The Phospholipid Flippase in Isolated Hog Gastric Vesicles : Evidence for Translocation of Endogenous Glycerophospholipids

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The gastric flippase has been found recently in isolated hog gastric vesicles. We found here that the flippase ATP-dependently translocates endogenous phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine from the outer (cytosolic) to inner (luminal) leaflet of the vesicle membrane bilayer or to the vesicle interior. In the experiments, vesicles were incubated in the presence and absence of ATP, and change in the distribution of endogenous phospholipids between the outer and inner leaflets was determined. Furthermore, the vesicle diameter was measured by a quasi-elastic laser light scattering method. The diameter significantly decreased during the translocation, indicating that the glycerophospholipids are translocated into the vesicle interior. The decrease in the size depended on ATP hydrolysis and was inhibited by flippase inhibitors such as 2-methyl-8-(phenylmethoxy)imidazo-[1,2-*a*]pyridine-3-acetonitrile (SCH 28080) and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS). The gastric flippase, which moves into the apical membrane of the parietal cell upon stimulation, may be a candidate that provides the cytoprotective hydrophobic barrier of adsorbed monolayer on the apical membrane covering the phospholipid bilayer and the luminal surface of transmembrane proteins.

Keywords : gastric flippase / proton pump / cytoprotection / phosphatidylcholine / phosphatidylserine / gastric acid

1. Introduction

The gastric parietal cell has intracellular lumen surrounded by the canalicular membrane. Gastric acid is initially secreted into the lumen by gastric proton pump, H⁺,K⁺-ATPase, which is present in the canalicular membrane of the parietal cell. The canalicular membrane must have a cytoprotective barrier against the very strong acid. One of the most plausible mechanisms for the barrier may be an adsorbed monolayer on the apical membrane, forming

the tri-layer and rendering the luminal surface hydrophobic (Hills, 1993 and 1996; Bernhard *et al.* 1995). We found the gastric flippase in isolated gastric vesicles that also contain the gastric proton pump (Suzuki *et al.* 1997). The flippase translocates ATP-dependently fluorescent analogues of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) from the outer (cytosolic side) to inner leaflet (luminal side) of the vesicle bilayer or further into the vesicle interior (Suzuki *et al.* 1997). If this flippase secretes phospho-

lipids into the vesicle interior (luminal space), the flippase is able to engage in formation of the hydrophobic surface lining.

Therefore, we studied here 1) whether endogenous phospholipids of gastric vesicles are translocated by the flippase, 2) whether the direction of translocation of endogenous phospholipids is the same as that had been reported by Suzuki *et al.* (1997) using the fluorescent probes which were incorporated almost only in the outer leaflet of vesicle bilayer, producing the overwhelming mass gradient of the probe from the outer to the inner leaflet, and 3) whether the flippase transports these glycerophospholipids from the outer (cytosolic) leaflet of the bilayer to the vesicle interior (luminal space).

2. Experimental

2.1 Materials and solutions

Phospholipase C from *Bacillus cereus* (grade I, sphingomyelinase < 0.05 %) was obtained from Boehringer Mannheim (Mannheim, Germany). Sphingomyelinase from *Bacillus cereus* (phospholipase C < 1 %) and tricyclodecan-9-yl-xanthogenate (D609, a phospholipase C inhibitor (Kiss & Tomono, 1995)) were obtained from Sigma (Tokyo, Japan). 2-Methyl-8-(phenylmethoxy)imidazo-[1,2-*a*]pyridine-3-acetonitrile (SCH 28080) was obtained from Schering-Plough Co (Bloomfield, NJ, U.S.A.). 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) was obtained from Dojin Co. (Kumamoto, Japan). Gastric vesicles (GI fraction) were prepared from hog gastric mucosae that were obtained from a local slaughterhouse as described elsewhere (Takeguchi *et al.*, 1983). Vesicles were stored below -80 °C until use.

For phospholipid transport study, typically, 1 ml of solution containing 100 µg/ml tightly-

sealed gastric vesicles, 130 mM NaCl, 4 mM MgCl₂, 3 µg/ml creatine kinase, 2 mM creatine phosphate, and 40 mM Tris/HCl (pH 7.40) was incubated at 37 °C for 60 min in the presence or absence of 2 mM ATP, where creatine kinase and creatine phosphate were added to keep a constant ATP concentration.

2.2 Determination of the distribution of endogenous phospholipids in outer and inner leaflets of the gastric vesicle bilayer

To degrade glycerophospholipids in the outer layer of the vesicle bilayer, the vesicle solution was incubated, unless indicated, for 20 min at 37 °C in the presence of 4 units of phospholipase C. This method was previously applied for the present gastric vesicle system and its validity was established (Olaissou *et al.*, 1985). For degradation of sphingomyelin, 0.5 unit of sphingomyelinase plus 100 µg/ml of D609 were used. D609 was used to inhibit phospholipase C that was present in the reagent of sphingomyelinase as a contaminant. The reaction was terminated by the addition of 3.75 ml of chloroform-methanol mixture (1 : 2 (v/v)) to the 1 ml solution. This method did not distinguish whether the remaining phospholipids after the lipase treatment existed in the inner leaflet of the vesicle bilayer or in the vesicle interior.

2.3 Phospholipid analysis

Phospholipid was extracted from the above reaction mixture as described elsewhere (Bligh & Dyer, 1959). The mixture containing chloroform and methanol was shaken for 2 min and stood for 10 min at room temperature. Then, 1.25 ml of chloroform and 1.25 ml of distilled water were added to the mixture and shaken for 30s. After centrifugation (1,200 × g) for 5 min, the upper layer was removed carefully and discarded. To remove aqueous phosphorus from

Fig. 1 Degradation of the gastric vesicle phospholipids with phospholipase C.

Vesicles at 100 μ g/ml were pre-incubated for 60 min at 37 °C in the presence (●) and absence (○) of 2 mM ATP, which was followed by another incubation with 4 unit/ml of phospholipase C for indicated various times. After 20 min incubation with phospholipase C, 5 mM sodium cholate was added. The total phospholipids were determined, and expressed as % of the initial total amount measured after 60 min pre-incubation in the absence of ATP. The data present means $\pm$ S.E.M. of 3-4 experiments. ### p <0.001 (Dunnnett's correction).

the lower layer, 2 ml of chloroform-methanol-water (3 : 48 : 47 (v/v)) was added to the lower layer, and then the mixture was shaken and centrifuged. The resulting upper layer was discarded. This removal was repeated three times. The final lower layer was collected in a tube and organic solvent was evaporated under nitrogen gas stream. The phospholipid was dissolved into 20 μ l of chloroform, spotted on a thin layer chromatography (TLC) plate (silica gel 60, 20 $\times$ 20 cm, Merck) and developed with methyl acetate / n-propanol / chloroform / methanol / 0.25 % KCl (25 : 25 : 25 : 10 : 9 (v/v)). The developed phospholipids were colored and determined (Gustavsson, 1986).

The total phospholipid phosphorus was deter-

mined as described elsewhere (Ames, 1966). The translocation activity of total glycerophospholipids was represented as the difference between the amounts of undegraded phospholipids incubated in the absence and presence of ATP.

2. 4 Determination of the vesicle diameter

The vesicle diameter was determined by a quasi-elastic laser light scattering method (Morii *et al.*, 1984) using LPA3000 system (Otsuka Electronics Co., Osaka, Japan) in a solution containing 100 μ g/ml of vesicles, 130 mM NaCl, 4 mM MgCl₂, 40 mM Tris/HCl (pH 7.40) and 2 mM ATP or 5'-adenylylimidodiphosphate (AMPPNP) at 25 °C.

2. 5 Statistical analysis

Data are presented as means $\pm$ S.E.M. Difference between two groups was analyzed by Student's *t* test for unpaired data. Difference between more than two groups was analyzed by one-way analysis of variance (ANOVA), and correction for multiple comparisons was made by using Dunnnett's multiple comparison. Statistical significance was assumed at p <0.05.

3. Results

3. 1 Distribution of phospholipids in the gastric vesicle bilayer

Here, we used the vesicles that were pre-incubated for 60 min in the absence of ATP. The vesicles were found to contain 653 ± 27 nmol phospholipid / mg protein (n=4). Fig. 1 shows the remaining undegraded total phospholipids in gastric vesicles as a function of the incubation time with phospholipase C that degrades glycerophospholipids (PC+PE+PS) (Fig. 1, open circles). The amount of undegraded total

Table 1 The composition of major phospholipids in gastric vesicles.

Phospholipid composition (mol % of total phospholipids)	
PC	20.9 ± 1.1
PE	23.8 ± 1.6
PS	11.8 ± 0.9
SM	38.2 ± 0.5

Phospholipids (PC, phosphatidylcholine ; PE, phosphatidylethanolamine ; PS, phosphatidylserine ; SM, sphingomyelin) were separated by the method of thin-layer chromatography, then colored and quantified. The values present means ± S.E.M. of 3-4 experiments.

phospholipids showed a plateau phase within 10 min, indicating that glycerophospholipids in the outer membrane was degraded in a short time (Olaissou *et al.*, 1985). After 20 min incubation with phospholipase C, 5 mM sodium cholate was added, which caused solubilization of the membrane resulting in a complete degradation of glycerophospholipids. From the values of the remaining total phospholipids at 0 and 40 min, the initial amount of glycerophospholipids before degradation was found to constitute 61.8 ± 0.5 % (mean ± S.E.M, n=4) of the total phospholipids.

The composition (PC, PS and PE) was determined by the TLC analysis in 2 different preparations ; vesicles incubated for 60 min in the absence of ATP (preparation 1) and vesicles incubated for 60 min in the absence of ATP followed by a 20-min incubation with phospholipase C (preparation 2). The analysis of preparation 1 gave that PC, PE and PS constituted 20.9, 23.8 and 11.8 mol % of the total phospholipids (Table 1). Combining the results obtained for preparation 1 and 2, the relative distribution of the inner leaflet for each phospholipid was calculated : PC in the inner leaflet (and/or the vesicle interior) constituted 12.5 % of the total PC (Table 2) and the majority of PC was in the outer leaflet (87.5 %). Similarly, PE and PS in

Table 2 The transbilayer distribution of major phospholipids in gastric vesicles.

Each phospholipid distribution in the inner leaflet (%) ; [inner leaflet/(inner and outer leaflets)] × 100		
	– ATP	+ATP
PC	12.5 ± 1.1	15.6 ± 0.7*
PE	15.2 ± 0.5	23.3 ± 2.4*
PS	18.4 ± 1.7	22.0 ± 2.6
SM	66.2 ± 1.3	63.4 ± 1.3

The relative distributions of PC, PE, PS and SM in the inner leaflet of the vesicle bilayer are expressed as % of the sum of each phospholipid in the inner and outer leaflets. The distributions were measured after the incubation in the absence (middle column) and presence (right column) of ATP for 60 min at 37 °C . The values present means ± S.E.M. of 3-4 experiments.

* $p < 0.05$ significantly different from the corresponding values measured in the absence of ATP for each phospholipids.

the inner leaflet constituted 15.2 % and 18.4 % of the total PE and PS, respectively.

We also determined the distribution of sphingomyelin (SM) in the vesicle membrane using sphingomyelinase and D609 in experiments similar to those shown in Fig. 1 for glycerophospholipids. It constituted 38 mol % of the total phospholipids (Table 1) and 66 % of SM was present in the inner leaflet and 34 % was in the outer leaflet of the bilayer (Table 2). Fig. 2 shows the distribution of phospholipids in the inner and outer leaflets of the vesicle membrane bilayer expressed as % of the total phospholipids.

3. 2 Translocation of endogenous glycerophospholipids of gastric vesicles by the ATP-dependent flippase

Here, we used vesicles that were pre-incubated for 60 min in the presence of ATP. The amount of the total vesicle phospholipids incubated in the presence of ATP was the same as that incubated in the absence of ATP. Fig. 1 (closed circles) shows the remaining total phos-

Fig. 2 The distribution of phospholipids in the inner and outer leaflets of the gastric vesicle bilayer.

Vesicles at 100 μ g/ml were incubated for 60 min at 37 °C in the absence of ATP, which was followed by another incubation with 4 units/ml of phospholipase C or 0.5 unit/ml of sphingomyelinase+10 μ g/ml of D609 for 20 min. Phospholipids (PC, phosphatidylcholine ; PE, phosphatidylethanolamine ; PS, phosphatidylserine ; SM, sphingomyelin) were separated and quantified by a TLC method and the trans-bilayer distribution was expressed as mol % of the total phospholipids. Data are means $\pm$ S.E.M. of 3-4 experiments.

pholipids as a function of the degradation time with phospholipase C. At 10 and 20 min degradation times, the values obtained for the vesicle preparation incubated in the presence of ATP (closed circles) were significantly greater than those incubated in the absence of ATP (open circles), indicating that the translocation of phospholipids occurs in the presence of ATP from the outer to inner leaflet of the vesicle membrane (or further into the vesicle interior). Similar experiments using sphingomyelinase and D609 showed that SM was not significantly translocated from the outer to inner leaflet in the presence of ATP (Table 2).

Using the preparation incubated in the presence of ATP for 60 min followed by another incubation in the presence of phospholipase C

Fig. 3 The translocation of phospholipids from outer to inner leaflet of the vesicle membrane bilayer (or into the vesicle interior).

Vesicles were pre-incubated for the indicated various times in the presence (●) or absence (○) of 2 mM ATP at 37 °C, which was followed by 20-min incubation with phospholipase C. Then the remaining total phospholipids were determined and expressed as % of the initial amount of phospholipids measured after 60-min pre-incubation in the absence of ATP.

* $p < 0.05$ and *** $p < 0.001$.

for 20 min, we analyzed the composition of PC, PE and PS in the inner leaflet of the bilayer by the TLC method. The results are shown in Table 2. Comparison of the results obtained from the experiments in the absence of ATP (Table 2) shows that PC and PE were significantly translocated from the outer to inner leaflet (or further into the vesicle interior) in the presence of ATP. For example, 12.5 % of PC was present in the inner leaflet and 87.5 % of PC in the outer leaflet when incubated in the absence of ATP. The amount of the relative distribution of PC in the inner leaflet significantly increased to 15.6 % when incubated in the presence of ATP. The present results indicate that the flippase shows the low selectivity between glycerophospholipids (PC, PE and maybe PS).

Fig. 3 shows the remaining total phospho-

Fig. 4 Effect of inhibitors on the ATP-dependent translocation of glycerophospholipids in gastric vesicles.

Vesicles were pre-incubated in the absence and presence of ATP for 60 min at 37 °C together with various concentrations of flippase inhibitors SCH 28080 (○) or DIDS (●), which was followed by another 20-min incubation with 4 unit/ml of phospholipase C. The difference of the remaining total phospholipids between the samples incubated in the presence and absence of ATP is expressed as mol % of control that is the amount obtained from the experiment without the inhibitors.

lipids in the gastric vesicles as a function of the pre-incubation time in the absence and presence of ATP, which was followed by 20-min incubation with phospholipase C. The presence of ATP in the incubation solution significantly increased the amount of glycerophospholipids in the inner leaflet of the vesicle membrane and the ATP-dependent increase depended on the incubation time (Fig. 3, closed circles). The amounts of this ATP dependent increase at 60 min depended on vesicle preparations ranging from 4.3 % to 9.1 % of the total phospholipids, its average being 6.1 ± 0.4 % of total phospholipids, $n=38$ in 4 preparations.

Fig. 4 shows concentration-dependent effects of inhibitors, SCH 28080 and DIDS, on this ATP-dependent glycerophospholipids transport. The

Fig. 5 Change in the vesicle diameter during incubation under different conditions.

The mean diameter of vesicles was measured at 25 °C before and after the 60-min incubation in a solution containing 100 $\mu\text{g}/\text{ml}$ of protein, 130 mM NaCl, 4 mM MgCl_2 and 40 mM Tris/HCl (pH 7.40) in the presence of 2 mM ATP or AMPPNP. When indicated, MgCl_2 was deleted from the solution, or 10 μM SCH 28080 or 10 μM DIDS was supplemented. The mean diameter of vesicles before the incubation was 221 ± 3 nm ($n=6$). The difference between the values of the diameter measured before and after 60-min incubation was defined as the net change in the diameter, and negative values show decrease in the size. The data are from 5 experiments.

** $p < 0.01$ (t -test) and ## $p < 0.01$ (Dunnett's correction).

values of IC_{50} for SCH 28080 and DIDS were 0.14 and 0.78 μM , respectively, which are comparable with the previous results obtained using a fluorescent probe of PC, IC_{50} being 0.14 and 3 μM , respectively (Suzuki *et al.*, 1997).

3. 3 Change in the vesicle diameter during the ATP-dependent phospholipid translocation

The size of present hog gastric vesicles was previously studied by us (Morii *et al.*, 1984) and the size distribution was found to be extremely homogenous. The averaged size was 221 ± 3

nm ($n=6$). Fig. 5 shows that the vesicle size does not change after the 60-min incubation in the presence of ATP when Mg^{2+} is removed from the solution. But, it significantly decreased in the presence of Mg^{2+} and ATP by about 5 %. The decrease was inhibited by 10 μ M SCH 28080 and 10 μ M DIDS, indicating that the inhibition of the flippase diminishes the change in the diameter. When AMPPNP was used in place of ATP, the change in the diameter did not occur. In these experiments, changes in the diameter were measured in the solution devoid of KCl to avoid activation of H^+ , K^+ -ATPase. To further avoid possible effect of ion movements, we added 10 μ M monensin in the solution. The addition did not affect the initial vesicle size, and the incubation in the presence of ATP for 60 min decreased the size irrespective of the presence of monensin, indicating that the size decreases found in the present study were not due to the ion movements (data not shown).

4. Discussion

Major fractions of the total PC, PE and PS are present in the outer leaflet of the gastric vesicle bilayer (Fig. 2), which is in agreement with the previous report (Olaissou *et al.*, 1985). The present results have demonstrated that the flippase in the vesicles translocates endogenous glycerophospholipids from the outer to inner leaflet of the vesicle bilayer or further into the vesicle interior. This gastric flippase transports both PC and PE (and possibly PS, although this is not statistically significant), which is different from so far known ATP-dependent flippases. For example, aminophospholipid flippase (translocase) exists in various cell types and selectively translocates PS and PE but not PC from the cell surface to cytoplasmic leaflet, forming asymmetric distribution of membrane

phospholipids (Auland *et al.*, 1996 ; Tang *et al.*, 1996). During blood coagulation or cell apoptosis, PS appears on cell surface and its exposed PS acts as pro-coagulation fields or phagocytosis recognition signals (Fadok *et al.*, 1992 ; Bratton *et al.*, 1997). As another example, human MDR2 (=MDR3)/P-gp translocates only PC and is involved in PC secretion into bile (Ruetz & Gros, 1995 ; van Helvoort *et al.*, 1996).

Another important finding in this study is that the size of gastric vesicles significantly decreases in the process of ATP-dependent translocation of glycerophospholipids (Fig. 5). If the transported phospholipids from the outer leaflet to the inner leaflet were not secreted in the vesicle interior, the vesicle size would not change. Therefore, the present results show that the glycerophospholipids are translocated into the vesicle interior. The energy of ATP may be used for the secretion of phospholipids.

In this study we used isolated gastric vesicles that originated gastric tubulovesicles, which contain the proton pump and the flippase. The tubulovesicles fuse with the apical membrane of the parietal cell upon stimulation of acid secretion by histamine. Thus, the gastric flippase in the apical membrane may be able to secrete glycerophospholipids. As previously suggested by others (Bernhard *et al.*, 1995 ; Hills, 1996), the secreted glycerophospholipids may provide the hydrophobic barrier of adsorbed monolayer on the apical membrane covering the phospholipid bilayer and the luminal surface of transmembrane proteins against the secreted hydrogen ions.

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