+} and/or Mg^{2+}). The presence of such enzyme would explain some of the observations presented above, such as the lack of a swelling effect of ATP at lower ATP concentrations (Fig. 1) or the massive accumulation of Ca^{2+} in the tissue incubated in the presence of both ATP and Ca^{2+}. It appears reasonable to assume that the volume of cells would gradually return to values found in controls once the added ATP was completely hydrolysed. The P_i formed by the splitting of ATP would then allow calcium phosphate to precipitate in the cells, particularly at pH values above 7.0.

Properties of a (Ca^{2+}–Mg^{2+})-activated ATPase from kidney tubules

The above data suggested the need for more information on the properties of the Ca^{2+}-activated ATPase from kidney tubular cells, keeping in mind recent observations on the presence of contractile proteins with a Ca^{2+}-activated ATPase

activity in several cellular species[3,5,20–22]. Accordingly, a convenient procedure for the extraction of such enzyme from the kidney cortex material was sought. Banga and Szent-Györgyi[23] described in 1941 a procedure whereby a protein with a high flow birefringence could be isolated from kidney and liver tissue. This procedure consisted in first exhaustively extracting the coarsely homogenized tissues with isotonic NaCl, followed by an extraction of the insoluble residue with Edsall's hypertonic fluid, and subsequently extracting the residue with Edsall's fluid containing urea. Preliminary experiments readily ascertained the presence of a Ca^{2+}-activated ATPase in the supernatant obtained from kidney cortex by the first extraction with Edsall's fluid; the plastic protein, renosin, extracted by Edsall's fluid with urea, did not show any ATPase activity stimulated by Ca^{2+}, but was very active as a $(Na^+ + K^+)$-activated ATPase. In order to avoid possible contamination of the Ca^{2+}-activated ATPase by such enzyme(s) stemming from cells other than kidney tubules, care was taken to use as a starting material for the enzyme extraction a purified preparation of the tubules, devoid of glomeruli, red blood cells, or blood vessels. Therefore, the procedure for the preparation of kidney tubules was carefully checked. Of the tubule pellet prepared by the usual procedure[2,9] only the middle portion was used; the uppermost layer contained broken cells and erythrocytes, whereas the lowest layer was rich in glomeruli. These tubules were then extracted as described in Methods. The properties of the obtained preparation appear to be rather similar to those of the Ca^{2+}-activated ATPase first reported by Wheeler and Whittam[24] and studied more extensively by Parkinson and Radde[10], although different preparative procedures were used. Therefore, only differences between the various preparations, and additional information, will be given here in detail.

In agreement with the data of Parkinson and Radde[10], the hydrolysis of ATP by the present preparation was found to be activated more by Mg^{2+} than by Ca^{2+}. Na^+ (125 mM) or K^+ (25 mM), did not activate the enzyme, and ouabain (0.1 and 0.5 mM) did not inhibit its activity. The fact that the preparation was not inhibited by oligomycin (10 μg/ml) indicates that the studied enzyme is not identical with the Ca^{2+}-activated ATPase isolated from mitochondria. The present preparation also showed a maximal activation by about 2 mM Ca^{2+} or Mg^{2+}, and an inhibition when the concentration of these cations was raised to 5 mM. The pH optimum of the Ca^{2+}-activated enzyme coincided with the value given by Parkinson and Radde[10], *i.e.* about 7.8 (Fig. 6). It will be noted, however, that the pH dependence of the

TABLE VII

RATE OF SPLITTING OF NUCLEOTIDES BY AN EXTRACT OF KIDNEY TUBULES

Experiments were performed at pH 7.4, 37 °C, in the presence of an extract of tubules (2 mg protein/ml), 5 mM Ca^{2+}, and 5 mM of each of the nucleotides. Incubation time, 30 min.

Nucleotide	*Nucleotidase activity (μmoles P_i/mg protein per h)*
ATP	0.970
GTP	0.879
UTP	0.575
ITP	0.572
ADP	0.825

Mg^{2+}-activated enzyme differed from that found for the Ca^{2+} stimulation. As to the substrate specificity, the present study showed the enzyme to be a rather non-specific nucleotidase hydrolysing ATP, ADP, ITP, GTP, and UTP. As compared with ATP, the rates of hydrolysis of the other nucleotides tested were markedly lower (Table VII).

Evidence in favor of the localization of this enzyme in the membrane region of kidney tubular cells was obtained by comparing the ATPase activity in subcellular fractions (Table VIII). The highest activity was found in the membrane and light microsome fractions. No differences were observed in the distribution of the Ca^{2+}-ATPase and $(Na^+ + K^+)$-ATPase activities in the subcellular fractions, in agreement with data of Wheeler and Whittam[24].

The major difference between the present enzyme preparation and that reported by Parkinson and Radde[10] appears to consist in the specific activity, the enzyme studied here being less active.

In the course of this investigation it was noticed that under some experimental conditions a turbidity developed in the assay mixture when the enzyme was brought into contact with substrate *plus* Ca^{2+}. Data summarized in Table IX show that at pH 7.2 the presence of both Ca^{2+} and ATP produced a several-fold increase in the

TABLE VIII

DISTRIBUTION OF THE ATPase ACTIVITIES IN SUBCELLULAR FRACTIONS OF KIDNEY TUBULES

Portions of subcellular fractions were used for determination of $(Na^+ + K^+)$-ATPase activity. Further portions were extracted with Edsall's solution (1 ml/100 mg) and the extract employed for the assay of Ca^{2+}-ATPase activity. Results are the mean of three values.

Subcellular fraction	*ATPase activity (μmoles P_i/mg protein per h)*	
	Ca^{2+}-ATPase	*$(Na^+ + K^+)$-ATPase*
Nuclear fraction	0.11	2.15
Mitochondrial fraction	0.07	0.45
Membrane fraction	0.24	4.88
Heavy microsomes	0.16	4.72
Light microsomes	0.39	6.45

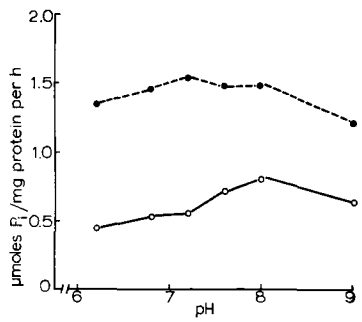

Fig. 6. Effect of pH on the activation of the ATPase activity by Mg^{2+} and Ca^{2+}. The enzyme activity was assayed as described in Methods, the pH being varied by appropriate mixtures of Tris and TES (*N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid) buffers. ○, Ca^{2+} (5 mM); ●, Mg^{2+} (5 mM).

turbidity of the mixture (measured by the absorbance at 550 nm). This phenomenon, reminiscent of superprecipitation first described for actomyosin, was found to be specific for both Ca^{2+} and ATP; no increase in turbidity was observed when ADP or GTP replaced ATP. It should be noted here that superprecipitation was described for the Ca^{2+}-activated ATPase of erythrocytes[3]. At pH 8.2, the addition of ATP *plus* Ca^{2+} did not produce any turbidity of the clear enzyme solution. The enzyme was not freely soluble at pH 6.2.

TABLE IX

EFFECT OF Ca^{2+}, ATP, AND ADP ON THE ABSORBANCE OF THE EXTRACT OF KIDNEY MICROSOMES

The analyzed mixture contained tissue extract (2 mg protein/ml) and further additions. Changes in absorbance were measured at 550 nm. Results are given in absorbance units. Each value is the mean of analyses obtained with two preparations.

Additions (final concn)	*Absorbance*
None	0.16
Ca^{2+} (5 mM)	0.34
ATP (5 mM)	0.39
ADP (5 mM)	0.12
ATP (5 mM) + Ca^{2+} (5 mM)	0.72
ADP (5 mM) + Ca^{2+} (5 mM)	0.21

DISCUSSION

It has been shown above that in Ca^{2+}-free media, external ATP produced a marked effect on the cellular volume and ionic distribution of renal tubular cells. The following characteristics of this phenomenon were noted. The effect of ATP on the cell volume and ionic distribution (1) is abolished by the presence of Ca^{2+} (*viz.* Tables I, II, and Fig. 4) or, to a lesser degree, of Mg^{2+} (Table V); (2) is independent of the active, ouabain-sensitive system for Na^+ transport (Fig. 3 and Table III); (3) is dependent on pH, whereas at all pH values tested (pH 6.2 to 8.2) external ATP produced an increase in cell Na^+, Cl^-, and Ca^{2+}, and a loss of K^+, only at about 7.2 was a cellular swelling observed (Table II); and (4) appears to be specific for ATP; no such action of some other nucleotides has been found (Table IV).

These characteristics clearly relate the effect of ATP on the cellular volume to the ouabain-insensitive (Na^+-independent) volume control of renal cells, which also showed[1,2] dependence on external Ca^{2+} and pH, as well as on cellular ATP. Furthermore, the described phenomenon shows marked similarities to recent observations on the role of Ca^{2+} and ATP in determining the physical properties and also the ionic permeabilities and regulation of cell volume of erythrocytes[4–6] and in the TA3 strain of ascites tumor cells[7,8]. With the ascites tumor cells suspended in Ca^{2+}-free media, external ATP brought about a transient swelling of the cells, associated with changes in the membrane permeability to Na^+ and K^+. Either Ca^{2+} or Mg^{2+} were found to be effective in abolishing the ATP-induced cell swelling. A study of the interactions of ATP and Ca^{2+} with erythrocyte ghosts led to the conclusion[4–6] that these substances interacted with a (fibrillar) protein[20,21] with Ca^{2+}-ATPase activity located at the inner face of the membrane[3–5] and thus, produced changes of membrane permeability to ions and uncharged molecules as well as changes of the

mechanical properties of the membrane (contraction). A contraction of ghosts of porcine erythrocytes in the presence of Ca^{2+} and ATP has been reported earlier by Wins and Schoffeniels[25].

A role of ATP and Ca^{2+} in the determination of the physical properties of the membrane of renal tubular cells has been postulated previously[1,2,26] on the basis of investigations of the properties of the ouabain-insensitive (Na^+-independent) system for the regulation of cell volume. Such mechanism has now also been suggested for the ouabain-insensitive extrusion of Na^+ and volume control in various cell species (see ref. 1 for a review). A myosin-like protein has also been described to be present in the membranes of liver cells[27] and thrombocytes[28]. In bovine sperm cells, which appear to be unable to control their volume by an ouabain-insensitive mechanism and therefore swell (at pH 7.0) when the Na^+ extrusion was blocked by ouabain or absence of Na^+, external Ca^{2+} prevented the swelling and also preserved the cellular motility[29,30]. The present results provide further evidence in favor of the mechanochemical hypothesis of the ouabain-insensitive control of cell volume and point to a specific interaction of ATP and Ca^{2+} with membrane component(s).

Localization of the ATP action at the membrane appears to be borne out by the following observations: (a) external ATP was rapidly hydrolyzed by intact renal tubules (Fig. 5), and this activity was inhibited by Ca^{2+} (Table VI); (b) the highest activity of a Ca^{2+}-activated ATPase was found in the membrane fraction of the cells (Table VIII); (c) external ATP entered the cells only to a small extent (Results, *Hydrolysis of ATP by renal tubules*); (d) several authors found that ATP markedly increased the binding of $^{45}Ca^{2+}$ and P_i by a membrane fraction of renal tubular cells[31], and erythrocytes[32,33]; and (e) filamentous elements, resembling fibers of smooth muscle, have been described[34] to be present near the basal cell membrane of the proximal renal tubule.

Several mechanisms of an action of external ATP on the ionic distribution and cell volume of renal cells, and the effect of Ca^{2+} thereon, might be visualized:

(i) ATP might directly affect the active extrusion of Na^+ from the cells, thus producing a steady-state ionic distribution in the cells similar to that observed when the (Na^+–K^+)-ATPase is inhibited by ouabain[2]. Such possibility appears to be remote in view of the fact that ATP acted both in the presence of ouabain concentrations sufficient to block completely the Na^+–K^+ exchange in renal cells (Table III), or in the absence of Na^+ (Fig. 3); furthermore, the absence of external Ca^{2+} was mandatory in order to demonstrate the effect of ATP. No action of ATP on the active extrusion of Na^+ has been found in erythrocytes[6] or ascites tumor cells[8].

(ii) The passive membrane permeability to Na^+ and/or K^+ might be affected by external ATP. This possibility arises in view of the observation that at pH 6.2 in a Ca^{2+}-free saline, external ATP did markedly increase the cell Na^+ and produced a loss of K^+ (Table III) without affecting the cell volume. A direct effect of external ATP on cell permeability has been demonstrated for erythrocyte ghosts[6] and also for ascites tumor cells[8].

(iii) ATP, by interaction with membrane component(s) and Ca^{2+} would affect the physical properties of this structure. Changes of cell volume might then be produced either by a contractile system, or, alternatively, the interaction of ATP, Ca^{2+}, and membrane component(s) would determine the passive distensibility of the structure. The cell volume would thus reflect the balance of passive ionic flows along

the osmotic gradient owing to intracellular non-diffusible components, and hydrostatic forces determined by the physical properties of the membrane. Such view is compatible with the ATP-dependent binding of Ca^{2+} by membrane fractions of renal cells[31], the presence of a Ca^{2+}-activated ATPase, and particularly the ATP-specific superprecipitation phenomenon displayed by this preparation (Table IX), as well as data available on the relationship between ATP, Ca^{2+}, and membrane components especially of erythrocytes[3–6]. In particular, the diminished Ca^{2+} binding by ghost proteins due to external ATP is of interest.

The second and third possibilities, stated above, do not appear to be mutually exclusive. In fact, conformational changes produced by an interaction of membrane components with ATP and Ca^{2+}, demonstrated by the superprecipitation phenomenon, might well be responsible for changes of both the permeability of the membrane and also of its mechanochemical properties. In this context it should be noted that in erythrocytes the Ca^{2+}-induced increase in K^+ permeability was found to be ATP dependent[35]. Further experiments in this direction are clearly desirable. Studies are also required to elucidate why external ATP actually decreased the cellular swelling in the absence of Ca^{2+} at pH 8.2 (Fig. 2); possibly, this observation might be related to the fact that at pH 8.2, no superprecipitation of the ATPase preparation was demonstrable.

The effect of ATP on the membrane properties does not appear to be related to the capability of this nucleotide to chelate Ca^{2+}. Two arguments are pertinent here. First, EDTA, or EGTA (ethyleneglycol-bis-(β-amino-ethyl ether)-*N*, *N'*-tetra-acetic acid), both of which are much more powerful chelators of Ca^{2+} than ATP or GTP, did not affect the cell volume in slices[36] or isolated tubules (tissue water, kg/kg dry wt, $\pm$ S.E., $n = 5$: control, 3.47 $\pm$ 0.08; 0.1 mM EDTA, 3.51 $\pm$ 0.06; 0.1 mM EGTA, 3.57 $\pm$ 0.09). Secondly, and this point is considered to carry more weight, GTP, as opposed to ATP, did not affect the cell volume (Table IV) although it was hydrolyzed by the Ca^{2+}-activated nucleotidase (Table IX) and its stability constant K_s of the calcium chelate ($pK_s = 3.58$, see ref. 6) is of the same order as that of ATP ($pK_s = 3.77$).

The views presented above imply the suggestion that the Ca^{2+}-activated ATPase, present at or near the membrane of renal cells (ref. 10 and present communication) is involved in the investigated effect of ATP on the membrane properties. The pH dependence of the enzyme and its ionic requirement are very similar to those observed for the effect of ATP on the tubules. The possibility should be investigated further whether the enzyme might act by phosphorylating a membrane component, as suggested from studies of the effect of ATP on Ca^{2+} binding[31]. The envisaged role of the Ca^{2+}-activated ATPase in determining the physical properties of the cell membrane does not exclude the possibility that the enzyme is also involved in the transport of Ca^{2+}, as suggested for the role of the Ca^{2+}-activated ATPase of the kidney cortex[10] and the placenta[37].

The presence of a Ca^{2+}-activated ATPase in the membrane fraction of renal cells represents a feasible explanation for such observations as the ATP-induced accumulation of cell Ca^{2+} (Tables II, III), or the lack of cellular swelling at low external concentrations of ATP (Fig. 1). It has been shown previously[2] that a marked cellular accumulation of Ca^{2+} was dependent on the availability in the medium of phosphate; the hydrolysis of ATP would supply sufficient phosphate at the cell

membrane to allow Ca^{2+} to be taken up, and this process would be enhanced at pH values higher than 7.2. Furthermore, the rate of ATP hydrolysis by intact tubules appears to be sufficient to account for the fact that a swelling effect on cells could not be demonstrated when the starting concentration of external ATP was below 2 mM.

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