AN ATPase FROM DOG GASTRIC MUCOSA: CHANGES OF OUTER pH IN SUSPENSIONS OF MEMBRANE VESICLES ACCOMPANYING ATP HYDROLYSIS

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SUMMARY A microsomal Mg-ATPase from the gastric mucosa of dog, cat and frog has a K_m for ATP in the region 20-25 μM and by the value of this coefficient can be differentiated from the mitochondrial Mg-ATPase. The microsomal Mg-ATPase from dog gastric mucosa can be stimulated by gramicidin, nigericin and valinomycin in a KCl medium. This Mg-ATPase seems to be located in the ion impermeable membrane of microsomal vesicles and ATP hydrolysis driven changes of the outer pH can be observed. The data are consistent with the ATP hydrolysis driven entry of H^+ ions across the vesicle membrane.

INTRODUCTION Ganser and Forte (1) have described a K^+ stimulated Mg-dependent ATPase (Mg-ATPase) in microsomes of bullfrog oxyntic cells. More recently they have reported (2) that certain ionophores enhance the ATPase activity in the presence of K^+ ions. If the ATPase is located on membrane vesicles which have a low permeability to K^+ ions (as their data suggest) and to other ions in general, it should be possible to investigate the ion transport properties of this system.

In this paper we show that an ATPase system with similar properties can be isolated from dog gastric mucosa and describe ATP hydrolysis driven changes of the outer pH (pH_o) in suspensions of microsomal vesicles.

MATERIALS AND METHODS Oligomycin was purchased from Sigma, valinomycin from Calbiochem, FCCP and gramicidin D from Boehringer and nigericin was a gift from Eli Lilly.

Dog gastric mucosa was obtained from control animals (Beagles 18-22 Kg) used in toxicological studies. The antral and cardiac regions were cut away and the glandular mucosa was removed from the muscle by scraping. It was chopped finely in a medium containing 250 mM sucrose buffered at pH 7.4 with 10 mM Tris HCl (sucrose Tris HCl) to give a suspension containing 20-25 g wet weight of tissue per 100 ml. The tissue was homogenised for 5 seconds in a Silverson tissue homogeniser followed by 5 strokes at 1500 - 2000 rpm in a loose fitting teflon glass homogeniser. Differential centrifugation was

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Abbreviations Used: FCCP, Carbonyl cyanide p-trifluoro methoxyphenyl hydrazone

carried out to give residues at 500g x 10 minutes, 20,000g x 20 minutes and 150,000g x 90 minutes. The residue obtained at 20,000g was washed and resuspended in the sucrose Tris HCl medium and used as the mitochondrial fraction. The 150,000g residue (microsomal fraction) was washed in sucrose Tris HCl medium and resuspended in the same medium except when prepared for experiments involving pH measurements in which case the microsomal fraction was suspended in 250 mM sucrose. All manipulations were done at 0-4°. Fractions from other species were prepared similarly.

The reaction cell (volume 4 ml) and electrode system used in measurements of pH was as described by Mitchell and Moyle (3). ATP hydrolysis driven changes of pH_0 were measured by the ATP pulse technique described by Mitchell and Moyle (4). Microsomal fraction (approximately 2.5 mg protein) was added to the chamber containing 150 mM KCl, 5 mM glycyl glycine and 2 mM $MgCl_2$ under aerobic conditions. Measurements were made in the pH_0 range 6.1 - 6.15 at 25°. In this pH range no net acid is produced when ATP is hydrolysed to ADP and Pi (5). ATP was added as 10 µl of a solution containing 10 mM Na_2ATP and 10 mM $MgCl_2$ referred to as the MgATP solution. Valinomycin, FCCP, gramicidin D, nigericin and oligomycin were added as solutions in methanol (1-10 µl).

Measurements of Mg-ATPase activity used for calculating K_m for ATP were made in a medium containing 150 mM KCl, 5 mM glycyl glycine and 5 mM $MgCl_2$ in the pH_0 range 7.0 - 7.1 at 25° using the pH method (6,7). Frozen samples of mitochondria and microsomes were used in these experiments (mitochondrial fractions were also sonicated for 30 seconds) but checks showed that fresh samples gave similar results.

Experiments on the effect of ionophores on the K^+ stimulation of ATPase activity were done at 37° and pH 7.4 with freshly prepared microsomal fraction. The basal medium contained 25 mM sucrose, 20 mM Tris HCl, 5 mM $MgCl_2$ and 2 mM Na_2ATP. The phosphate released was measured by the method of Yoda and Hokin (8). Succinate dehydrogenase and K^+ stimulated p-nitrophenyl phosphatase were measured as described (1). 5′-nucleotidase was measured in a medium containing 50 mM Tris HCl (pH 8.0), 0.2 mM $MgCl_2$ and 0.02 mM [^{3}H] 5′-AMP. The reaction was terminated by addition of Dowex 1 and the adenosine formed in the reaction estimated as described by Somerville *et al* (9). Protein concentration was measured by the method of Lowry *et al* (10).

RESULTS AND DISCUSSION Table 1 shows that the ATPase systems from microsomal and mitochondrial fractions isolated from the gastric mucosa of dog, cat and frog can be distinguished by the value of K_m for ATP. For the microsomal fractions a value near 2 x 10^{-5}M was obtained whilst mitochondrial fractions gave a value near 1 x 10^{-4}M similar to the value obtained by Mitchell and

TABLE 1. The K_m for ATP of Mg-ATPase in Microsomal and Mitochondrial Fractions of Gastric Mucosa

Source of Gastric Mucosa	TISSUE FRACTION Microsomes	Mitochondria
Dog	$2.07 \pm 0.25 \times 10^{-5}$M(6)	1.05×10^{-4}(2)
Cat	$2.45 \pm 0.31 \times 10^{-5}$M(6)	8.7×10^{-5}(3)
Frog (*R. temporaria*)	2.0×10^{-5}(1)	Not measured

Mg-ATPase activity was measured by the pH method (6,7) under the conditions described in the Methods. Values reported are averages with standard deviations where applicable (the number of determinations is shown in parenthesis).

Moyle (7) for rat liver mitochondrial ATPase under similar conditions. Measurements of K_m for ATP made on 2 microsomal preparations from dog pancreas gave values of 1.8×10^{-5}M and 1.9×10^{-5}M. However we could not detect an ATPase with a low K_m for ATP under these conditions in dog liver, rat liver, dog kidney or dog antrum.

In a typical experiment the microsomal fraction from dog gastric mucosa containing 8% of the protein of the total homogenate possessed 7.4% of the total succinate dehydrogenase activity, 42% 5'-nucleotidase and 58% K^+ stimulated p-nitrophenyl phosphatase, suggesting that this fraction is derived largely from cell membranes. However, in agreement with Ganser and Forte (1) no stimulation of the ATPase system of microsomal fractions was observed with the addition of Na^+ ions in the presence of K^+ ions and the activity was insensitive to ouabain. The Mg-ATPase activity of this fraction can therefore be distinguished from the Na^+/K^+ ATPase normally found in plasma membrane fractions of animal cells.

Table 2 shows that gramicidin, valinomycin and nigericin enhance the Mg-ATPase activity of the microsomal fraction when assayed in the presence of K^+ ions. This result establishes a similarity between this mammalian system and the amphibian system studied by Ganser and Forte (2). Similar observations can also be made with microsomal fractions from cat gastric mucosa.

Figure 1 shows the time-course of the observed outer pH change (ΔpH_0) when additions of FCCP and valinomycin were made to suspensions of microsomal fraction in a KCl medium in the pH_0 range 6.1 - 6.2. Following the addition of valinomycin a slow acidification of the medium was observed (trace a) and

TABLE 2. The Effect of Ionophores on the Mg-ATPase Activity of Microsomal Fractions from Dog Gastric Mucosa

Additions	ATPase ACTIVITY In Basal Medium	20 mM KCl Added	20 mM NaCl Added
None	26.5	30.1	27.4
Gramicidin 10 µg/ml	21.4	42.9	23.4
Valinomycin 10 µg/ml	24.4	45.1	25.8
Nigericin 10 µg/ml	25.9	43.6	28.3

Mg-ATPase activity was measured by the release of Pi as described in the Methods. The activity is expressed in µmoles Pi/mg protein per hr.

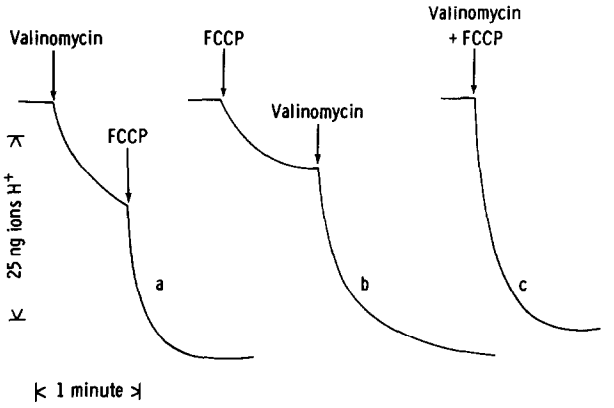

Figure 1 shows the time-course of ΔpH_0 when valinomycin (1.25 µg/ml) and FCCP (4.5×10^{-6}M) were added as indicated by the arrows, to suspensions of microsomal vesicles (prepared in 250 mM sucrose) in the KCl medium described in methods. Protein concentration 0.55 mg/ml.

the subsequent addition of FCCP resulted in a more rapid acidification. Similar observations were made (trace b) when the order of addition was reversed, whilst the simultaneous addition of both reagents produced a single rapid acidification (trace c). Since the microsomal fraction was prepared in a low K^+ medium it seems probable that addition of valinomycin (trace a) allowed the entry of K^+ ions into membrane vesicle structures but exit of a counter ion (H^+ in this case) was limited by the permeability properties of the membrane. When this restraint was removed by addition of FCCP a rapid equilibration occurred. The experiment indicates that a proportion of the

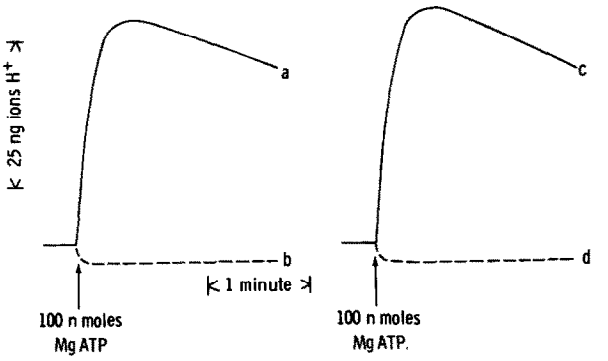

Figure 2 shows the time-course of ATP hydrolysis driven ΔpH_0 in a suspension of microsomal vesicles in the KCl medium described in methods (trace a). The changes recorded in trace b were in the presence of Triton X100 (500 µg/ml). Similar changes were recorded (traces c and d) in the presence of oligomycin (2.5 µg/ml). MgATP (100 nmoles) was added as indicated. Protein concentration 0.67 mg/ml.

microsomal fraction consists of membrane vesicles of low permeability to H^+, Cl^- and K^+, the major ions present.

Figure 2 (trace a) shows the time-course of ΔpH_0 when MgATP (100 nmoles) was added to a suspension of microsomal vesicles in the pH_0 range 6.1 - 6.15. Following the addition of MgATP the medium became more alkaline reaching a maximum in about 30 seconds and the subsequent decay of ΔpH_0 was very slow. Trace b records the pH change when MgATP (100 nmoles) was added to a suspension of vesicles treated with Triton X100 (500 µg/ml). Under these conditions the vesicles are disrupted but since the ATPase activity is also inhibited by Triton X100 this control may only be used to assess the small pH shift occurring on injection of the MgATP solution. Similar changes were observed when this experiment was repeated in the presence of oligomycin (traces c and d). Since oligomycin had no affect we conclude that the pH_0 changes were not due to mitochondrial contamination of the microsomal vesicles.

When membrane vesicles were disrupted by Triton X100 (Figure 3, trace a) an alkalinisation of the medium was observed (ΔpH_A). A second addition of Triton X100 (trace b) resulted in an acidification of the medium as expected from the acidity of the Triton X100 solution. This experiment indicated that the inner phase of the microsomal vesicles under these conditions was more alkaline than the suspending medium. Figure 3 (trace c) shows the time-course of the pH_0 change following the addition of 100 nmoles MgATP. When Triton X100 was added after 40 seconds a rapid acidification of the medium was observed. However, the final pH observed was displaced from the original baseline (ΔpH_C). Trace d shows the pH change (ΔpH_D) when MgATP was added to

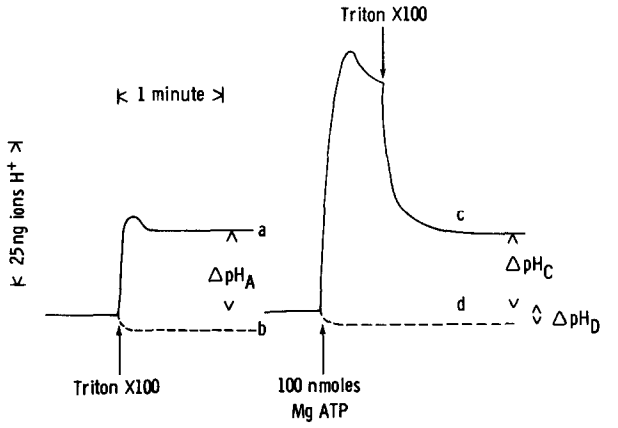

Figure 3 shows (trace a) the pH_0 change when Triton X100 (500 µg/ml) was added to a suspension of microsomal vesicles and trace b shows the effect of a second addition of Triton X100. Trace c shows the ATP hydrolysis driven ΔpH_0 and the rapid acidification following the addition of Triton X100 and trace d shows the pH change when MgATP was added in the presence of Triton X100. Protein concentration 0.65 mg/ml. Oligomycin (2.5 µg/ml) was present and the KCl medium was used as described in methods.

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the medium after disruption of the vesicles by Triton X100. Since $\Delta pH_A \simeq \Delta pH_C + \Delta pH_D$ we conclude that net formation of a basic substance does not contribute significantly to the pH_0 changes observed when MgATP is added to a suspension of microsomal vesicles under these conditions. The results are consistent with the ATP hydrolysis driven entry of H^+ ions or exit of OH^- ions across the membrane of the vesicles catalysed by the membrane Mg-ATPase. (For the purpose of discussion we will refer to the pH_0 changes as an ATP hydrolysis driven entry of H^+ ions).

Figure 4 shows the effect of valinomycin and FCCP on pH_0 changes following the addition of 100 nmoles MgATP. Comparing traces a and b the extent of ΔpH_0 and the rate of alkalinisation of the medium were not significantly altered in the presence of valinomycin but an increase was observed in the rate of decay of ΔpH_0. When FCCP was added to a vesicle suspension previously treated with valinomycin (trace c) the ΔpH_0 observed in response to MgATP was greatly reduced. After 60 seconds pH_0 had returned to a baseline, displaced from the original baseline because of the acidity of the MgATP solution. Figure 4 (trace d) shows that FCCP alone reduced the extent of ΔpH_0 by a small amount and the decay rate of ΔpH_0 was increased. Observations similar to those reported for trace c were made in the presence of FCCP and valinomycin (trace e).

It is noteworthy that the ATP hydrolysis driven entry of H^+ ions into

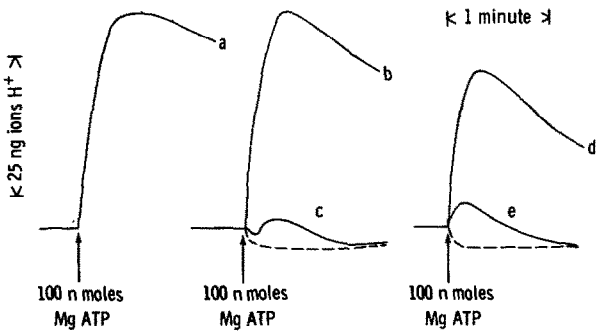

Figure 4 shows the effect of valinomycin and FCCP on ATP hydrolysis driven ΔpH_0. Trace a records the pH_0 change in the KCl medium with oligomycin (2.5 µg/ml) present. The changes in pH_0 with additions of 2.5 µg/ml valinomycin (trace b), 9 x 10^{-6}M FCCP (trace d) and valinomycin (2.5 µg/ml) plus 9 x 10^{-6}M FCCP (traces c and e) to this medium are also recorded. The dashed traces show the pH change when MgATP was added in the presence of Triton X100 (500 µg/ml). Protein concentration 0.67 mg/ml.

microsomal vesicles has properties which differ from the ATP hydrolysis driven proton translocation observed in sonic particles of mitochondria (5,11,12). In order to observe the translocation of H^+ ions across the membrane of submitochondrial particles it is necessary to provide a mechanism for the movement of a counter ion which collapses the electrical component of the proton motive potential. This can be accomplished by the addition of valinomycin in a K^+ medium. The finding that valinomycin has no affect on the extent of ΔpH_0 when MgATP is added to a suspension of microsomal vesicles suggests that the entry of H^+ ions accompanying ATP hydrolysis is an electrically neutral process. FCCP alone does not abolish the ATP hydrolysis driven pH_0 changes, thus the membrane vesicles must be impermeable to the counter ion. It therefore seems improbable that the counter ion is a mobile charged substance present in the membrane as proposed to explain the "backlash" phenomenum in mitochondria (13).

Experiments in this paper and similar experiments by Ganser and Forte (2) indicate that entry of K^+ ions into the microsomal vesicles is important in maintaining optimal ATPase activity. It seems possible therefore that K^+ ion exit from the vesicles accompanies H^+ ion entry during the hydrolysis of ATP. Experiments are in progress aimed at differentiating this type of mechanism from a mechanism involving the electrically neutral ATP hydrolysis driven entry of H^+ ions and Cl^- ions (the major anion present in these experiments).

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