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THE EFFECT OF IONIC STRENGTH ON A Mg^{2+}-ATPase AND ITS RELEVANCE TO THE DETERMINATION OF $(Na^+ + K^+)$-ATPase

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SUMMARY

A ouabain-insensitive Mg^{2+}-ATPase present in a microsomal fraction prepared from the dog submandibular gland was studied. This Mg^{2+}-ATPase was inhibited by increasing concentrations of NaCl, KCl, RbCl and CsCl. The addition of an osmotically equal amount of sucrose was without effect. This inhibition was obtained over a pH range of from 6.3 to 8.8. The Mg^{2+}-ATPase present in microsomes treated with NaI showed a similar inhibition. These results indicate that it is advisable to keep the ionic strength constant in solutions used to obtain $(Na^+ + K^+)$-ATPase activities.

INTRODUCTION

$(Na^+ + K^+)$-ATPases have been extensively studied because of their probable role in ion transport. These studies are usually performed on cellular fractions containing numerous other enzymes in addition to the $(Na^+ + K^+)$-ATPase. One of the contaminating enzymes is the Mg^{2+}-ATPase. Since Mg-ATP is the substrate for the $(Na^+ + K^+)$-ATPase, it is not possible to measure the activity of this enzyme without also activating the Mg^{2+}-ATPase. Several different methods have been used to calculate the $(Na^+ + K^+)$-ATPase in preparations containing both enzymes. One common procedure is to take the difference between the ATPase activities in the presence of Na^+, K^+ and Mg^{2+} and in the presence of Mg^{2+} alone as the $(Na^+ + K^+)$-activated ATPase activity [1, 2]. Because the usual concentrations of NaCl and KCl are in the regions of 150 and 5 mM, respectively, it can be appreciated that there are significant differences in ionic strength under these two conditions. Another procedure for calculating the $(Na^+ + K^+)$-ATPase activity is to equate it with the ouabain-sensitive activity [3, 4]. Since the ouabain does not contribute significantly to the ionic strength of these solutions, the difference measurement is done at essentially constant ionic strength. A third procedure for measuring $(Na^+ + K^+)$-ATPase activity is to take the difference in activities between solutions containing $Na^+ + K^+ + Mg^{2+}$ and those containing $Na^+ + Mg^{2+}$ alone [5]. Since the KCl concentration is usually much less than the NaCl concentration, these solutions are also of nearly constant ionic strength.

Finally, salts (other than NaCl) have also been used to maintain ionic strength during the measurement of the Mg^{2+}-ATPase activity [6].

Heretofore, there has not been good reason to favor one of these procedures over the others, and in fact the average of three of these has been used as the reference ATPase activity [7]. However, it has been our experience that these procedures yield consistently different results for the $(Na^+ + K^+)$-activated ATPase present in microsomes prepared from the dog submandibular gland. The present study is an outgrowth from this observation. These experiments indicate that the above procedures for determining the $(Na^+ + K^+)$-stimulated ATPase activities are not equivalent because the Mg^{2+}-ATPase is sensitive to ionic strength.

METHODS

The microsomal fraction was prepared as described in detail elsewhere [8]. Most preparations were used with out NaI treatment. Those preparations that were treated with NaI were washed twice with 1 mM Tris–EDTA and then were suspended in 1 mM Tris–EDTA (pH 7.4) rather than the NaEDTA previously used.

Standard determinations of ATPase activities were performed as was previously described in detail [8, 9] except that the standard incubation solutions contained 5 mM $MgSO_4$, 50 mM Tris–H_2SO_4 buffer (pH 7.5) and 5 mM Tris–ATP as well as the cations and anions to be described in the Results. The final reaction volume was 2 ml and each tube was run in duplicate. The reaction was terminated after incubation for an hour at 37 °C by the addition of 1 ml 15 % trichloroacetic acid. The liberated phosphate was measured by the method of Fiske and SubbaRow [10] and protein by the method of Lowry et al. [11].

RESULTS

The Mg^{2+}- and the $(Na^+ + K^+ + Mg^{2+})$-ATPase activities in untreated microsome fractions were measured in the presence and absence of 10^{-4} M ouabain. The average values from 30 determinations are given in Table I. There was no significant

TABLE I

Mg^{2+} AND $(Na^+ + K^+)$-ATPase ACTIVITIES IN THE PRESENCE AND ABSENCE OF OUABAIN

Reaction mixture for $(Na^+ + K^+)$-ATPase contained 5 mM $MgSO_4$, 5 mM Tris–ATP (pH 7.5), 50 mM Tris–H_2SO_4 (pH 7.5), 150 mM NaCl and 5 mM KCl. For the Mg^{2+}-ATPase, the NaCl and KCl were omitted. The ouabain concentration was 10^{-4} M. Incubation was for 1 h at 37 °C. The values are the means ±S.E. from 30 measurements.

Enzyme	Activity (μM P_i per mg protein per h)	
	Without ouabain	With ouabain
Mg^{2+}	16.0±1.1	15.7±1.1*
$Na^+ + K^+ + Mg^{2+}$	48.0±3.0	11.2±0.9**

* Not significantly different from the Mg^{2+}-ATPase activity in the absence of ouabain.

** The difference between this activity and the Mg^{2+}-ATPase activity was significant at better than the 0.001 level.

difference between the Mg^{2+}-ATPase activities in the presence and absence of ouabain. On the other hand, the average ATPase activity in the presence of $(Na^+ + K^+ + Mg^{2+} + \text{ouabain})$ was significantly ($P < 0.001$) different from that with Mg^{2+} alone. In every instance, the $Na^+ + K^+ + Mg^{2+} + \text{ouabain}$ activity was less than the Mg^{2+} activity, and the average value of the ratio of the ATPase activity in the presence of $Na^+ + K^+ + Mg^{2+} + \text{ouabain}$ to that in the presence of Mg^{2+} alone was 0.68 with a 95 % confidence interval of 0.78–0.58. Similar results were obtained if SO_4^{2-} in each of the stock solutions was replaced with Cl^-. In addition, fresh microsome preparations as well as microsomes that were frozen for periods between 1 and 33 days yielded similar results.

Effect of pH

The effect of pH on the ratio of the $(Na^+ + K^+ + Mg^{2+} + \text{ouabain})$-ATPase activity to the Mg^{2+}-ATPase activity was measured after titrating the stock Tris buffer to the pH values indicated in Table II (two experiments) and by using a combination Pipes-Tris buffer (one experiment). The NaCl, KCl and ouabain concentrations were the same as in Table I. The ATPase activity in the presence of $Na^+ + K^+ + Mg^{2+} + \text{ouabain}$ was less than that in the presence of Mg^{2+} alone at each of the pH values tested. Thus, the degree of $Na^+ + K^+$ stimulation was different depending on whether the Mg^{2+}-ATPase or the $(Na^+ + K^+ + Mg^{2+} + \text{ouabain})$-ATPase activity was taken as the reference base (Table II).

TABLE II

VARIATION OF THE ATPase ACTIVITIES RATIOS WITH pH

The values are from a representative experiment. The solutions were the same as those described in Table I except that the Tris buffer was titrated to the indicated pH values before incubation (2 experiments) or the Tris buffer was replaced with Pipes–Tris buffer (1 experiment).

Enzyme	ATPase activity (μM P_i per mg protein per h)			
pH:	6.3	7.0	7.8	8.8
Mg^{2+}	10.3	10.5	12.0	14.0
$(Na^+ + K^+ + Mg^{2+})$*	6.4	6.6	8.3	9.4
$(Na^+ + K^+ + Mg^{2+})$	26.4	25.2	20.4	16.8

* In the presence of 10^{-4} M ouabain.

Effect of monovalent cations and sucrose

Fig. 1 summarizes the effect of varying the cation concentration on the ATPase activities. Curve 1 shows that the ATPase activity decreased in a similar fashion with increasing concentrations of KCl, LiCl, CsCl and RbCl. In addition, the results obtained with these cations in the presence of ouabain, in the presence of 5 mM KCl, and in the presence of 5 mM KCl and ouabain were not significantly different from Curve 1. Thus it is concluded that these cations are equally effective in inhibiting the Mg^{2+}-ATPase and that they cannot substitute for Na^+ in activating the $(Na^+ + K^+)$-ATPase. This latter finding is consistent with our earlier results [8].

The results obtained with NaCl were similar to the results obtained with the other salts with one exception: the fact that the $(Na^+ + Mg^{2+})$-ATPase was always

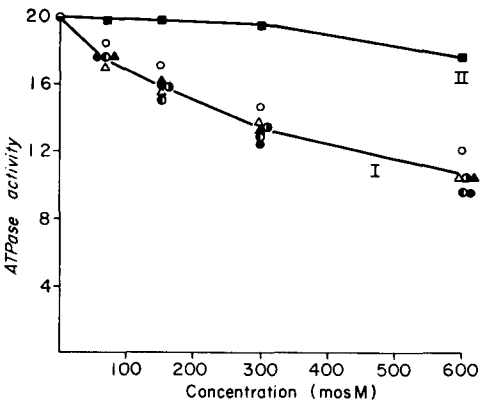

Fig. 1. The effect of cations on the ouabain insensitive ATPase. All incubation solutions contained 5 mM $MgSO_4$, 5 mM Tris–ATP (pH 7.5), 50 mM Tris–H_2SO_4 (pH 7.5) and 5 mM KCl. In addition, varying amounts of either NaCl (○), LiCl (◐), CsCl (◑), RbCl (●), KCl (△), NaCl (plus 10^{-4} M ouabain) (▲) (Curve I), or sucrose (■) (Curve II) were added. (The KCl was omitted from those solutions towhich NaCl but not ouabain was added.) The ATPase activities units are μM P_i per mg protein per h. The experiment was repeated twice.

slightly greater if ouabain was not present (Curve I, Fig. 1).

Curve II in Fig. 1 was obtained by the addition of sucrose to the standard incubation medium. The sucrose concentrations were chosen to be the same osmolarity as the cation solutions plotted in Curve I. The difference between Curves I and II indicates that the inhibition obtained with the monovalent cations (at least in the physiological range, $\leqslant$ 300 mosM) cannot be attributed to an effect of osmotic strength per se.

The data in Table III were obtained by varying the NaCl concentration of the incubation medium between 0 and 150 mM. In addition, either LiCl or RbCl was added so that the sum of the concentrations of the salts that were added to the standard incubation solution was 150 mM. In this case, the ratio remained depressed at the level of 150 mM NaCl.

These results (Fig. 1, Table III) indicate that variations in ionic strength are probably responsible for the observed inhibition of the Mg^{2+}-ATPase.

TABLE III

Mg^{2+}-ATPase ACTIVITY MEASURED AT CONSTANT IONIC STRENGTH

The solutions were the same as those described in Table I except that the NaCl concentrations were varied between 0 and 150 mM and either LiCl or RbCl was added so that [NaCl] + [LiCl] (or [RbCl]) = 150 mM. Similar results were obtained in two other experiments. The $(Na^+ + K^+ + Mg^{2+})$-ATPase was measured in the presence of 10^{-4} M ouabain. The Mg^{2+}-ATPase activity was 14 μM per mg protein per h.

Activities ratio	NaCl concentration (mM):	0	50	100	150
$\frac{(Na^+ + K^+ + Mg^{2+})}{Mg^{2+}}$		0.69	0.63	0.63	0.63

Effect of monovalent cations and sucrose on NaI-treated microsomes

The results obtained with NaI-treated microsomes were similar to those obtained with the untreated microsomes. In every instance, the ATPase activity was decreased by increasing ionic strength. The averaged results from the two experiments are shown in Fig. 2. The measurements were made as in Fig. 1 except that 10^{-4} M ouabain was added to the standard solutions. The ATPase activities of microsomes incubated in the 600 and 300 mM sucrose plus standard solutions were 103 and 102 %, respectively, of the Mg^{2+}-ATPase activity.

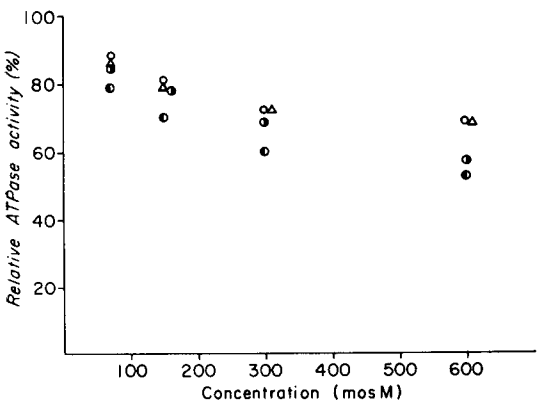

Fig. 2. The effect of cations on the ouabain insensitive ATPase from NaI-treated microsomes. The experimental conditions were as in Fig. 1 except that NaI-treated microsomes were used. The effects of increasing concentrations of NaCl (○), CsCl (◑), KCl (△) and LiCl (◐) were tested. The 100 % Mg^{2+}-ATPase activity was 6.0 μM P_i per mg protein per h. The experiment was performed twice.

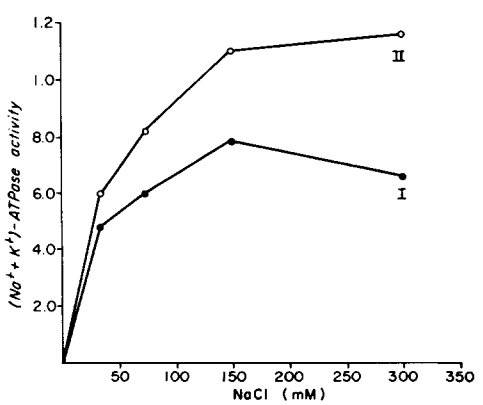

Fig. 3. Na activation of the Mg^{2+}-ATPase at constant KCl concentration. The Mg^{2+}-ATPase was measured as described in Table I. In addition, KCl (5 mM) and increasing amounts of NaCl were added to incubation solutions in the presence and absence of 10^{-4} M ouabain. The Mg^{2+}-ATPase activity (Curve I) or the $(Na^+ + K^+ + Mg^{2+})$-ATPase activity in the presence of ouabain (Curve II) was then subtracted from the $(Na^+ + K^+ + Mg^{2+})$-ATPase activity to obtain the activation curves at each NaCl concentration. The experiment was repeated twice.

Na^+-activation curves

Na^+-activation curves for the non-NaI-treated microsomes were obtained under two conditions. In the first (Fig. 3), the NaCl concentration was varied between 0 and 150 mM in the presence of 5 mM KCl in the standard incubation medium. Under this condition, the ionic strength of the incubation solutions increased with increasing NaCl concentration. The $(Na^+ + K^+)$-activated ATPase was measured as the difference between each value and the Mg^{2+}-ATPase (Curve I) and as the difference between the values obtained in the presence and absence of ouabain at each NaCl concentration (Curve II). In the latter method the effect of ionic strength on the Mg^{2+}-ATPase is corrected for at each test point but the ionic strengths at each of the test points are different.

The second set of Na^+-activation curves were obtained under conditions where the ionic strength in all solutions were kept constant by varying the concentration of either KCl or LiCl (Fig. 4). Curve I in Fig. 4 was obtained while keeping the sum [NaCl]+[KCl] = 150 mM. Curve II was obtained while keeping the sum [NaCl]+[KCl]+[LiCl] = 155 mM with [KCl] constant at 50 mM. Curve III was obtained while keeping the sum [NaCl]+[KCl]+[LiCl] = 155 mM with [KCl] constant at 5 mM. Similar curves were obtained when CsCl replaced LiCl.

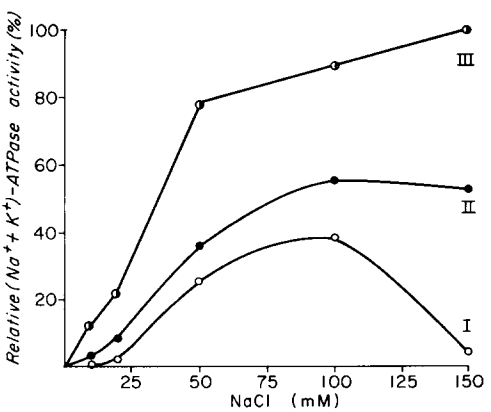

Fig. 4. Na activation of the Mg^{2+}-ATPase at constant KCl concentration and constant ionic strength. All solutions contained 5 mM $MgSO_4$, 5 mM Tris–ATP (pH 7.5) and 50 mM Tris–H_2SO_4 (pH 7.5). In addition, in Curve I NaCl and KCl were added so that their sum was 150 mM. In Curve II the KCl concentration was 50 mM and the sum [NaCl]+[KCl]+[LiCl] was 155 mM. In Curve III, the KCl concentration was constant at 5 mM and the sum was again 155 mM. Similar results were obtained if CsCl was used to keep ionic strength constant.

DISCUSSION

These experiments demonstrate that at least in the present system, ionic strength is an important parameter in the measurement of the $(Na^+ + K^+)$-stimulated ATPase since increases in ionic strength resulted in an inhibition of the Mg^{2+}-ATPase (Tables I and III and Fig. 1). Thus the degree of $Na^+ + K^+$ stimulation varied depending on whether it was calculated as the difference between the $(Na^+ + K^+ + Mg^{2+})$-ATPase and the Mg^{2+}-ATPase activities or as the ouabain-sensitive activity.

The present results indicate that the latter calculation is more accurate since the solutions in the presence and absence of ouabain have essentially the same ionic strength. This value would also be expected to be the larger of the two calculated activities.

The present results also indicate that Na^+-activation curves for the $(Na^+ + K^+)$-ATPase should be done at constant ionic strength. Figs 1 and 4 indicate that any of the monovalent alkali metal cations can be used for this purpose except K^+. The activation curve in the presence of K^+ is complicated by an inhibitory effect at higher KCl concentrations (Fig. 4).

In addition the degree of inhibition of Mg^{2+}-ATPase was consistently higher if NaCl was tested with ouabain rather than without ouabain.

Previous studies on the effects of ionic strength on the microsomal Mg^{2+}-ATPase have yielded inconsistent results. Ahmed and Thomas [5] found that the addition of salts did not affect the Mg^{2+}-ATPase activity in rat brain microsomes. There was also no significant alkali metal cation effect on the Mg^{2+}-ATPase from red blood cells [12]. On the other hand, Bray [13] has published findings similar to ours in a study on skeletal muscle fiber segments, and Schwartz and Laseter [14] have published similar findings on a microsomal fraction prepared from cardiac muscle (Expt 1 in their Table I). However, their data indicated that the phenomenon was lost following aging for 21 days or more while aging was without effect in the present experiments. In addition, Atkinson and Lowe [15] concluded that NaI-treated microsomes prepared from pig brains could be categorized into two groups: Types α and β. This Type β preparation demonstrated an inhibition of the Mg^{2+}-ATPase with increasing concentrations of NaCl (and thus of ionic strength), while this effect was barely detectable in the Type α preparations. Thus, it is possible that the inconsistent results from the ionic strength studies to date may be due to variabilities in the properties of the microsomes prepared from various tissues. Regardless of the explanation, maintenance of constant ionic strength in the test solutions would assure valid results in any case.

Finally, it should be realized that the present results are of significance even if the intracellular sites of origin of the Mg^{2+}-ATPase and the $(Na^+ + K^+ + Mg^{2+})$-ATPase are different. In this regard, Robinson[16] and Rendi and Uhr [17] have reported vanishingly small levels of Mg^{2+}-ATPase activity as their microsome fractions were treated to remove mitochondrial contamination. This indicates that the Mg^{2+}-ATPase may originate in mitochondria [18]. On the other hand, it is clear that in red blood cells, there is a non-mitochondrial Mg^{2+}-ATPase [12]. In the present study, the fact that the NaI-treated microsomes responded in a similar fashion as non-treated microsomes to increases in ionic strength (Figs 1 and 2), indicates that the present findings have general applicability since NaI treatment has been shown to decrease mitochondrial contamination [9, 19]. Thus, an ionic strength-sensitive Mg^{2+}-ATPase may be present in many microsomal preparations and this possibility should be recognized when measuring $(Na^+ + K^+)$-stimulated ATPase activities.

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