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PROTECTIVE EFFECT OF EGTA ON RAT GASTRIC MEMBRANES ENRICHED IN (H^+-K^+)-ATPASE

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Rat gastric membranes enriched in (H^+-K^+)-ATPase, when prepared in the presence of 1 mM ethyleneglycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid, showed the ability to accumulate H^+ ions upon addition of ATP, KCl, and valinomycin. The membranes were largely impermeable to K^+ and Cl^-. In contrast, the rat membranes prepared without the Ca^{2+} chelator lost the ability to develop a pH gradient because of the membrane leakiness to H^+. A majority of these membrane vesicles became also permeable to K^+. We suggest that the calcium chelator preserved the gastric membrane permeability barrier during isolation by inhibiting various Ca^{2+}-dependent phospholipases in rat gastric mucosa.

INTRODUCTION

Gastric microsomal membranes from the rat like from other species (1-4) have been reported to contain (H^+-K^+)-ATPase (5), but no information is available about the transport activity of the rat membranes. It has been observed recently that isolated rat gastric membranes were uniquely unstable (6). For instance, (H^+-K^+)-ATPase activity in the membranes decreased with a $t_{1/2}$ of about 30 min at 37°. The decay of the gastric ATPase in the membranes, however, did not involve proteolytic digestion of the membrane peptides, and was largely prevented by addition of a millimolar level of EGTA or $LaCl_3$ (6). These observations indicate the presence of Ca^{2+}-dependent enzymes, possibly phospholipases, and their destructive activity in isolated rat gastric membranes. In this study we will show that the presence of EGTA even during preparation of the membranes is essential to preserve the membrane permeability barrier to H^+ and K^+.

Abbreviation; EGTA, Ethyleneglycol-bis-(β-aminoethyl ether)N,N'-tetracetic acid. Hepes, (N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid. Pipes, (Piperazine-N,N'-bis[2-ethanesulfonic acid]).

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MATERIALS AND METHODS

Rat gastric mucosal scrapings were suspended in 10 volumes of a solution containing 250 mM sucrose, 2 mM $MgCl_2$, 1 mM EGTA, and 2 mM Hepes/Tris, pH 7.4. The tissues were homogenized with 20 strokes of a motor-driven (∿1,500 rpm) teflon pestle in a Potter-Elvehjem homogenizer. The microsomal membranes were isolated by differential centrifugation (7) and further resolved by density gradient sedimentation (60 min at 100,000 x g) over a gradient of 30 and 40% sucrose (W/W) containing 2 mM MgCl , 1 mM EGTA, and 5 mM Hepes/Tris, pH 7.4. The membrane band above 30% sucrose was collected. To study the effect of EGTA, sometimes the membranes were prepared using the same media free of EGTA.

Accumulation of H^+ ions inside of the membrane vesicles was estimated by the uptake of ^{14}C-aminopyrene (8). Typically, 15 µl of the membrane suspension (15 or 20 µg protein) with or without 10 µg of valinomycin were mixed with 500 µl of a buffer containing 150 mM KCl, 1 mM $MgSO_4$, 1 mM ATP, 10 mM Pipes, pH 7.0, and 3 µM ^{14}C-aminopyrene (99.5 mCi/mmol). After incubation at 22° for an indicated time, the mixture was filtered over a Millipore filter (HAWP 0.45 µM). The ^{14}C-radioactivity trapped within the membrane vesicles was counted using 10 ml of Instagel (Packard) scintillation cocktail.

(H^+-K^+)-ATPase activity was measured in 1 ml media containing 40 mM Tris/acetate, pH 7.4, 180 mM sucrose, 2 mM $MgCl_2$, and 2 mM ATP with or without 20 mM KCl or other additions as indicated. Addition of salts was accomplished by replacing sucrose isoosmotically. Reaction was at 37° for 5 min and was terminated by adding 1 ml of 10% trichloroacetic acid and 0.1 g HCl-washed charcoal. After removal of charcoal, inorganic phosphate was measured colorimetrically (9). Protein was determined by the Lowry method (10). ^{14}C-aminopyrene was obtained from New England Nuclear. Male Upjohn Sprague-Dawley rats (∿230 g) were used throughout the experiments.

RESULTS

(H^+-K^+)-ATPase in rat gastric membranes prepared in the presence of EGTA was stimulated differentially by ionophores and NH_4^+ ion (Table 1). Valinomycin increased K^+-dependent ATP hydrolysis by about 35% as compared to the value observed with KCl alone while nigericin or NH_4Cl (10 mM) brought about a five-fold stimulation of the ATP hydrolysis. NH_4^+ acts as a membrane-permeable substitute for K^+ (4, 11) by virtue of its conversion to NH_3. These observations indicate that the rat gastric membranes were largely impermeable to K^+. The weak stimulation of (H^+-K^+)-ATPase by valinomycin in the membranes further indicates limited permeability of the rat gastric membranes to Cl^-, since the efficiency of valinomycin to transfer a bulk of K^+ ions depends on the membrane permeability to the counterion, Cl^- in this case, necessary to dissipate electrical potential.

The rat gastric membranes prepared in the absence of EGTA showed about a 20% reduction in the specific activity of K^+-dependent ATP hydrolysis

Table I

Comparison of (H^+-K^+)-ATPase activity in rat gastric membranes prepared in the presence or absence of EGTA

	K^+-dependent ATP hydrolysis by rat gastric membranes	
	Not treated	EGTA treated
	μmol/hr · mg protein	
20 mM KCl	50	19
10 mM KCl + 10 mM NH_4Cl	78	95
20 mM KCl + Nigericin (10 μg)	79	93
20 mM KCl + Valinomycin (10 μg)	58	26

The rat gastric membranes were suspended in sucrose 250 mM-Hepes/Tris 2 mM-$MgCl_2$ 2 mM at a final protein concentration of 3 mg/ml. The reaction medium contained about 30 μg membrane protein. K^+-Dependent ATP hydrolysis was obtained by subtracting a value in K^+-free medium. The data represent the mean of three determinations and experimental errors were less than 5%. Other experimental conditions were described in detail under Materials and Methods.

(NH_4^+-stimulated) as compared to those prepared with the calcium chelator (Table 1). Also, KCl alone activated almost 65% of the ATP hydrolysis observed in the membranes with NH_4^+ or nigericin. The data indicate that a majority of the rat gastric membrane vesicles, when prepared without EGTA, became permeable to K^+.

Proton transport activity of these rat gastric membranes was compared by measuring ^{14}C-aminopyrene uptake in the presence of ATP, KCl, and valinomycin (Fig. 1). The rat gastric membranes prepared in the presence of EGTA showed valinomycin-dependent aminopyrene uptake. At equilibrium, the distribution ratio of ^{14}C-aminopyrene between the intra- and extravesicular water spaces reached about 2,000 assuming the former as 3 μl per mg membrane protein. The membranes prepared without EGTA, on the other hand, failed to show valinomycin-dependent ^{14}C-aminopyrene uptake. The inability of the membranes to develop a pH gradient should be attributed to the leakiness of the membranes to H^+, since the membranes still retained a majority of (H^+-K^+)-ATPase activity.

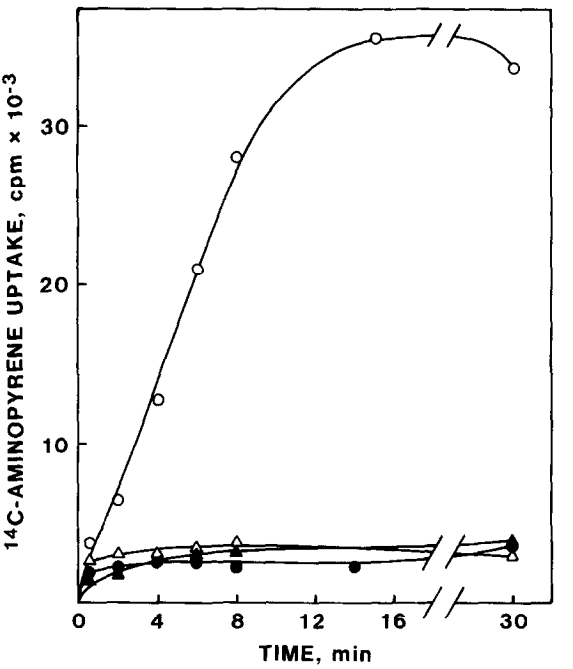

Fig. 1. Time-course profiles of ^{14}C-aminopyrene uptake by rat gastric membranes prepared in the absence (●,▲) or presence of (o,△) of 1 mM EGTA as described under Materials and Methods. The uptake was measured in the media containing about 15 µg membrane protein, 150 mM KCl, 10 mM Pipes, pH 7.4, 1 mM $MgSO_4$, and 1 mM ATP with (o,●) or without (△,▲) valinomycin (10 µg/ml). Other experimental details were described under Materials and Methods.

DISCUSSION

This study demonstrated the advantage of using EGTA during isolation of rat gastric membranes. Although we have not yet explored the possible target enzymes of the EGTA action, it is certain that phospholipases were one class of the enzymes inhibited by the calcium chelator. Rat gastric mucosa have been reported to contain high levels of phospholipase A_1, A_2, C, and other lipolytic enzymes (12-15). The hydrolysis products of these enzymes, lysophospholipids and free fatty acids, are not only detergents, but potent inhibitors of gastric (H^+-K^+)-ATPase (6). One noteworthy point is that hog gastric membranes are known to be quite stable even without the EGTA treatment (7). In view of our current postulation, such difference in the stability of the rat and hog gastric membranes may be due to possible differences either in the level of the lipases in their respective mucosa or in the membrane susceptibility to the action of the lipases.

The rat gastric membranes prepared only in the presence of EGTA showed permeability properties qualitatively similar to the hog or bull frog gastric membranes (4, 11). For instance, the membranes were largely impermeable to K^+ and were able to develop a pH gradient in the presence of valinomycin, KCl, and ATP. One minor difference noted in this study, however, was that the rat gastric membranes were much less permeable to Cl^- than the others on the basis of valinomycin-activation of (H^+-K^+)-ATPase. Addition of Ca^{2+} ions in the assay media (5×10^{-6} M) had no effect on the degree of valinomycin activation of the ATPase in the rat membranes indicating that the removal of Ca^{2+} per se was not responsible for the low Cl^- permeability of the membranes.

REFERENCES

1. Ganser, A. L., and Forte, J. G. (1973) Biochim. Biophys. Acta 307, 169-180.
2. Spenney, J. G., Saccomani, G., Spitzer, H. L., and Sachs, G. (1974) Arch. Biochem. Biophys. 161, 456-471.
3. Forte, J. G., Ganser, A. L., Beesley, R., and Forte, T. M. (1975) Gastroenterology 69, 175-189.
4. Sachs, G., Spenney, J. G., and Lewin, M. (1978) Physiol. Rev. 58, 106-173.
5. Shaltz, L. J., Bools, C., and Reimann, E. M. (1981) Biochim. Biophys. Acta 673, 539-551.
6. Im, W. B., and Blakeman, D. P. (1982) Fed. Proc. 41, 6423 Ab.
7. Saccomani, G., Stewart, H. B., Shaw, D., Lewin, M., and Sachs, G. (1977) Biochim. Biophys. Acta 465, 311-330.
8. Rabon, E., Chang, H., and Sachs, G. (1978) Biochemistry 17, 3345-3353.
9. Tsai, C. M., Chen, K-Y., and Canellakis, E. S. (1975) Biochim. Biophys. Acta 401, 196-212.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 165-275.
11. Forte, J. G., Machen, T. E., and Öbrink, K. J. (1980) Ann. Rev. Physiol. 42, 111-126.
12. Wassef, M. K., Lin, Y. N., and Horowitz, M. I. (1979) Biochim. Biophys. Acta 528, 318-330.
13. Lin, Y. N., Wassef, M. K., and Horowitz, M. I. (1979) Arch. Biochem. Biophys. 193, 213-220.
14. Wassef, M. K., and Horowitz, M. I. (1981) Biochim. Biophys. Acta 665, 234-243.
15. Levy, E., Goldstein, R., Freier, S., and Shafrir, E. (1981) Biochim. Biophys. Acta 664, 316-326.