Biochimica et Biophysica Acta, 394 (1975) 281–292

BBA 76987

OUABAIN-INSENSITIVE Na^+ STIMULATION OF AN Mg^{2+}-DEPENDENT ATPase IN KIDNEY TISSUE

F. PROVERBIO, MADALINA CONDRESCU-GUIDI and G. WHITTEMBURY

Centro de Biofísica y Bioquímica, Instituto Venezolano de Investigaciones Científicas, IVIC, P.O. Box 1827, Caracas (Venezuela)

(Received November 29th, 1974)

SUMMARY

1. Freshly prepared microsomal fractions of the outermost cortex of guinea pig kidney show an Mg^{2+}-dependent ATPase activity which is partially inhibited by 100 mM NaCl, LiCl, KCl, RbCl, CsCl, NH_4Cl or choline chloride.

2. If the microsomal preparation is aged by storage at 4 °C for 10–15 days, the Mg^{2+}-dependent activity shows stimulation by Na^+ and Li^+ but not by K^+, Rb^+, Cs^+, NH_4^+ or choline.

3. Stimulation is similar with sodium salts of Cl^-, HCO_3^-, CH_3COO^-, Br^-, SO_4^{2-} or methylsulphonate.

4. Stimulation is insensitive to 1 mM and 10 mM ouabain.

5. Stimulation is unaltered by the presence of 0.5 mM ethyleneglycol-bis-(β-aminoethyl ether)*N,N'*-tetracetic acid.

6. Stimulation is 100 % inhibited by 2 mM ethacrynic acid, a concentration which inhibits only 30% of the Mg^{2+}-dependent ATPase and 50 % of the $(Na^+ + K^+)$-stimulated ATPase.

7. Some of these characteristics coincide with those of an ouabain-resistant, K^+-independent, ethacrynic acid-sensitive mode of Na^+ extrusion out of guinea pig kidney cortex cells.

INTRODUCTION

There is general agreement that kidney cells possess a cardiac glycoside (ouabain)-sensitive, Na^+-extruding mechanism which exchanges intracellular Na^+ for extracellular K^+, obtaining its energy from hydrolysis of ATP [1–7]. As has been demonstrated in other tissues [8–10] in the kidney a $(Na^+ + K^+)$-stimulated ATPase with a similar sensitivity to ouabain is thought to be linked to this mode of Na^+ extrusion [11–15]. However, in kidney slices such an mechanism seems insufficient to account for the observation that Na^+ can be extruded out of Na^+ loaded cells with Cl^- and water in the absence of external K^+ despite the presence of ouabain concentrations which should have the $(Na^+ + K^+)$-stimulated ATPase completely inhibited [2,4–7]. This alternative mode of Na^+ extrusion is stimulated by angiotensin

[16], and inhibited by ethacrynic acid [5] (which, in red cells, curtails an ouabain-insensitive Na^+ efflux [17]) to which the $(Na^+ + K^+)$-stimulated ATPase is only partially sensitive [14]. Na^+ extrusion is completely inhibited if ouabain and ethacrynic acid are added together [5]. In dog and rat kidney, a single Na^+ pump dependent on the $(Na^+ + K^+)$-stimulated ATPase also seems to be insufficient to account for the observation that about half of the filtered Na^+ continues being reabsorbed under conditions in which the $(Na^+ + K^+)$-stimulated ATPase has been completely inhibited by large doses of cardiac glycosides [18, 19]. Moreover, in the rabbit kidney this ouabain-insensitive fraction of Na^+ reabsorption can be suppressed if ouabain and ethacrynic acid are added together at moderate concentrations which inhibit the $(Na^+ + K^+)$-stimulated ATPase activity by only 46 % [20]. Authors vary in the interpretation of the observations (cf. ref. 21), some of which have been recently challenged [22]. Some authors have suggested the possible existence of a second Na^+-extruding mechanism [5, 14, 16, 18–20, 23]. The latter would also derive its energy from hydrolysis of ATP, since both modes of Na^+ extrusion are inhibited by 2,4-dinitrophenol and anoxia [5, 24] and 2,4-dinitrophenol and anoxia inhibit the production of cellular ATP [25]. On the other hand, the inhibitory action of ethacrynic acid on the ouabain-insensitive transport of water and electrolytes in kidney slices is thought to be unspecific and related to other actions of ethacrynic acid, namely, inhibition of glycolysis and electron transport chain in mitochondria and reduction of cellular ATP levels in kidney tissues [21, 43].

In view of these possibilities, we decided to look for an ouabain-insensitive, Na^+-stimulated ATPase in kidney tissue, paying particular attention to the effect of storage, which has been shown to inhibit the Mg^{2+}-dependent ATPase but to enhance the activity of $(Na^+ + K^+)$-stimulated ATPase [26].

MATERIALS AND METHODS

Preparation of microsomal fractions. Outermost slices of kidney cortex of healthy adult male guinea pigs weighing about 500 g were obtained as described [3]. Each gram of tissue was immediately homogenized at 0 °C in 10 ml of a solution of 0.25 M sucrose, 20 mM Tris · HCl (pH 7.2) and 1 mM EDTA, according to the techniques already described [14, 26]. Briefly, the homogenate was centrifuged for 20 min at 10 000 × *g*, the supernatant recentrifuged for 1 h, at 100 000 × *g*, and the sediment from the last centrifugation suspended in 5 ml of the sucrose/Tris/EDTA solution and used for the study of the ATPase activity in fresh or aged preparations.

The ageing process consisted of leaving the preparations in the refrigerator at 4 °C in the same extraction medium for several days.

Assay of the ATPase activity. 20 µl of the microsomal suspension (containing about 6 mg protein/ml) were preincubated at 37 °C for 10 min in the presence of (final concentrations) 100 mM imidazole buffer (pH 7.2), 5 mM $MgCl_2$ and, according to the experimental design, requisite amounts of NaCl and/or ouabain. KCl, other salts and ethacrynic acid were also used when required. The reaction was started by adding to the medium Na^+-free Tris · ATP (2 mM final concentration). The final volume was 1 ml. After 30 min, the incubation was terminated by the addition of 1 ml of ice cold 6 % $HClO_4$ to the incubation tubes. The samples

were chilled and centrifuged. The liberated phosphate was determined in the deproteinized solution [27]. Preliminary experiments demonstrated that, under our incubation conditions, a linear relationship existed between the liberation of phosphate and the time of incubation. The protein content of the original suspension was measured by means of Folin reagent [28]. All samples were run in triplicate or quadruplicate. ATPase activity is expressed as nmol of phosphorus produced per mg of protein per min after subtraction of a blank run in parallel either without the 20 μl of microsomal suspension or without ATP.

Ouabain (Strophanthin-G), EGTA and Na^+-free Tris · ATP were purchased from the Sigma Chemical Company, St. Louis, Mo., U.S.A. Ethacrynic acid was generously provided by Merck, Sharp and Dohme, Rahway, N.J., U.S.A. All other reagents were from E. Merck AG., Darmstadt, Germany.

RESULTS

Electronmicroscopic observation confirmed that fresh and aged preparations consisted almost exclusively of microsomes which appeared smaller and often ruptured in aged preparations (see also ref. 44). The presence of plasma membrane fragments in our preparation is indicated by the presence of 5'-nucleotidase activity (EC 3.1.3.5) (Table VII) and of ouabain-sensitive ($Na^+ + K^+$)-stimulated ATPase activity (EC 3.6.1.3) (Table I), two widely accepted plasma-membrane marker enzymes [45]. A very low succinate dehydrogenase activity (utilized as a marker for mitochondrial contamination [46]) was found in our preparation. Thus our microsomal fraction showed an activity which was at most 5–10 % of the activity shown by the 10 000 $\times g$ pellet fraction. This and the experimental results shown in Table VI substantiates the conclusion that our preparation has a very low mitochondrial contamination.

Table I shows results of measurements of the ATPase activity of a freshly prepared microsomal suspension. The difference between the ATP hydrolyzing

TABLE I

ATPase ACTIVITY OF FRESH MICROSOMAL FRACTION OF OUTERMOST KIDNEY CORTEX

Fresh preparations were incubated for ATPase activity. In the experiments summarized in this and in the following tables, the following procedure was used. 20 μl of microsomal preparation (containing about 6 mg protein/ml) were added to 1 ml of the incubation medium containing as final concentration in mM, imidazole (pH 7.2), 100; $MgCl_2$, 5; ATP · Tris, 2; NaCl, KCl and ouabain 100, 10 and 1, respectively, when required. After 10 min of preincubation at 37 °C, the reaction was started by adding the ATP to the medium and it was stopped after 30 min by addition of 1 ml of ice cold 6 % perchloric acid to the incubation tubes. The samples were chilled, centrifuged and assayed for the presence of P_i. In this and in the following tables the values are expressed as the mean ±S.E. In the present table $n = 20$ (6 animals).

Incubation medium	ATP hydrolysed (nmol/mg protein per min)
Mg^{2+}	96±2
$Mg^{2+} + Na^+ + K^+$	126±2
$Mg^{2+} + Na^+ + K^+$ + ouabain	86±3

activity observed in the presence of Mg^{2+} alone (Mg^{2+}-dependent ATPase) and that activated by $Na^+ + K^+$ in addition to Mg^{2+} (total ATPase) represents the well known ($Na^+ + K^+$)-stimulated ATPase activity which, as shown in Table I, is totally inhibited by the presence of 1 mM ouabain (cf. refs 11–15). The activities obtained are in the range of those previously reported for similar preparations [14]. Working with fresh preparations, addition of Na^+ (with Cl^-), without K^+, to the medium with Mg^{2+}, does not further stimulate the ATPase activity. On the contrary, as

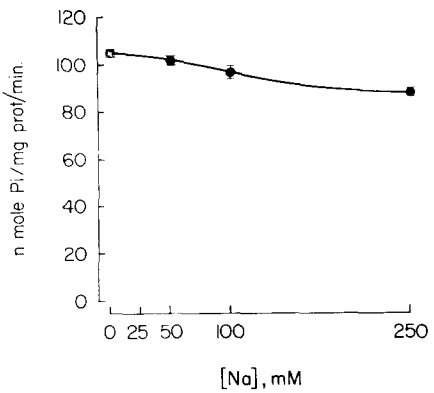

Fig. 1. Effect of increasing Na^+ concentration (as NaCl) on the Mg^{2+}-dependent ATPase activity of fresh microsomal preparation. In the experiments summarized in this and in the following figures the reaction was carried out as indicated in Methods. Mg^{2+} concentration was 5 mM. Values are expressed as mean ±S.E. In the present figure, $n = 4$. Similar results were obtained when the experiment was repeated on 5 animals.

illustrated in Fig. 1, addition of increasing concentrations of NaCl inhibits progressively the activity of the Mg^{2+}-dependent ATPase. Inhibition by Na^+ of the Mg^{2+}-dependent ATPase has been described before [29, 30]. The inhibitory effect observed in the present experiments does not seem to be Na^+-specific, since it is also observed in the presence of 100 mM LiCl, KCl, RbCl, NH_4Cl or choline chloride (Table II).

TABLE II

EFFECT OF 100 mM CHLORIDE SALT OF DIFFERENT CATIONS ON THE Mg^{2+}-DEPENDENT ATPase ACTIVITY OF FRESH MICROSOMAL FRACTIONS

Mg^{2+} concentration was 5 mM ($n = 4$).

Incubation medium	ATP hydrolysed (nmol/mg protein per min)
Mg^{2+}	95.0 ± 2.6
Mg^{2+} + NaCl	80.9 ± 2.6
Mg^{2+} + LiCl	83.4 ± 2.0
Mg^{2+} + KCl	72.6 ± 3.1
Mg^{2+} + RbCl	73.9 ± 2.9
Mg^{2+} + CsCl	72.0 ± 5.1
Mg^{2+} + NH_4Cl	75.2 ± 2.6
Mg^{2+} + choline chloride	81.7 ± 1.4

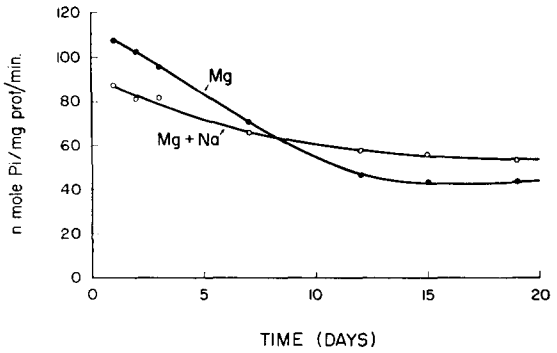

Fig. 2. Effect of storage on the Mg^{2+}-dependent ATPase activity in the absence (●) and in the presence (○) of 100 mM NaCl. Mg^{2+} concentration was 5 mM. After extraction, the membranes were stored at 4 °C in a refrigerator in the same extraction medium and assayed for ATPase activity at different days of ageing ($n = 3$). Similar results were obtained when the experiment was repeated on 6 different animals.

It is possible that this inhibitory effect of Na^+ on the Mg^{2+}-dependent ATPase activity is due to changes in the ionic strength of the incubation medium.

Fig. 2 illustrates the effect of storage in the refrigerator on the Mg^{2+}-dependent ATPase activity. As is known [26], we observed that the Mg^{2+}-dependent ATPase activity decreased as a function of the days of storage of the preparation at 4 °C. We were lucky to find that inhibition of Mg^{2+}-dependent ATPase activity by addition of Na^+ (which was observed with the fresh preparation) subsided with days of storage at 4 °C. After 12 days, the Mg^{2+}-dependent ATPase activity showed significant stimulation by addition of Na^+ to the incubation medium. It may be seen in Fig. 3 that this stimulation by Na^+ was proportional to the Na^+ concentration in the range explored (see also Fig. 4) and that it was insensitive to the presence of 1 mM ouabain. This ouabain concentration is at least ten times higher than the

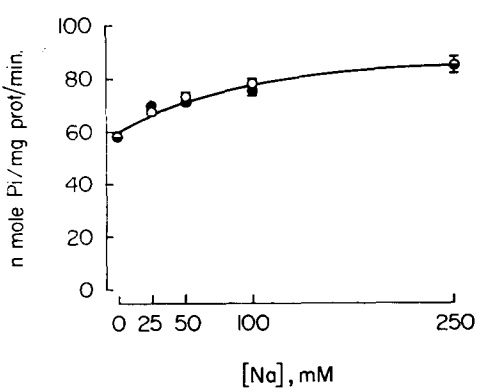

Fig. 3. Effect of increasing Na^+ concentration (as NaCl) on the Mg^{2+}-dependent ATPase activity of preparations aged for 10 days. Mg^{2+} concentration was 5 mM. Assays were performed in the presence (○) or absence (●) of 1 mM ouabain ($n = 4$). Similar results were obtained when the experiment was repeated on 5 animals.

TABLE III

EFFECT OF 100 mM CHLORIDE SALT OF DIFFERENT CATIONS ON THE Mg^{2+}-DEPENDENT ATPase ACTIVITY OF A PREPARATION AGED FOR 12 DAYS

All assays were performed in the presence of 1 mM ouabain. Mg^{2+} concentration was 5 mM ($n = 4$).

Incubation medium	ATP hydrolysed (nmol/mg protein per min)
Mg^{2+}	52.1 ± 0.7
Mg^{2+} + NaCl	64.7 ± 0.3 $P < 0.001$
Mg^{2+} + LiCl	60.0 ± 0.6 $P < 0.001$
Mg^{2+} + KCl	55.1 ± 1.0 n.s.
Mg^{2+} + RbCl	52.6 ± 0.6 n.s.
Mg^{2+} + CsCl	56.2 ± 2.6 n.s.
Mg^{2+} + NH_4Cl	52.0 ± 4.0 n.s.
Mg^{2+} + choline chloride	55.1 ± 1.3 n.s.

concentration that suppresses completely the $(Na^+ + K^+)$-stimulated ATPase activity [12, 14]. Since our aim was to characterize a ouabain insensitive ATPase, 1 mM ouabain was regularly used in all experiments performed to study the stimulation by Na^+ of the Mg^{2+}-dependent ATPase. Table III shows that 100 mM LiCl also produced stimulation of the Mg^{2+}-dependent ATPase activity ($P < 0.001$) of aged microsomal fractions. On the other hand, other cations, namely K^+, Rb^+, Cs^+, NH_4^+ and choline$^+$ did not show a significant stimulatory effect.

The results shown in Fig. 4 indicate that up to a concentration of 100 mM (of Na^+ or Li^+) increasing the concentrations of either Na^+ or Li^+ produced similar stimulation of the Mg^{2+}-dependent ATPase activity of aged microsomal preparations. The apparent K_m for either cation, calculated using a Lineweaver-Burk plot of Fig. 4, is 15 mM. It may be seen in Table IV that, at a concentration of 100 mM, the

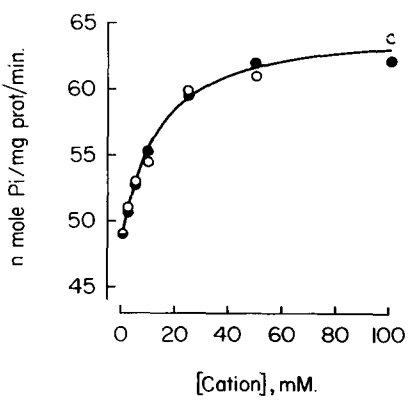

Fig. 4. Effect of increasing concentrations of Na^+ (○) and of Li^+ (●) as Cl^- salts on the Mg^{2+}-dependent ATPase activity of preparations aged for 15 days. Mg^{2+} concentration was 5 mM. All assays were performed in the presence of 1 mM ouabain ($n = 3$). Similar results were obtained when the experiment was repeated on 3 animals.

TABLE IV

EFFECT OF 100 mM SODIUM SALT OF DIFFERENT ANIONS ON THE Mg^{2+}-DEPENDENT ATPase ACTIVITY OF A PREPARATION AGED FOR 15 DAYS

All assays were performed in the presence of 1 mM ouabain. Mg^{2+} concentration was 5 mM ($n = 4$).

Incubation medium	ATP hydrolysed (nmol/mg protein per min)
Mg^{2+}	47.9±2.4
Mg^{2+}+NaCl	58.0±0.9
Mg^{2+}+$NaHCO_3$	58.7±3.2
Mg^{2+}+sodium acetate	57.2±1.6
Mg^{2+}+NaBr	60.4±2.4
Mg^{2+}+Na_2SO_4	54.0±1.9
Mg^{2+}+sodium methylsulphonate	58.4±2.1

stimulatory effect of Na^+ was independent of the anion present in the incubation medium. The optimum pH of stimulation by Na^+ lay between pH 6.5 and 7.2 (not shown).

We performed experiments to ascertain whether the Na^+-stimulation of the Mg^{2+}-dependent ATPase of the aged microsomal preparation was related to an (Mg^{2+}+Ca^{2+})-dependent ATPase which could be activated by the presence of endogenous Ca^{2+} in the microsomal preparations. For this purpose, aged microsomal preparations were incubated in the presence and absence of 0.5 mM ethyleneglycol-bis-(β-aminoethyl ether)*N*,*N*′-tetraacetic acid (EGTA), a chelating agent with strong preference for Ca^{2+} over Mg^{2+}. As may be seen in Table V, the presence of EGTA did not change either Mg^{2+}-dependent or (Mg^{2+}+Na^+)-ATPase activities. Therefore, it seems safe to conclude that the ouabain-insensitive Na^+ stimulation described here is unrelated to a Ca^{2+} ATPase as described in the literature [31, 32]. This is further supported by the observation that, in our preparation, the cation-stimulating sequence is different from the Ca^{2+} ATPases described in the literature [31, 32]. An (Mg^{2+}+Ca^{2+})-ATPase has been described which is stimulated more by K^+ and Na^+ than by Li^+ and choline$^+$ in red cells [31] and not by Na^+ in the kidney [32].

TABLE V

EFFECT OF THE PRESENCE OF 0.5 mM EGTA ON THE Mg^{2+}-DEPENDENT ATPase ACTIVITY OF PREPARATIONS AGED FOR 20 DAYS

All assays were performed in the presence of 1 mM ouabain. Mg^{2+} concentration was 5 mM. Na^+ concentration was 100 mM ($n = 8$). Similar results were obtained when the experiment was repeated in 3 different animals.

Additions	ATP hydrolysed (nmol/mg protein per min) Incubation medium		
	Mg^{2+}	Mg^{2+}+Na^+	Δ Na^+
None	35.8±2.6	44.7±0.9	8.9
EGTA	35.7±1.4	48.1±2.0	12.4

TABLE VI

EFFECT OF THE PRESENCE OF 15 μM OLIGOMYCIN AND OF 100 μM 2,4-DINITROPHENOL ON THE Mg^{2+} AND ON THE ($Mg^{2+}+Na^+$)-ATPase ACTIVITIES IN PREPARATIONS AGED FOR 20 DAYS

All assays were performed in the presence of 1 mM ouabain. Mg^{2+} concentration was 5 mM and Na^+ concentration was 100 mM (as NaCl) ($n = 4$). Similar results were obtained when the experiment was repeated in 3 different animals.

Additions	ATP hydrolysed (nmol/mg protein per min)		
	Incubation medium		
	Mg^{2+}	$Mg^{2+}+Na^+$	Δ Na^+
None	31.4±0.9	41.1±2.0	9.7
Oligomycin	28.4±1.8	42.1±0.3	13.7
2,4-Dinitrophenol	34.6±0.9	41.7±2.7	7.1

The possibility that the Na^+ stimulation of the Mg^{2+}-dependent ATPase was due to the presence of mitochondrial Mg^{2+}-ATPase activity in the microsomal preparation seems remote, since the Na^+ stimulation of the Mg^{2+}-dependent ATPase activity of aged fractions was not altered by either 15 μM oligomycin or 100 μM 2,4-dinitrophenol (Table VI). These agents are known to inhibit and to stimulate, respectively, the mitochondrial Mg^{2+}-ATPase activity [33–35].

The 5′-nucleotidase activity of aged preparations did not show any stimulation by Na^+ or by ($Na^+ + K^+$) as is shown in Table VII, when 2 mM AMP was used as substrate instead of ATP.

The effect of ethacrynic acid was studied in fresh and aged preparations. This agent is known to inhibit only partially the Mg^{2+} and the ($Mg^{2+}+Na^+ + K^+$)-ATPase activities [14]. Fig. 5 shows results of experiments performed with fresh preparations. In agreement with the observations illustrated in Fig. 1, the progressive inhibitory effect of increasing concentrations of Na^+ on the Mg^{2+}-dependent ATPase was reproduced in the absence of ethacrynic acid. With ethacrynic

TABLE VII

ATPase AND 5′-NUCLEOTIDASE ACTIVITIES IN MICROSOMAL PREPARATIONS AGED FOR 17 DAYS

Ouabain (1 mM) was present in the assay in the presence of Mg^{2+} and of $Mg^{2+}+Na^+$. AMP (2 mM) substituted for ATP in the 5′-nucleotidase assay. Mg^{2+} concentration was 5 mM: Na^+ concentration was 100 mM (as NaCl) and K^+ concentration was 10 mM (as KCl) ($n = 3$). Similar results were obtained when the experiment was repeated in 3 animals.

Substrate	ATP or AMP hydrolysed (nmol/mg protein per min)		
	Incubation medium		
	Mg^{2+}	$Mg^{2+}+Na^+$	$Mg^{2+}+Na^+ + K^+$
ATP	43.5±0.8	48.7 ±0.1	58.0±1.3
AMP	12.3±0.7	12.1 ±0.7	10.4±0.1

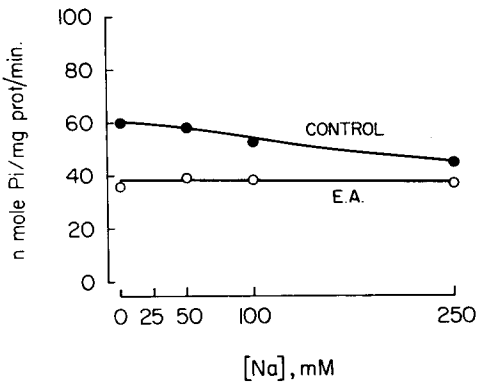

Fig. 5. Effect of 2 mM ethacrynic acid (E.A.) on the Mg^{2+}-dependent ATPase activity of fresh preparations assayed at different Na^+ concentrations (as NaCl). Mg^{2+} concentration was 5 mM. Values are the means of 3 samples run without ethacrynic acid (●) and with ethacrynic acid (○). Similar results were obtained when the experiment was repeated on another animal.

TABLE VIII

EFFECT OF 2 mM ETHACRYNIC ACID ON THE Mg^{2+} AND ($Mg^{2+}+Na^+$)-ATPase ACTIVITIES OF PREPARATIONS AGED FOR 20 DAYS

All assays were performed in the presence of 1 mM ouabain. Mg^{2+} concentration was 5 mM, Na^+ concentration (as NaCl) was 100 mM ($n = 4$). Similar results were obtained when the experiment was repeated in 3 different animals.

Incubation medium	ATP hydrolysed (nmol/mg protein per min)
Mg^{2+}	32.1 ± 0.1
Mg^{2+} + ethacrynic acid	23.0 ± 0.6
$Mg^{2+} + Na^+$	47.4 ± 1.6
$Mg^{2+} + Na^+$ + ethacrynic acid	23.6 ± 0.5

acid the Mg^{2+}-dependent ATPase activity was lowered to a value that was not further inhibited by increasing Na^+ concentrations. Table VIII summarizes results of the experiments in aged preparations. The Na^+ stimulation of the Mg^{2+}-dependent ATPase described earlier in this paper is observed again without ethacrynic acid. On the other hand, addition of 2 mM ethacrynic acid resulted in total inhibition of this Na^+ stimulation. Ethacrynic acid also inhibited the Mg^{2+}-dependent ATPase activity by about 28 %. In another series of experiments, the sensitivities to ethacrynic acid of three fractions of the total ATPase activity were compared, namely (a) the Mg^{2+}-dependent ATPase activity, (b) the Na^+-stimulated activity, which was calculated by subtracting Mg^{2+}-ATPase activity from the activity in the presence of $Mg^{2+}+Na^+$ and (c) the ($Na^+ + K^+$)-stimulated activity, which was calculated by subtracting $Mg^{2+}+Na^+$ activity from the activity observed in the presence of $Mg^{2+}+Na^+ + K^+$. For this purpose, aged membranes were incubated in the medium containing Mg^{2+}, or $Mg^{2+}+Na^+$, or $Mg^{2+}+Na^+ + K^+$ in the presence of different concentrations of ethacrynic acid. The results expressed as

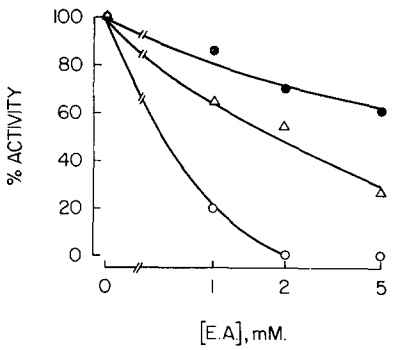

Fig. 6. Effect of increasing concentrations of ethacrynic acid (E.A.) (log scale) on the Mg^{2+}-dependent (●), Na^+-stimulated, ($Mg^{2+}+Na^+$) minus Mg^{2+} activities (○) and ($Na^+ + K^+$)-stimulated, ($Mg^{2+}+Na^+ + K^+$) minus ($Mg^{2+}+Na^+$) activities (△) ATPase activities of a preparation aged for 20 days. Mg^{2+} concentration was 5 mM, Na^+ concentration was 100 mM (as NaCl) and K^+ concentration was 10 mM (as KCl). Ouabain (1 mM) was present in the assay of the Mg^{2+} and ($Mg^{2+}+Na^+$) activities. No ouabain was added when the ($Mg^{2+}+Na^+ + K^+$)-activity was assayed ($n = 3$). Similar results were obtained when the experiment was repeated on 2 different animals.

percentage of each of the activities are shown in Fig. 6. Note that the Na^+-stimulated ATPase activity is the most sensitive and the Mg^{2+}-dependent ATPase activity the least sensitive to the action of ethacrynic acid. The ($Na^+ + K^+$)-stimulated ATPase occupies an intermediate position. It may also be noticed that a concentration of 2 mM ethacrynic acid totally inhibited the Na^+-stimulated ATPase activity (see also Table VIII). At this concentration of ethacrynic acid, the Mg^{2+}-dependent activity was inhibited by only 30 % and the ($Na^+ + K^+$)-stimulated activity was inhibited by 53 %.

DISCUSSION

The main finding of the present work is the observation of an ouabain-resistant stimulation of the Mg^{2+}-dependent ATPase activity by Na^+. This new form of ATPase activity is not observed in fresh preparations. It appears in kidney cortex microsomal preparations kept at 4 °C for several days. This Na^+ stimulation seems to be unrelated to the presence of Ca^{2+} remnant in the microsomal preparation. It does not seem to be the expression of the activity of a mitochondrial ATPase or of a 5'-nucleotidase.

An Mg^{2+}-dependent, Na^+-stimulated ATPase has been found in crab nerve [36] and mammalian brain and kidney [37, 38] at concentrations of ATP in the mM range and in rat erythrocyte ghosts [39] and brain microsomes [40] at ATP concentrations in the μM range. The high sensitivity to cardiac glycosides of the stimulation by Na^+ of the Mg^{2+}-dependent ATPase observed by these authors [36–40] represents the main difference with the system described in this paper. In our experiments 1 mM, or even 10 mM, ouabain did not inhibit the Na^+ stimulation of the Mg^{2+}-dependent ATPase activity.

This new form of stimulation of the Mg^{2+}-dependent ATPase activity has the following characteristics. (a) It is stimulated by Na^+. (b) It is stimulated by Li^+.

(c) It does not require K^+ for its activity. (d) It does not require a particular anion. (e) It is insensitive to the presence of ouabain. (f) It is more sensitive to ethacrynic acid than the $(Na^+ + K^+)$-stimulated ATPase and than the Mg^{2+}-dependent ATPase (Fig. 6). All these characteristics suggest either that we are in the presence of a different type of ATPase activity that is masked or inhibited in the fresh preparation, or that the Mg^{2+}-dependent or the $(Na^+ + K^+)$-stimulated ATPase undergoes transformations to give an ATPase with new characteristics. It seems reasonable to assume that the days of storage in the cold might be necessary for unmasking the Na^+-stimulated ATPase activity, for suppressing the action of a hypothetical inhibitor or for allowing the presumed transformations of ATPase to occur.

The six characteristics listed above fit very well into some of the conditions that should be fulfilled by the energy source of the system responsible for the second mode of Na^+ extrusion described in guinea pig kidney cortex slices [5, 14, 24]. Briefly, in this mode Na^+ is extruded out of the cells without requiring the presence of K^+ on the outside. This mode of Na^+ extrusion is ouabain insensitive and is suppressed by 2 mM ethacrynic acid under conditions in which the first mode of Na^+ extrusion (i.e. extrusion of Na^+ in exchange for K^+) is still operative. This concentration of ethacrynic acid completely inhibits the Na^+-stimulated ATPase activity but only partially inhibits the $(Na^+ + K^+)$-stimulated ATPase (Fig. 6). The stimulation by Li^+ of the Mg^{2+}-dependent ATPase fits with the observation that Li^+-loaded cells are able to extrude Li^+ with Cl^- and water in the presence of ouabain [21]. In spite of the similar characteristics of the second mode of Na^+ extrusion and of the Na^+-stimulated ATPase, one cannot relate both activities with certainty. Several questions remain unanswered as yet. (a) Is the Na^+-stimulated ATPase indeed present in the cell membrane fraction and not in some endoplasmic reticulum contaminant of our microsomal preparation? (b) Is the activation of the ATPase produced in the intact cell by intracellular Na^+? (c) Is the Na^+ activation of the Mg^{2+}-dependent ATPase stimulated (or inhibited) by specific drugs which stimulate (or inhibit) the second mode of Na^+ extrusion? (d) Is this Na^+-stimulated ATPase present in other tissues that also have an Na^+-extruding mechanism with the characteristics of that denoted as mode two in the kidney? (e) Can changes produced in the living organism in mode two of Na^+ extrusion be correlated with changes in the activity of the Na^+-stimulated ATPase described here?

Although the activity of this Na^+-stimulated ATPase is clearly established, it constitutes only a fraction of Mg^{2+}-dependent and $(Na^+ + K^+)$-stimulated ATPases. Much work remains to be done to increase the yield of Na^+-stimulated ATPase activity and to learn about the possible physiological role of the observations reported in this paper. This might help to understand better the action mechanism of diuretics [41] which so differently inhibit $(Na^+ + K^+)$-stimulated ATPase [41, 42]. In view of our observation that addition of Na^+ may either inhibit or stimulate Mg^{2+}-dependent ATPase activity, it is important to take observed inhibition or stimulation into consideration when defining $(Na^+ + K^+)$-stimulated ATPase. For example, in Table I one could take either 30 or 40 nmol/mg protein per min as due to $(Na^+ + K^+)$-stimulated ATPase activity.

It is a pleasure to acknowledge the stimulating criticism of Professor H. J. Schatzmann. Thanks are also due to Dr Margarita Pérez-González and Mr Roberto Ochoa for their help in the course of this work. We also acknowledge the superb assistance of Mr Henry Linares, Mrs María A. Silva and Mr R. Andreu. This work was supported in part with grant for project No. 0296 from CONICIT.

REFERENCES

1 Whittam, R. and Willis, J. S. (1963) J. Physiol. Lond. 168, 158–177
2 Kleinzeller, A. and Knotkova, A. (1964) J. Physiol. 175, 172–191
3 Whittembury, G. (1965) J. Gen. Physiol. 48, 699–717
4 Whittembury, G. and Fishman, J. (1966) Pflügers Arch. 307, 138–153
5 Whittembury, G. and Proverbio, F. (1970) Pflügers Arch. 316, 1–25
6 Macknight, A. D. C. (1968) Biochim. Biophys. Acta 150, 263–270
7 Willis, J. S. (1966) Biochim. Biophys. Acta 163, 506–515
8 Skou, J. C. (1965) Physiol. Rev. 45, 596–617
9 Glynn, I. M. (1964) Pharmacol. Rev. 16, 381–407
10 Bonting, S. L. (1970) in Membranes and ion transport (Bittar, E. E., ed.), Vol. 1, pp. 257–364, Wiley, New York
11 Whittam, R. and Wheeler, K. P. (1961) Biochim. Biophys. Acta 51, 622–624
12 Wheeler, K. P. and Whittam, R. (1964) Biochem. J. 93, 349–363
13 Charnock, J. S. and Post, R. L. (1963) Australian J. Exp. Biol. Med. Sci. 41, 547–560
14 Proverbio, F., Robinson, J. W. L. and Whittembury, G. (1970) Biochim. Biophys. Acta 211, 327–336
15 Katz, A. I. and Epstein, F. H. (1967) J. Clin. Invest. 46, 1999–2011
16 Munday, K. A., Parsons, B. J. and Poat, J. A. (1971) J. Physiol. 215, 269–282
17 Hoffman, J. F. and Kregenow, F. M. (1966) Ann. N. Y. Acad. Sci. 137, 566–576
18 Torretti, J., Hendler, E., Weinstein, E., Longnecker, R. E. and Epstein, F. H. (1972) Am. J. Physiol. 222, 1398–1405
19 Ross, B., Leaf, A., Silva, P. and Epstein, F. H. (1974) Am. J. Physiol. 226, 624–629
20 Ebel, H. (1974) Naunyn-Schmiedeberg's Arch. Pharmacol. 281, 301–314
21 Kleinzeller, A. (1972) in Metabolic pathways (Hokin, L. E., ed.), Vol. 6, pp. 91–131, Academic Press, New York
22 Podevin, R. A. and Boumendil-Podevin, E. F. (1972) Biochim. Biophys. Acta 282, 234–249
23 Robinson, J. W. L. (1972) in Na^+-linked transport of organic solutes (Heinz, E., ed.), pp. 140–145, Springer-Verlag, Berlin
24 Whittembury, G. (1968) J. Gen. Physiol. 51, 303s–314s
25 Caldwell, P. C. (1970) in Membranes and ion transport (Bittar, E. E., ed.), Vol. 1, pp. 433–462, Wiley, New York
26 Robinson, J. W. L. (1970) J. Physiol. Lond. 206, 41–60
27 King, E. J. (1932) Biochem. J. 26, 292–297
28 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
29 Gutman, Y., Wald, H. and Czaczkes, W. (1973) Pflügers Arch. 345, 81–92
30 Cole, C. H. and Dirks, J. H. (1971) Can. J. Physiol. 49, 63–69
31 Schatzmann, H. J. and Rossi, G. L. (1971) Biochim. Biophys. Acta 241, 379–392
32 Parkinson, D. K. and Radde, I. C. (1971) Biochim. Biophys. Acta 242, 238–246
33 Lardy, H. A., Johnson, D. and McMurray, W. C. (1958) Arch. Biochem. Biophys. 78, 587–597
34 Kagawa, Y. and Racker, E. (1966) J. Biol. Chem. 241, 2467–2474
35 Gemba, M. and Ueda, J. (1971) Jap. J. Pharmacol. 21, 263–270
36 Skou, J. C. (1960) Biochim. Biophys. Acta 42, 6–23
37 Skou, J. C. (1962) Biochim. Biophys. Acta 58, 314–325
38 Fujita, M., Nagano, K., Mizuno, N., Tashima, Y., Nakao, T. and Nakao, M. (1968) Biochem. J. 106, 113–121
39 Czerwinski, A., Gitelman, H. J. and Welt, L. G. (1967) Am. J. Physiol. 213, 786–792
40 Neufeld, A. H. and Levy, H. M. (1969) J. Biol. Chem. 244, 6493–6497
41 Baer, J. E. and Beyer, K. H. (1972) in Progr. Biochem. Pharmacol. (Edwards, C., ed.), Vol. 7, pp. 59–93, Karger, Basel
42 Gyori, A. Z., Brendel, U. and Kinne, R. (1972) Pflügers Arch. 335, 287–296
43 Epstein, R. W. (1972) Biochim. Biophys. Acta 274, 128–139
44 Robinson, J. W. L., Mirkovitch, V., Borgeaud, J. and Campiche, M. (1973) Biomedicine 18, 206–212
45 Pennington, R. J. (1961) Biochem. J. 80, 649–654
46 DePierre, J. W. and Karnovsky, M. L. (1973) J. Cell Biol. 56, 275–303