Regulation of the gastric microsomal ($H^+ + K^+$)-transporting ATPase system by the endogenous activator

Effect of phospholipase A_2 treatment

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Pig gastric microsomal ($H^+ + K^+$)-stimulated ATPase activity was nearly abolished within 10 min of digestion with phospholipase A_2 at room temperature. The enzyme activity could be largely restored by a cytosolic activator protein partially purified from the gastric cells. The K^+ sensitivity and turnover of ^{32}P -labelled intermediates produced by the control and the activator-reconstituted microsomal ($H^+ + K^+$)-stimulated ATPase were closely similar but were widely different to those from treated membranes without activator reconstitution. The data suggest an essential requirement for the endogenous activator for gastric ($H^+ + K^+$)-stimulated ATPase function.

The gastric H^+,K^+ -ATPase system has been identified as the pumping mechanism for the transport of H^+ across the secretory membranes of the parietal cells (Forte *et al.*, 1980; Ray & Fromm, 1981). Therefore, identification of any cellular factors or events that influence the H^+ pump is expected to increase our understanding of the mechanism of action and regulation of this important physiological process.

An endogenous activator protein has recently been reported (Ray, 1978) to greatly enhance the gastric microsomal H^+,K^+ -ATPase system in a number of different species. The activator from one species was found to stimulate the gastric H^+,K^+ -ATPase and K^+ -stimulated *p*-nitrophenyl phosphatase activities from another species and vice-versa, suggesting a unified intracellular mechanism for controlling the gastric secretory process (Ray, 1978). Several subsequent reports (Sen & Ray, 1980; Ray & Sen, 1981; Ray *et al.*, 1980) emphasized the importance of the activator protein in the regulation of the gastric H^+,K^+ -ATPase system.

The present study was undertaken to further explore the role of the endogenous activator in the regulation of the H^+ -transporting ATPase of gastric microsomes using phospholipase A_2 as a tool. It was demonstrated that digestion of gastric microsomes with phospholipase A_2 at room temperature (21°C)

for 5–10 min obliterates the H^+,K^+ -ATPase activity, which could be largely restored by the partially purified activator protein. The steady-state level and turnover of the ^{32}P -labelled intermediates produced by the control, phospholipase A_2 -treated and the activator-restored enzymes were also studied. The data suggested that the endogenous activator is an extrinsic protein that could be easily removed by phospholipase A_2 digestion. Furthermore, the activator protein appears to be critically involved in maintaining the functional integrity, K^+ -sensitivity and turnover of the enzyme.

Methods and materials

Isolation of gastric microsomes

Purified pig gastric microsomal membranes were isolated by the method of Ray (1978). Briefly, the fundic mucosa of pig was desquamated and scraped (Forte *et al.*, 1972) to collect the oxyntic-cell-enriched fractions. The mucosal cell homogenate in 250 mM-sucrose/0.25 mM-EDTA/0.2 mM-Pipes (1,4-piperazinediethanesulphonic acid) buffer (pH 6.8) was centrifuged at 8000 *g* for 5 min. The pellet was extracted three times by centrifugation as described above. All supernatants were pooled together and layered over 40 ml of 37% (w/v) sucrose in 84 ml screw-cap tubes and centrifuged at 150 000 *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

Abbreviation used: H^+,K^+ -ATPase, ($H^+ + K^+$)-transporting ATPase.

activator protein for K^+ -stimulated ATPase (Ray, 1978). The membrane bands were collected and centrifuged at 100000 g for 90 min. The pellet after resuspension in the homogenizing medium was used for our study. Proteins were assayed by the method of Lowry *et al.* (1951).

Preparation of partially purified activator protein

The soluble supernatant of the pig gastric cells was dialysed exhaustively against 2 mM-Pipes buffer (pH 6.8), containing 0.2 mM-dithiothreitol, at 5°C for 48 h with two changes. The dialysed supernatant was then carefully titrated with 1 M-acetic acid to pH 4.8 at 4°C. The precipitate was collected by centrifugation, dissolved in dialysing medium and used as the source of the partially purified activator for the K^+ -stimulated ATPase (Sen & Ray, 1980).

Digestion of microsomes with phospholipase A₂

Contamination of the bee venom phospholipase A₂ (Sigma) by any proteolytic activity was checked under identical conditions of microsome digestion using a highly sensitive procedure (Wiesner & Troll, 1982). The method of Wiesner & Troll (1982) uses 2-methoxy-2,4-diphenylfuran-3(2H)-one-conjugated casein as substrate, which produces highly fluorescent peptides after proteolysis. No proteinase activity could be detected against a trypsin standard. Consistent with other reports (Saccomani *et al.*, 1979) the bee venom phospholipase A₂ was found to be specific for the *sn*-2-position of the phosphatidylcholine and phosphatidylethanolamine. Furthermore, phospholipase A₂ treatment gave a single band of mol.wt. 22000 on sodium dodecyl sulphate/polyacrylamide gels.

Gastric microsomes (1 mg) were digested at 21°C with 130 units of phospholipase A₂ (about 0.1 mg of protein) in a total volume of 1 ml containing 250 mM-sucrose, 2 mM-CaCl₂ and 20 mM-Tris (pH 7.4). After 10 min the reactions were stopped by 30-fold dilution with ice-cold 250 mM-sucrose/0.2 mM-EDTA/20 mM-Tris (pH 7.4) and immediate centrifugation (150000 g, 60 min). After resuspension in the buffer as described above the membranes were collected by centrifugation. Pellets were suspended in 250 mM-sucrose/0.2 mM-EDTA/0.2 mM-Pipes buffer (pH 6.8) and used for the present study. Control membranes were run in parallel without any phospholipase A₂.

Assay of ATPase

The ATPase was assayed as previously described (Sen & Ray, 1980) in a hypo-osmotic medium. The incubation mixture contained, in a total volume of 1 ml, 50 μmol of Pipes (pH 6.8), 1 μmol of MgCl₂, 2 μmol of Tris/ATP, 10 μg of membrane protein with or without about 50 μg of the activator protein and in the presence or in the absence of 5 μmol of KCl.

After 10 min pre-incubation 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) trichloroacetic acid. The P_i was assayed by the procedure of Sanui (1974).

Assay of the phosphorylated intermediate

The ³²P-labelled intermediates were assayed by a modification of the procedure described previously (Ray & Forte, 1976). Briefly, about 50 μg of the control of phospholipase A₂-treated microsomes were incubated at 0–4°C for 30–60 s in 200 μl of an incubation medium consisting of 2.5 μmol of Pipes (pH 6.8), 1 μmol of MgCl₂, 125 nmol of [γ -³²P]ATP (New England Nuclear; 5.5 × 10¹⁰ c.p.m./mol) with or without 5 μmol of KCl. The reactions were stopped by an equal volume of ice-cold 14% trichloroacetic acid. The membrane precipitate was isolated and thoroughly washed on Millipore filter paper discs (0.45 μm), presoaked in the washing medium. The washing medium consisted of 7% ice-cold trichloroacetic acid containing 1 mM unlabelled ATP and 10 mM-Na₂HPO₄. The filter papers were then washed with 10 ml of 95% ethanol, dried and counted for radioactivity.

Results and discussion

The data in Table 1 demonstrate that digestion of purified pig gastric microsomes with phospholipase A₂ at 21°C abolishes the H⁺,K⁺-ATPase activity. Under similar conditions the gastric microsomal phosphatidylethanolamine and phosphatidylcholine were severely attacked by phospholipase A₂ and the concentrations (μmol/100 mg of protein; means ± S.D.) were 4.9 ± 0.8 and 9.8 ± 1.0 respectively, compared with the corresponding control values of 19.2 ± 1.0 and 24.2 ± 1.0. Other membrane lipids remained unaffected. The activity of the phospholipase A₂-treated enzyme was largely restored by the partially purified activator protein (Table 1). Since the bee venom phospholipase A₂ did not have any detectable proteolytic activity (see the Methods and materials section) the data suggest that the phospholipase A₂ only disrupts the association of the activator protein with the H⁺,K⁺-ATPase complex without affecting the immediate lipid microenvironment of the enzyme to any appreciable extent. The data also suggest that the activator is an extrinsic protein. Our previous reports with mild trypsin (Ray & Sen, 1981) or ethanol (Sen & Ray, 1980; Ray *et al.*, 1980) treatment of gastric microsomes are consistent with the idea of the extrinsic nature of the activator protein.

It was demonstrated previously that the mechanism of gastric H⁺,K⁺-ATPase reaction consists primarily of two steps: an Mg²⁺-dependent kinase

Table 1. Effects of endogenous activator on the gastric microsomal H^+,K^+ -ATPase activity before and after phospholipase A_2 treatment at 21°C

The activity restored by the endogenous activator remained unaltered when the phospholipase A_2 -treated membranes were supplemented with phosphatidylcholine or phosphatidylethanolamine before their assay with the activator. Bovine serum albumin was completely ineffective as a substitute for the activator protein. The control microsomes were not exposed to phospholipase A_2 but otherwise were treated under identical conditions. Details of the phospholipase A_2 treatment and ATPase assay are given in the Methods and materials section. Data are means $\pm$ S.D. ($n = 8$).

Treatment	H^+,K^+ -ATPase activity ($\mu\text{mol}/\text{mg per h}$)			
	Without activator		With activator	
	Mg^{2+} alone	$\text{Mg}^{2+} + \text{K}^+$	Mg^{2+} alone	$\text{Mg}^{2+} + \text{K}^+$
Control microsomes	10.7 ± 1.5	47.8 ± 1.4	6.6 ± 1.3	63.1 ± 4.6
Phospholipase A_2 (21°C for 10 min)	12.9 ± 0.8	16.3 ± 0.3	4.6 ± 0.6	49.8 ± 1.0

step and a K^+ -dependent phosphatase step. The rate-limiting step in the turnover of the ^{32}P -labelled intermediates and consequent hydrolysis of ATP is the K^+ -dependent phosphatase reaction (Ray & Forte, 1976). The present data demonstrate that the phospholipase A_2 -treated microsomes produce a level of ^{32}P -labelled intermediates only about 40% of that in the untreated membranes (Figs. 1a and 2a). Furthermore, K^+ decreases the intermediate level at a greatly reduced rate compared with the control (Fig. 1a). The endogenous activator not only elevates the intermediate level (Figs. 1b and 2a) but also restores the K^+ -sensitivity to near the control level (Fig. 1b). These data strongly suggest that the activator protein is intimately involved in the function of the total ATPase reaction, i.e. both at the kinase and at the phosphatase steps.

The turnover of the ^{32}P -labelled intermediate (Fig. 2b) associated with the phospholipase A_2 -treated and untreated membranes in the presence and in the absence of the activator protein show that the treated microsomes without any added activator have an extremely slow turnover compared with the rest. Thus, the steady-state ^{32}P -labelled intermediate level of control, control plus activator- and the activator-reconstituted phospholipase A_2 -treated membranes (Fig. 2b) is reduced to nearly a new low steady-state value within 5 s of addition of the unlabelled ATP, whereas the intermediates of the treated membranes are decreased, with a $t_{1/2}$ of about 90 s. The data (Figs. 1 and 2) clearly demonstrate that the activator protein plays a critical role in the function of the gastric H^+,K^+ -ATPase system.

The ^{32}P -labelled intermediate produced by the gastric microsomes after treatment with phospholipase A_2 for various lengths of time reached a new low steady-state level (Fig. 1a) within 5 min of treatment and the level was maintained for at least 10 min (T. K. Ray & J. Nandi, unpublished work).

Fig. 1. Effects of K^+ on the steady-state phosphorylated intermediate levels produced by control and phospholipase A_2 (PLA_2)-treated gastric microsomes (a) and reduction by K^+ of ^{32}P -labelled intermediate levels produced by the control and PLA_2 -treated (10 min) membranes in presence of the endogenous activator (b) (a) Symbols: O, control, without K^+ ; ●, control, with K^+ ; Δ, PLA_2 treatment (10 min), without K^+ ; ▲, PLA_2 treatment, with K^+ . (b) Symbols are the same as for (a). Details of assay are given in the Methods and materials section.

Fig. 2. Turnover of the ^{32}P -labelled intermediate generated by the control and phospholipase A_2 -treated microsomes in the presence and in the absence of the endogenous activator

Symbols: ○, control without activator; ●, control with activator; △, phospholipase A_2 -treatment (10 min), without activator; ▲, phospholipase A_2 treatment, with activator. The control and phospholipase A_2 -treated microsomes were phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ at room temperature in the presence and absence of the activator protein ($50\mu\text{g}$) without any added K^+ . Details of the experimental conditions are given in the Methods and materials section. At 60s, when the steady-state (□) has been reached, unlabelled ATP (10mM) was added as shown by an arrow and the rate of change was monitored. The steady-state levels (nmol/mg; means $\pm$ S.D.; $n=6$) of the control, control plus activator, phospholipase A_2 -treated and phospholipase A_2 -treated plus the activator were 527 ± 17 , 662 ± 10 , 252 ± 54 and 400 ± 60 respectively.

This new level of phosphorylation associated with the phospholipase A_2 -treated membranes was closely similar to those attained by the control or activator-reconstituted microsomes in the presence of

K^+ (Figs. 1a and 1b) or unlabelled ATP (Fig. 2). Such insensitivity of the ^{32}P -labelling of the treated membranes to K^+ or ATP might suggest a portion (about 40%) of the total phosphorylated membrane to be unrelated to the gastric H^+, K^+ -ATPase system. Extremely slow turnover of the ^{32}P -labelled membranes (Fig. 2) as well as virtual absence of stimulation (Table 1) of the gastric H^+, K^+ -ATPase system by K^+ after phospholipase A_2 treatment would support such an idea. Furthermore, lack of extractability of the radioactivity from ^{32}P -labelled membranes by 95% ethanol (see the Methods and materials section) suggests that the labels are primarily associated with the protein. Further studies are needed to define the precise nature and role of the slow-turnover phosphoproteins associated with the phospholipase A_2 -treated microsomes.

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