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ENDOGENOUS PROTEASE MEDIATED MANIFESTATION OF A Ca^{2+}-STIMULATED ATPase IN PURIFIED DOG GASTRIC MICROSOMES

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An endogenous soluble protease has been demonstrated to unmask a Ca^{2+}-stimulated ATPase activity in purified dog gastric microsomes. The presence of ATP during protease treatment appears essential for the manifestation of the gastric Ca^{2+}-stimulated ATPase activity. The endogenous protease appears to have trypsin-like activity, since soybean trypsin inhibitor completely blocks the protease effect. Manifestation of the Ca^{2+}-stimulated ATPase occurs without affecting the microsomal ($H^+ + K^+$)-ATPase activity and associated H^+ uptake ability. The unmasked Ca^{2+}-stimulated ATPase appears insensitive to calmodulin. Possible roles of the enzyme in the regulation of gastric H^+ transport have been discussed.

Introduction

We have recently reported [1–3] the unmasking of a Ca^{2+}-stimulated ATPase from pig gastric microsomes after trypsinization in presence of ATP. This discovery raised the question whether there is any endogenous proteolytic activity within gastric cells for the manifestation of the Ca^{2+}-stimulated ATPase under physiologic conditions. Furthermore, is the phenomenon unique for pig gastric microsomes or general for the gastric microsomes from other species as well? In order to answer those questions we carried out the present investigation using dog fundic mucosal cells. We report that similar to pig, the purified dog gastric microsomes also contain a latent Ca^{2+}-stimulated ATPase, which is manifested after trypsinization in presence of ATP. In addition, we have demonstrated that the soluble supernatant fraction of dog fundic cells contains an enzymic activity which, like trypsin, can unmask the microsomal Ca^{2+}-stimulated ATPase activity. Possible roles of the Ca^{2+}-stimulated ATPase in the regulation of gastric acid secretion have been discussed.

Methods and Materials

Isolation of gastric microsomes. Purified microsomal membranes from dog gastric mucosa were prepared by the method of Ray [4]. Briefly, the fundic mucosa of dog was desquamated and scraped [5] to collect the oxyntic cell enriched fractions. The mucosal cells were homogenized in 0.25 M sucrose containing 0.25 mM EDTA and 2.0 mM Pipes buffer (pH 6.8) by using a loose-fitting pestle in a Dounce homogenizer. The homogenate was centrifuged at $8000 \times g$ for 5 min. The process was repeated three times. All supernatants were pooled together and layered over 40 ml 37% (w/v) sucrose in 84 ml screw-cap tubes and centrifuged at $100000 \times g$ for 5 h in a type 35 Beckman angle rotor. The microsomal membrane band appeared at the interface of the clear soluble supernatant and 37% sucrose. The soluble supernatant was used as the source of the endogenous activator [4] and the protease for Ca^{2+}-stimulated

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Abbreviations: Pipes, 1,4-piperazinediethanesulfonic acid.

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ATPase. The membrane bands were collected, diluted with homogenizing medium and centrifuged at $100000 \times g$ for 90 min. The pellet was resuspended in the homogenizing medium at an appropriate protein concentration and used for the study. Proteins were assayed by the method of Lowry et al. [6] using bovine serum albumin as standard.

Treatment with soluble supernatant or trypsin. Treatment with trypsin (Sigma) was carried out at 30°C in 20 mM Tris-HCl buffer (pH 7.5) containing 250 mM sucrose with a weight ratio of trypsin to membrane protein 1:100 in presence and absence of 2 mM ATP as reported previously [1–3]. Control membranes were incubated in parallel without trypsin. After a preincubation period of 3 min, trypsin digestion was carried out for 5 min and terminated by adding soybean trypsin inhibitor equalling twice the weight of trypsin. Treatment with soluble supernatant (the ratio of microsome to supernatant protein, 1:1) was carried out under identical conditions as that of trypsin in presence of 2 mM Mg^{2+}. Control membranes were incubated in parallel with Mg^{2+} without any agents. About 0.5–1 mg membrane protein was used for each group. After the treatment the membranes were immediately assayed for the ATPase activity.

Measurement of ATP. The concentrations of ATP in the dialysed and non-dialysed soluble supernatant was measured by the luciferase method [7]. An aliquot of the cytosol was denatured by ice-cold 5% perchloric acid and the supernatant after removal of the protein was neutralized (pH 7.0) by KOH under ice-cold conditions. The $KClO_4$ precipitate was removed by centrifugation and the supernatant was assayed for ATP [7]. Vacuum-dried firefly lanterns (Sigma) was used as the source of luciferase.

Assay of ATPase. The K^+-stimulated ATPase was assayed as previously described [4] in an isotonic medium. The incubation mixture contained, in a total volume of 1 ml, 50 μmol of Pipes (pH 6.8), 200 μmol sucrose, 1 μmol $MgCl_2$, 2 μmol Tris-ATP, 10 μg membrane protein with or without about 50 μg of the activator protein [4,11] and in the presence or absence of 25 μmol of KCl. After 10 min preincubation at 37°C the reactions were started with 2 μmol of Tris-ATP and incubated for 15 min. The reactions were stopped by adding 1 ml of 12% (w/v) CCl_3COOH. The P_i was assayed by the procedure of Sanui [8].

For the assay of Ca^{2+}-stimulated ATPase, the reaction mixture contained, in a total volume of 1 ml: 5 μmol Pipes/Tris (pH 7.0), 1 μmol EGTA, 1 μmol $MgCl_2$, 2 μmol Tris-ATP, 10 μg membrane protein in presence and absence of 0.99 μmol of Ca^{2+}. All other incubation conditions were the same as mentioned above. A K_{app} of $1 \cdot 10^7$ was used to calculate the concentrations of free Ca^{2+} ($1 \cdot 10^{-5}$ M) of the Ca^{2+}-EGTA buffer [9].

Assay of H^+ uptake. The K^+-stimulated ATPase mediated transport of H^+ inside gastric microsomal vesicles was studied according to Lee and Forte [10] and as reported previously [11]. The method uses the fluorescence quenching of acridine orange as a measure of vesicular proton uptake.

Results and Discussion

We previously reported [1–3] that trypsinization of purified gastric microsomes in presence of ATP unmasks a Ca^{2+}-stimulated ATPase with high affinity for Ca^{2+}. The data [1–3] suggested that the manifestation of a latent gastric Ca^{2+}-stimulated ATPase occurred due to removal of some suppressor protein, which became vulnerable to tryptic attack following some ATP-induced conformational changes. The present data reveal that similar unmasking of a Ca^{2+}-stimulated ATPase occurs when the gastric microsomes are incubated with the soluble cytosolic fraction of the gastric cells (Fig. 1). Higher efficacy of the undialysed supernatant compared to the dialysed fraction (Fig. 1) might suggest a requirement of ATP for the process of gastric Ca^{2+}-stimulated ATPase manifestation, since ATP is essential for trypsin activation of the enzyme [1–3]. Our observations that the ATP level (nmol/mg protein) of the dialysed supernatant was much lower (4.5 ± 0.4) than the nondialysed ones (82.2 ± 2.9) strongly suggest such a possibility. In separate experiments we demonstrated that incorporation of 0.1 mM ATP during treatment of microsomes with dialysed supernatant does enhance the Ca^{2+}-stimulated ATPase from 2.7 ± 0.5 to 4.4 ± 0.3 ($n = 4$). Such lower effects of dialysed supernatant compared to the nondialysed ones (Fig. 1) might be due to

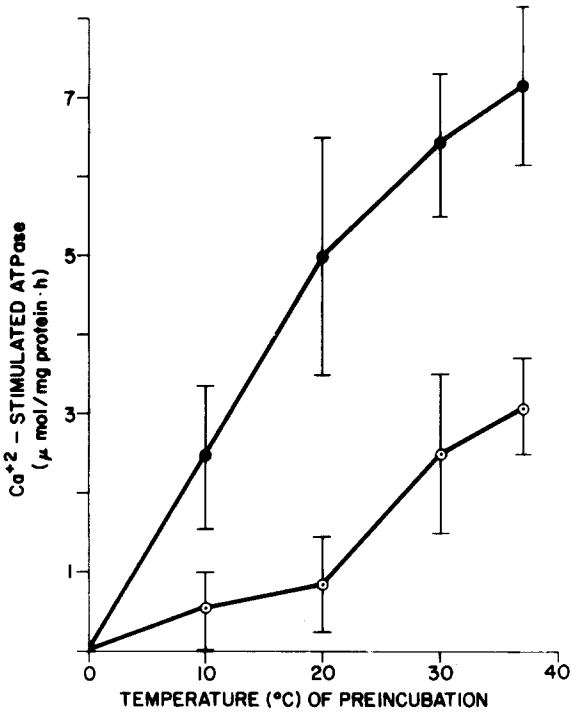

Fig. 1. Microsomes were incubated in 20 mM Pipes/Tris buffer (pH 7.4) containing 2 mM Mg^{2+}, dialysed (O——O) or nondialysed (●——●) supernatant for 5 min at the designated temperature. The ratio of membrane protein to supernatant was 1 : 1. The microsomes were then immediately assayed for ATPase. Details are given in Table I and Methods. Data are mean ±S.E. (n=4).

partial denaturation of the protease during prolonged (48 h) dialysis at 10°C [4]. Requirement of ATP for trypsin activation of the gastric Ca^{2+}-stimulated ATPase has been found to be less than 0.1 mM (unpublished results) and hence comparable to the present data (see above). Thus, the data (Fig. 1) suggest that there is an endogenous protease in the soluble supernatant of gastric cells which like trypsin is capable of unmasking a Ca^{2+}-stimulated ATPase in purified dog gastric microsomes in presence of ATP.

The fact that an endogenous protease having some similarity with trypsin is responsible for the unmasking of gastric microsomal Ca^{2+}-stimulated ATPase is further substantiated by our data in Table I. Thus, like trypsin the endogenous protease is heat labile and is completely inhibitable by soybean trypsin inhibitor (Table I).

It should be noted that there appears to be no calmodulin like activator for the gastric microsomal Ca^{2+}-stimulated ATPase in the soluble cytosolic fractoin. Thus, unlike calmodulin, which is heat stable, neither the boiled nor the dialysed supernatant is able to stimulate the Ca^{2+}-stimulated ATPase activity associated with the trypsinized microsomes (Table I). In view of the reports of widespread occurrence of calmodulin in

TABLE I

EFFECTS OF TREATMENT OF DOG GASTRIC MICROSOMES WITH TRYPSIN AND/OR SUPERNATANT UNDER VARIOUS CONDITIONS ON THE MICROSOMAL Ca^{2+}-STIMULATED ATPase ACTIVITY

Purified microsomes were treated in 20 mM Pipes/Tris buffer (pH 7.4) containing 2 mM Mg^{2+} with trypsin (trypsin-to-membrane ratio, 1 : 100) or soluble supernatant (membrane-to-supernatant ratio, 1 : 1) in presence and absence of 2 mM ATP for 5 min at 30°C. Trypsin digestion was stopped by adding soybean inhibitor equalling twice the weight of trypsin. Control microsomes were incubated under identical conditions without any agent. For the experiment designated as 'heated supernatant' the microsomes were incubated with a supernatant kept in an 85°C water bath for 5 min. At the end of incubation aliquots of the membranes were assayed for ATPase activity. Details of ATPase assay are given in Methods. Data are means ±S.E. from four separate experiments.

Conditions of Treatment	ATPase activity (μmol/mg per h)		
	Mg^{2+} alone	With $1 \cdot 10^{-5}$ M Ca^{2+}	ΔCa^{2+}
1 Control microsomes	19.22±0.72	18.30±0.62	0
2 Trypsin (without ATP)	18.96±1.0	19.33±0.86	0.37
3 Trypsin (with ATP)	21.19±0.27	23.94±0.35	2.75
4 Supernatant (nondialysed)	24.04±0.61	30.15±0.26	6.11
5 Heated supernatant	29.15±0.44	28.73±0.14	0
6 Supernatant plus trypsin inhibitor (25 μg)	19.98±0.47	19.98±0.31	0
7 Trypsinized (with ATP) plus heated supernatant	23.59±0.14	26.32±0.35	2.73
8 Trypsinized (with ATP) and assayed with supernatant (dialysed)	27.4 ±0.59	30.28±0.37	2.88

animal cells [12], these preliminary results showing absence of calmodulin like activity in the cytosolic fraction of gastric cells (Table I) is a curious finding. The calmodulin activity has been demonstrated to be associated with and probably identical to the heat-stable nondialysable activator protein of cyclic AMP phosphodiesterase [13,14]. It is interesting that although similar activator of cyclic AMP phosphodiesterase has been reported in the cytosolic fraction of gastric cells [15] no calmodulin like activity could be detected in the present study (Table I). It should be pointed out that a calmodulin-insensitive Ca^{2+}-stimulated ATPase associated with a low density plasma membrane fraction from rat corpus luteum has recently been reported [16]. Such lack of sensitivity to calmodulin has been attributed to saturation of the enzyme with tightly bound calmodulin [16]. Whether similar tight binding is responsible for the lack of calmodulin stimulation of the gastric Ca^{2+}-stimulated ATPase remains to be seen. In view of a recent report by Sarkadi et al. [17] it is also possible that trypsin treatment might have removed some limit peptide from the Ca^{2+}-pump protein with the generation of a maximally stimulated Ca^{2+}-ATPase which is totally insensitive to calmodulin. Hence the role of calmodulin in gastric microsomal Ca^{2+}-stimulated ATPase activity needs to be carefully investigated. It is noteworthy in this connection that calmodulin like activity in the cytosolic fraction from rat gastric cells, capable of activating a Ca^{2+}-dependent protein kinase associated with the purified rat gastric microsomes have recently been reported [18].

Presence of a latent Ca^{2+}-stimulated ATPase in purified gastric microsomes appears to be a general phenomenon since it is present in pig [1–3] and also in dog (present study). In view of the reported critical role of Ca^{2+} in gastric acid secretion [19,20] the present demonstration of a soluble endogenous protease capable of manifesting the gastric Ca^{2+}-stimulated ATPase makes this enzyme a likely candidate for its role in the regulation of gastric H^+ transport. This view will be consistent with the fact that low concentrations (20–50 μM) of Ca^{2+} inhibits the endogenous activator-stimulated microsomal K^+-stimulated ATPase [4], which has recently been identified as the proton pump [10,21,22]. Thus it appears possible that the activity of the gastric Ca^{2+}-stimulated ATPase may effectively regulate the H^+ transport ability of the K^+-stimulated ATPase system by limiting the concentration of Ca^{2+} within the domain of the H^+ pumping mechanism. An alternative possibility is that Ca^{2+} may be involved in the fusion of intracellular tubulovesicles with apical plasmalemma, known to accompany the secretagogue-induced gastric acid secretion [23] by the parietal cells. Rather high concentrations of Ca^{2+}, that would be necessary for any phospholipase A-induced membrane fusion will subsequently be pumped out by the gastric Ca^{2+}-stimulated ATPase.

Consistent with our earlier reports [1,2] the data in Fig. 2a demonstrate that the gastric microsomal K^+-stimulated ATPase activity remains active after unmasking of the microsomal Ca^{2+}-stimulated ATPase activity. Also, the K^+-ATPase mediated H^+ uptake ability of the microsomal vesicles remains unaffected (Fig. 2b). However, the relationship between the unmasked Ca^{2+}-stimulated ATPase and the K^+-stimulated ATPase in the regulation of gastric microsomal H^+ transport remains to be elucidated. It should be cau-

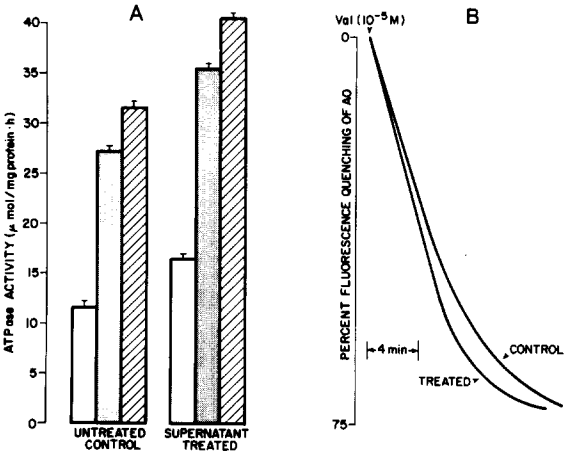

Fig. 2. Effects of pretreatment of microsomes with nondialysed supernatant for 5 min at 22°C on ATPase activity (Fig. 2a). Control microsomes were incubated under identical conditions without any supernatant. Details are given in Table I and Methods. Open bars, Mg^{2+} alone; dotted bars, Mg^{2+} plus K^+, hatched bars, Mg^{2+} plus K^+ plus $1 \cdot 10^{-5}$ M valinomycin (Val). Data are mean ±S.E. ($n = 4$). Fig. 2b shows the fluorescence quenching of acridine orange (AO) by the same control and treated microsomes as above.

tioned, however, that the gastric microsomes, although known to be derived primarily from the apical and tubulovesicular membranes of the parietal cells, also are likely to contain some membranes of nonparietal cell origin and the unmasked Ca^{2+}-stimulated ATPase activity may be localized in the latter membranes. Thus it is essential to characterize fully the origin of the membranes containing Ca^{2+}-stimulated ATPase activity for further assessment of its role in gastric H^+ transport.

Another intriguing question remaining to be answered is why the gastric microsomal Ca^{2+}-stimulated ATPase remains unmasked. It may be mentioned in this connection that increasing amount of evidence are appearing in the literature [24] suggesting that the endogenous proteases may be involved in the regulation of various metabolic processes in a number of different cell types by various mechanisms including activation of certain key enzymes. The phenomenon of ATP dependent unmasking of the gastric Ca^{2+}-ATPase by the endogenous protease in various functional states of the cells needs to be carefully studied in order to understand its physiological significance as a potential intracellular regulator of H^+ transport.

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