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A MICROSOMAL HCO_3^--STIMULATED ATPase FROM THE DOG SUBMANDIBULAR GLAND

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SUMMARY

A HCO_3^--stimulated, Mg^{2+}-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) present in a microsomal fraction prepared from dog submandibular glands was investigated. The bicarbonate concentration necessary for half maximal activation of the enzyme was about 2 mM and the optimum pH was 7.7. The enzyme was completely inhibited by SCN^- but only partially inhibited by ouabain or acetazolamide. The HCO_3^--stimulated ATPase activity was correlated with dinitrophenol stimulation and with succinate dehydrogenase activity. This suggests that the enzyme is of mitochondrial origin.

INTRODUCTION

The HCO_3^- concentration in the saliva of many species may be 50 mM or higher[1,2]. In the light of this unusual transport of HCO_3^- by salivary glands, it was of interest to see if they contained a HCO_3^--stimulated ATPase.

Previous investigators have described a HCO_3^--stimulated, Mg^{2+}-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) in microsomal fractions prepared from the gastric mucosa of various species[3–6]. These investigations have led to speculation that this enzyme may be involved with transcellular HCO_3^- transport[4,5,7]. However these experiments with microsomal fractions were done with the components of the carbonic acid system out of equilibrium[3–5]. Under these conditions, maintaining a constant pH will not keep the HCO_3^- concentration constant, as the change in the HCO_3^- concentration will be determined by the diffusion path for CO_2. The buffer value of the reaction medium will determine whether there is a concomitant pH change. Thus, it is necessary to compare the HCO_3^- activation of the enzyme with the carbonic acid system in equilibrium during the incubation period to that with the carbonic acid system out of equilibrium. Such a comparison has previously been made for a mitochondrial enzyme and important differences were observed[8].

Abbreviations: HEPES, hydroxyethylpiperazineethanesulfonic acid; PIPES, piperazine-bis(2-ethanesulfonic acid).

METHODS

The microsomal fraction was prepared as described in detail elsewhere (S. H. Hall, I. A. Siegel and K. T. Izutsu, unpublished). The minced glands were first homogenized in a Waring blender for 60 s with 9 vol. (w/v) of an ice-cold solution containing 0.1% deoxycholate, 250 mM sucrose and 1 mM EDTA (pH 7.3). This homogenate was centrifuged at 14 000 × *g* for 20 min. The supernatant was then centrifuged at 105 000 × *g* for 1 h. The resulting pellet was resuspended in 6 vol. (w/v) of 0.05% deoxycholate, 250 mM sucrose and 1 mM EDTA (pH 7.3). The centrifugation procedures were then repeated and the final pellet was suspended in a volume of 1 mM EDTA (pH 7.2) equal to the original tissue weight (ml/g). This suspension was diluted with two volumes of distilled water before being added to the reaction vials. In several experiments the suspension was treated with a 2 M NaI procedure[9].

When no attempt was made to equilibrate the components of the CO_2 system, the ATPase activity was measured in a 2-ml reaction volume containing 5 mM $MgSO_4$, 1 mM EDTA (acid), 50 mM Tris–H_2SO_4 buffer (pH 8.0), 5 mM Tris–ATP and approximately 30 μg of microsomes (pH 8.0) with various concentrations of HCO_3^-. The effects of the various inhibitors discussed in the Results were measured under these conditions.

Where the HCO_3^--stimulated Mg^{2+}-ATPase activity was measured with the CO_2 system in equilibrium, the reaction solutions were the same as those above except that the Tris buffer was omitted. These solutions were equilibrated at 37 °C with the CO_2 tensions necessary to maintain pH constant for the chosen HCO_3^- concentrations. The Henderson–Hasselbalch equation[10] was used to calculate the appropriate CO_2 tensions. These tensions were obtained by varying the relative flow rates of moist CO_2 and O_2 at 37 °C with a clinical anesthesia machine. After a steady pH had been obtained, the microsomes were added and the tubes were stoppered with rubber caps. The tubes were then preincubated for 10 min and the reaction was started by adding 0.2 ml of a 50 mM Tris–ATP solution through a hypodermic needle.

The pH dependence of the HCO_3^--stimulated Mg^{2+}-ATPase was also measured with the CO_2 system in and out of equilibrium. The equilibrated tubes were treated as before except that the HCO_3^- concentration was kept constant and the CO_2 tension was varied to cover the desired pH range. In order to measure the pH dependence under nonequilibrium conditions, it was necessary to replace the 50 mM Tris buffer with a 20 mM Tris, 20 mM hydroxyethylpiperazineethanesulfonic acid (HEPES) and 20 mM piperazine-bis(2-ethanesulfonic acid) (PIPES) buffer system. The control tubes contained the same solutions as those above except that all tubes contained the appropriate buffers and NaCl was substituted for $NaHCO_3$.

The tubes were incubated for 60 min and the reaction was stopped by adding 1 ml of a 15% trichloroacetic acid solution. The liberated phosphate was measured by a modification of the method of Fiske and Subbarow[11] and the protein was determined according to Lowry *et al.*[12]. All tubes were run in triplicate and all experiments were repeated at least twice. The pH values of the individual tubes were measured (at 37 °C) both before and after incubation.

The succinate dehydrogenase activities of various cell fractions were also measured. Cell debris (700 × *g*), nuclei (7000 × *g*), mitochondria (14 000 × *g*),

microsomes (105 000 × *g*, both treated and untreated with 2 M NaI) and a supernatant fraction were prepared by centrifugation. The succinate dehydrogenase activities of these fractions (relative to the mitochondrial fraction) were then measured in a Gilford recording spectrophotometer using the indophenol method of Green *et al.*[13]. The addition of 3 mM $CaCl_2$ to the assay medium (with Tris buffer substituted for phosphate) did not affect the results.

RESULTS

Dependence on the HCO_3^- concentration

The HCO_3^- stimulation of the ATPase was dependent on the presence of Mg^{2+} and this activity was stimulated equally by the addition of either $NaHCO_3$ or $KHCO_3$. The HCO_3^--stimulated Mg^{2+}-ATPase activity varied sharply with the HCO_3^- concentration of the reaction medium (Figs 1 and 2). This was true whether or not the CO_2 system had been equilibrated. However, the curve obtained with the CO_2 system in equilibrium had a maximum at approximately 10 mM HCO_3^- and then decreased at

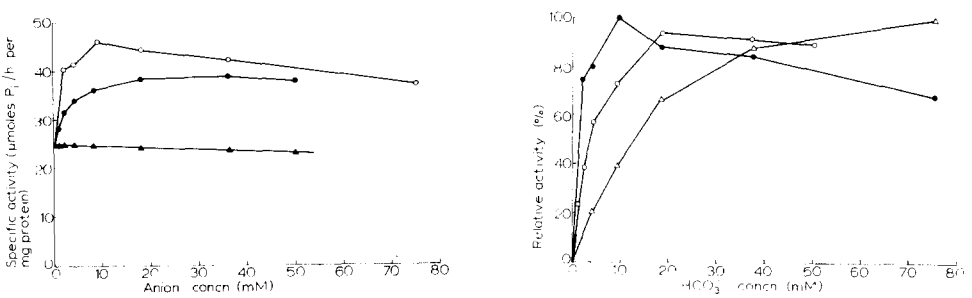

Fig. 1. Influence of anion concentration on ATPase activity. The triangles and circles indicate the results obtained with Cl^- and HCO_3^-, respectively. The solid triangles and circles are the averaged results obtained with Tris buffer and air in three experiments. The open circles are the results obtained with HCO_3^- and CO_2 as the buffer in two experiments. Mg^{2+} was present in all experiments. The Tris-buffered solutions had a pH of 8.0 ± 0.15 (range). The HCO_3^-–CO_2 solutions had a pH of 7.4 ± 0.2 (range).

Fig. 2. The HCO_3^--stimulated ATPase activity in various buffers. The solid circles are the results obtained with HCO_3^-–CO_2 buffer (pH 7.4). The open circles are the results obtained with 50 mM Tris as the buffer (pH 8.0). The triangles are the results obtained with 50 mM Tris as the buffer (initial pH 7.4). The HCO_3^--stimulated Mg^{2+}-ATPase activity *minus* the Cl^--stimulated Mg^{2+}-ATPase activity.

higher concentrations. In contrast, the curve (at pH 8.0) with the CO_2 system out of equilibrium plateaued as the HCO_3^- concentration increased. The HCO_3^- concentration necessary for half maximal activation of the enzyme was 1 mM when equilibrated with CO_2 and 3 mM when not equilibrated. The Tris buffer (50 mM) was unable to maintain a pH of 7.4 over the incubation period. Use of the Tris buffer at this pH resulted in a significantly different activation curve with a half maximal activation concentration of about 15 mM (Fig. 2). The pH of these solutions changed from the initial 7.4 setting to 7.1–7.9. The amount of phosphate liberated was proportional to the protein concentration and to the incubation period (between 10 and 120 min).

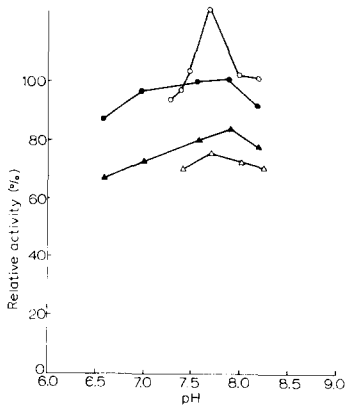

Fig. 3. The pH dependence of the HCO_3^--stimulated ATPase. The solid circles represent the relative ATPase activities (with Tris buffer) obtained with HCO_3^- (25 mM) + Mg^{2+} (5 mM). The solid triangles represent the results obtained with Mg^{2+} alone (Tris buffer). The open circles represent the results obtained with constant HCO_3^- (25 mM) + Mg^{2+} (5 mM) and various CO_2 tensions. The open triangles represent the results obtained from the same preparation but with Mg^{2+} and a HEPES–PIPES–Tris buffer. Further details are given in the Methods.

pH dependence of the HCO_3^--stimulated Mg^{2+}-ATPase

The activity of the HCO_3^--stimulated Mg^{2+}-ATPase varied with pH (Fig. 3). The optimum pH for the HCO_3^--stimulated Mg^{2+}-ATPase was 7.7 where a sharp peak was obtained in the equilibrated case. The NaCl-stimulated Mg^{2+}-ATPase activity and the Mg^{2+}-ATPase activity had a similar pH dependence.

Inhibition of the HCO_3^--stimulated Mg^{2+}-ATPase by various agents

Ouabain, acetazolamide and SCN^- were all able to inhibit this enzyme to some degree. NaSCN was an extremely effective inhibitor and at a 10 mM concentration completely obliterated enzymatic activity while a concentration of 1 mM resulted in a 60% inhibition. Ouabain and acetazolamide were nearly equally effective in inhibiting the enzyme. Ouabain (at $1 \cdot 10^{-3}$ or $1 \cdot 10^{-4}$ M) reduced the activity by 30%. Acetazolamide (at $1 \cdot 10^{-3}$ M) produced a 40% inhibition. Carbamylcholine in concentrations of from $5 \cdot 10^{-3}$ M to $5 \cdot 10^{-5}$ M was without effect.

The enzymatic activities of the cell fractions

ATPase and succinate dehydrogenase activites were found in all cell fractions except in the supernatant after centrifugation at 105 000 $\times$ *g*. The (Na^+–K^+)-ATPase specific activity was highest for the microsomal fraction while the succinate dehydrogenase activity was maximal in the mitochondrial fraction. The microsomal and nuclear succinate dehydrogenase activities were about 80% and 50%, respectively, of the mitochondrial activity. Stimulation of the Mg^{2+}-ATPase by dinitrophenol and by HCO_3^- was correlated with the succinate dehydrogenase activity in all fractions. Illustrative enzymatic activities for the mitochondrial fraction are given in Table I.

The effect of treatment with 2 M NaI

Treating the microsomal fraction with 2 M NaI sharply reduced the HCO_3^- stimulation. The relative activities of the various enzymes of interest for a representa-

TABLE I

CORRELATION BETWEEN HCO_3^--STIMULATED ATPASE ACTIVITY AND DINITROPHENOL STIMULATION AND SUCCINATE DEHYDROGENASE ACTIVITY

The incubation media contained 5 mM Tris–ATP, 1 mM EDTA (acid) and 50 mM Tris buffer (pH 8.0) in addition to the substances listed below. The ATPase activities in each column have had the Mg^{2+}-ATPase activity subtracted from them and are expressed as percentages of the Mg^{2+}-ATPase activity for that fraction. The succinate dehydrogenase activities are relative to that for the 14 000 × *g* fraction. Similar results were obtained in two experiments.

Enzymes (and salt concentrations, mM)	*Enzyme activity (%)*		
	S_3 (14 000 × g)	*Microsomes (105 000 × g)*	*Microsomes 2 M NaI (105 000 × g)*
Mg^{2+}-ATPase (5)	0	0	0
$(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase (150) + (5) + (5)	38	103	254
NaCl-stimulated Mg^{2+}-ATPase (25) + (5)	+ 7	− 4	−13
$NaHCO_3$-stimulated Mg^{2+}-ATPase (25) + (5)	+64	+36	+ 2
Mg^{2+}-ATPase + dinitrophenol (5) + (0.5)	+37	+20	+ 0
Succinate dehydrogenase (relative)	1.00	0.90	0

tive microsomal preparation are given in the second column of Table I. This same preparation was also treated with 2 M NaI and the resulting enzymatic activities are given in the third column. The decrease in the HCO_3^--stimulated Mg^{2+}-ATPase activity was correlated with a decrease in the dinitrophenol stimulation of the Mg^{2+}-ATPase and a decrease in the succinate dehydrogenase activity. In contrast, the NaI treatment increased the $(Na^+–K^+)$-ATPase specific activity 3-fold.

DISCUSSION

The present results indicate that a HCO_3^--stimulated ATPase may be demonstrated in microsomal fractions prepared from the dog submandibular glands. The activity of the ATPase as measured without equilibrating the CO_2 system closely approximates the activity following CO_2 equilibration. However there was consistently greater HCO_3^- stimulation if the CO_2 system was in equilibrium during the incubation period (Figs 1 and 3). This finding has also been reported for the mitochondrial enzyme[8].

The experiments to establish the HCO_3^- activation curve (with CO_2 equilibrated) for this enzyme were hampered by the fact that the solutions with low HCO_3^- concentrations were often unable to maintain constant pH during incubation. However this variation resulted in a range of the HCO_3^- concentration for half maximal activation of only 0.5–2.0 mM (Fig. 2).

Comparison with gastric mucosa

The present enzyme is similar to that found in gastric mucosa[3–5]. Both require Mg^{2+}, are inhibited by SCN^- and have similar activation curves. The gastric mucosa

Mg^{2+}-ATPase has a pH optimum of 8.3[3,5] compared to the present result of approximately 7.9. In the presence of HCO_3^-, the gastric mucosa ATPase optimum is at pH 7.4[5]. However, $1 \cdot 10^{-4}$ M or $1 \cdot 10^{-3}$ M ouabain resulted in 30% inhibition of the present enzyme whereas it was without effect on the gastric mucosa ATPase[3].

Comparison with mitochondrial ATPase

A HCO_3^--stimulated Mg^{2+}-ATPase has previously been demonstrated in mitochondrial fractions prepared from beef heart[14,15] and rat liver[8] homogenates. In addition, it has recently been suggested that the microsomal HCO_3^--stimulated Mg^{2+}-ATPase activity may be due to contamination of this fraction with mitochondria[16].

The present results are consistent with the mitochondrial contamination hypothesis in that the HCO_3^- stimulation in each of the fractions was correlated with the succinate dehydrogenase activity and with dinitrophenol stimulation and not with (Na^+–K^+)-ATPase activity (Table I). In addition, the fact that two NaI treated preparations with no detectable HCO_3^- stimulation also had no succinate dehydrogenase activity and also no dinitrophenol stimulation, appears to establish the mitochondrial origin of the present enzyme. A similar correlation between the HCO_3^--stimulated Mg^{2+}-ATPase activity and the succinate dehydrogenase activity in various cell fractions prepared from the gastric mucosa has also been observed[4].

The fact that the succinate dehydrogenase activity was not sharply limited to the mitochondrial fraction probably indicates that mitochondria were disrupted in the preparatory procedure. Such a result is not unexpected with tissues that are difficult to homogenize[17].

It is difficult to ascribe a role in transcellular bicarbonate transport to the present enzyme since it appears to be of mitochondrial origin.

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REFERENCES

1 A. S. V. Burgen and N. G. Emmelin, *Physiology of the Salivary Glands*, The Williams and Wilkins Company, Baltimore, 1961, p. 144.
2 J. A. Young, C. J. Martin, M. Asz and F. D. Weber, *Pflügers Arch. Eur. J. Physiol.*, 319 (1970) 185.
3 D. K. Kasbekar and R. P. Durbin, *Biochim. Biophys. Acta*, 105 (1965) 472.
4 V. D. Wiebelhaus, C. P. Sung, H. F. Helander, G. Shah, A. L. Blum and G. Sachs, *Biochim. Biophys. Acta*, 241 (1971) 49.
5 A. L. Blum, G. Shah, T. St. Pierre, H. F. Helander, C. P. Sung, V. D. Wiebelhaus and G. Sachs, *Biochim. Biophys. Acta*, 249 (1971) 101.
6 C. Koenig and J. D. Vial, *J. Histochem. Cytochem.*, 18 (1970) 340.
7 R. P. Durbin and D. K. Kasbekar, *Fed. Proc.*, 24 (1965) 1377.
8 D. D. Fanestil, A. Baird Hastings and T. A. Mahowald, *J. Biol. Chem.*, 238 (1963) 836.
9 S. Uesugi, A. Kahlenberg, F. Medzihradsky and L. Hokin, *Arch. Biochim. Biophys.*, 130 (1969) 156.
10 J. T. Edsall and J. Wyman, *Biophysical Chemistry*, Vol. 1, Academic Press, New York, 1958, pp. 555–558.

11 C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, 66 (1925) 551.
12 O. H. Lowry, N. I. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
13 D. E. Green, S. Mii and P. M. Kohout, *J. Biol. Chem.*, 217 (1955) 551.
14 M. E. Pullman, H. S. Penefsky, A. Datta and E. Racker, *J. Biol. Chem.*, 235 (1960) 3322.
15 E. Racker, *Fed. Proc.*, 21 (1962) 54.
16 A. I. Katz and F. H. Epstein, *Enzyme*, 12 (1971) 499.
17 P. Siekevitz, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. V, Academic Press, New York, 1962, p. 61.