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The Phospholipid Flippase Activity of Gastric Vesicles*

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We found that isolated gastric vesicles contain a novel Mg^{2+}-ATP-dependent phospholipid translocation (flippase) activity. Fluorescence analogue of phosphatidylcholine, 2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine, was ATP-dependently translocated from the outer (cytosolic) to inner (luminal) leaflet of the lipid membrane bilayer of hog gastric vesicles. The translocation was saturable and depended on time and the ATP concentration (K_m = 3.1 μM). The basal Mg^{2+}-ATPase activity of gastric vesicles in the absence of K^+ showed high (K_m = 1.6 μM) and low (K_m = 80 μM) affinities for ATP, indicating that the present flippase activity is driven mostly by the high affinity Mg^{2+}-ATPase activity. It required Mg^{2+} but not K^+. Verapamil, which is an inhibitor of mouse *mdr2* phosphatidylcholine flippase, did not inhibit the present flippase activity. Isolated sarcoplasmic reticulum vesicles that contain Ca^{2+}-ATPase did not show any flippase activity. Fluorescence analogues of phosphatidylserine and phosphatidylethanolamine were similarly translocated by the gastric flippase. These phospholipid flippase activities were inhibited by 2-methyl-8-(phenylmethoxy)imidazo[1,2-*a*]-pyridine-3-acetonitrile (SCH 28080) (IC_{50} = 0.14–0.25 μM), a specific K^+-ATPase inhibitor of gastric H^+,K^+-ATPase rich in gastric vesicles. IC_{50} value for the SCH 28080-inhibitable Mg^{2+}-ATPase activity was about 0.13 μM, indicating that the phospholipid translocation was driven mostly by the SCH 28080-sensitive Mg^{2+}-ATPase activity. Possible physiological roles of flippases were discussed in relation with the gastric acid secretory and cytoprotective mechanisms.

Dramatic morphological changes occur in the parietal cell during the gastric acid secretory cycle: that is (*a*) the fusion of intracellular tubulovesicles that contain proton pump (gastric H^+,K^+-ATPase) with the apical membranes upon stimulation of acid secretion and (*b*) subsequent membrane retrieval and recycling (1, 2). Cytoskeletal proteins such as actin, spectrin, and ankyrin have been shown to be involved in the fusion process (3, 4). In isolated gastric vesicles that were tightly sealed and originated from the tubulovesicles (5), 82% of phosphatidylcholine, 77% of phosphatidylethanolamine, and 60% of phosphatidylserine were located in the outer (cytosolic) leaflet of the gastric vesicle membrane bilayer (6). However, the dynamic aspect of phospholipid translocation between the inner and outer leaflets has not been studied.

Phospholipid translocation (flippase) activities so far known are ATP-dependent or independent (7). For example, aminophospholipid translocases in the erythrocyte and chromaffin granule membranes ATP-dependently flip-flop phosphatidylserine and phosphatidylethanolamine (8, 9), and a mouse *mdr2* gene product ATP-dependently translocates phosphatidylcholine (10) and secretes this phospholipid into bile (11). In the endoplasmic reticulum of eukaryotic cells, glycerophospholipids such as phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine are ATP-independently flip-flopped between the outer and inner leaflets of the membrane bilayer (12, 13).

We studied whether fluorescent analogues of phospholipids were translocated from the outer (cytosolic) to inner (luminal) leaflet of the lipid bilayer of isolated hog gastric vesicles in the presence and the absence of ATP, using the same experimental protocol employed for measuring the phosphatidylcholine translocation activity of mouse *mdr2* gene product that was expressed in secretory vesicles from a yeast mutant (10). We found Mg^{2+}-ATP-dependent phospholipid translocation in gastric vesicles. Interestingly, this flippase activity was inhibited by a low concentration of SCH 28080,[1] which is a specific K^+-competitive gastric H^+,K^+-ATPase inhibitor. This is the first evidence for the presence of the phospholipid flippase activity in gastric vesicles.

MATERIALS AND METHODS

Chemicals and Drugs—Fluorescent analogues of phosphatidylcholine (NBD-C_{12}-HPC), phosphatidylethanolamine (NBD-PE), and phosphatidylserine (NBD-PS) were obtained from Molecular Probes Inc. (Eugene, OR) or Avanti Polar Lipids Inc. (Alabaster, AL). SCH 28080 was obtained from Schering-Plough Co. (Bloomfield, NJ). Pyruvate kinase (500 units/mg at 37 °C, solution in 50% glycerol) and lactate dehydrogenase (850 units/mg at 37 °C, solution in 50% glycerol) were obtained from Boehringer Mannheim-Yamanouchi Co. (Tokyo, Japan); ATP from Oriental Yeast Co. (Tokyo, Japan), DIDS, phosphoenolpyruvate, Ap5A, and NADH were from Sigma. Other chemicals used were of the highest purity available.

Preparation of Donor Liposomes Containing NBD-labeled Phospholipid—Donor small unilamellar liposomes (DLs) were used to transfer NBD-labeled phospholipid into the gastric vesicle membrane. DLs containing 40 mol % of NBD-labeled phospholipid and 60 mol % of phosphatidylcholine were prepared by an ethanol injection method (10, 14). Ethanol solution of phospholipid mixture (1.3 μmol of NBD-labeled phospholipid and 2.0 μmol of phosphatidylcholine in 250 μl of ethanol) was injected at room temperature with a microsyringe into 3.1 ml of a

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[1] The abbreviations used are: SCH 28080, 2-methyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine-3-acetonitrile; NBD-C_{12}-HPC, 2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine; NBD-PS, 2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphoserine; Ap5A, P^1,P^5-di(adenosine-5′)pentaphosphate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; DL, donor liposome; AMPPNP, 5′-adenylyl β,γ-imidodiphosphate.

stirred buffer solution containing 100 mM NaCl and 10 mM Tris/HCl (pH 7.40) (its final ethanol concentration was 7.5%). Then DLs were dialyzed at 4 °C for more than 16 h against the same buffer solution. The final liposome concentration was about 0.4 μmol of NBD-labeled phospholipid/ml.

Preparation of Gastric Vesicles Containing NBD-Phospholipid—A gastric microsomal fraction prepared from hog gastric mucosa was used (15). When this microsomal fraction is one-step further purified by discontinuous density gradient ultracentrifugation, so-called gastric vesicles are obtained (15). As found for the case of DLs and secretory vesicles (10), NBD-labeled phospholipids were transferred from DLs into the mostly outer leaflet of the bilayer of the microsomes by simple incubation of a mixture of the microsomal fraction and DLs for 30 min on ice, where the concentration of NBD-phospholipid in the mixture was adjusted to about 5 nmol NBD-phospholipid/mg protein. Then this mixture was applied on the top layer of discontinuous density gradient solutions consisting of a 250 mM sucrose solution (top layer), a mixture of 3.1% Ficoll 400 and 250 mM sucrose (middle layer), and a mixture of 7% Ficoll 400 and 250 mM sucrose (bottom layer), and ultracentrifuged using a vertical rotor (Hitachi RPV50T, 132,000 × *g* for 1 h at 4 °C). This centrifugation separated the gastric microsomal fraction from DLs, accompanying further purification of the microsomes and resulting in production of gastric vesicles. Gastric vesicles (their diameter was about 0.2 μm) were recovered from the boundary between the middle and bottom layers. Small DLs (their diameter was about 0.03 μm) were recovered from the boundary between the top and middle layers. The tightness of the vesicle membrane was determined by the increase in K^+ stimulation of ATPase activity in the presence of KCl due to the ionophore, nigericin (16). The K^+-ATPase activity in the presence of 1 μg/ml nigericin, 20 mM KCl, 130 mM NaCl, 4 mM $MgCl_2$, 0.2 mM ATP, and 40 mM Tris/HCl (pH 7.40) in the presence of an ATP regeneration system (see the following section of "Assay of Mg^{2+}-ATPase Activity"), was 50.5 μmol P_i/mg·h at 37 °C, and in the absence of nigericin, 3.7 μmol P_i/mg·h. These results indicated that about 93% of vesicles were tightly sealed. Similar experiments at 25 °C gave 97%, indicating that almost all gastric vesicles were tightly sealed. The tightness of the vesicle membrane was also evaluated by their ability to accumulate proton into the vesicles; the rate of active H^+ uptake of NBD-labeled vesicles was almost the same as that of unlabeled control vesicles. Gastric vesicles containing NBD-labeled phospholipid were stored at −85 °C. Protein concentration was determined by the method of Lowry *et al.* (17) with bovine serum albumin as standard.

When indicated, isolated sarcoplasmic reticulum vesicles containing NBD-C_{12}-HPC were used in place of gastric vesicles. Sarcoplasmic reticulum vesicles were prepared from rabbit leg and back muscles (18) and were incubated with DLs and separated by ultracentrifugation. The labeling efficiency of sarcoplasmic reticulum vesicles with NBD-C_{12}-HPC was the same as that of gastric vesicles, and the integrity of sarcoplasmic reticulum membrane bilayer was not damaged by this labeling.

Assay of Phospholipid Translocation—Gastric vesicles (typically, 20 μg/ml) labeled with NBD-phospholipid were preincubated at 37 °C for 10 min in 2 ml of solution containing 130 mM NaCl, 4 mM $MgCl_2$, and 40 mM Tris/HCl (pH 7.40) in a fluorescence cuvette. Unless indicated, K^+ was absent in this solution. Fluorescence emission from NBD-labeled phospholipid associated with the outer and inner leaflets of the membranes bilayer of tight gastric vesicles was monitored using a Shimadzu Spectrofluorophotometer (RF-5000) equipped with a temperature-controlled water jacket and a magnetic stirrer. Fluorescence intensity was recorded at a wavelength of 530 nm with a 10-nm slit width using an excitation wavelength of 470 nm with a 5-nm slit width. After another incubation for the predetermined periods (10–480 min) in the absence and the presence of ATP and chemicals indicated, the fluorescence intensity was recorded (F_T). Then 20 μl of 1 M sodium dithionite in 1 M Tris (pH 10) was added to reduce the fluorescent aryl-nitro group of NBD-labeled phospholipid associated with the outer leaflet of the vesicle membrane bilayer, and the fluorescence intensity was recorded after 10 min (F_D). Membrane-impermeable sodium dithionite reduced only the NBD-labeled phospholipid present in the outer leaflet (10). Then NBD-phospholipid fluorescence associated with the inner leaflet of the vesicle membrane bilayer was quenched by sodium dithionite after disrupting the vesicles by the addition of 80 μl of a 25% (w/v) Triton X-100, and the fluorescence intensity was recorded (F_0). The fraction of NBD-labeled phospholipid in the outer leaflet was calculated by the following equation (10): percentage of outer leaflet = $100\ [(F_T - F_D)/(F_T - F_0)]$, and that of inner leaflet was calculated by: percentage of inner leaflet = $100\ [(F_D - F_0)/(F_T - F_0)]$.

When indicated, the translocation (flippase) activity, as evidenced by increase in "% of inner leaflet" was measured as a function of ATP concentrations (2–1,000 μM), where the ATP concentration was kept constant during the incubation period of 20 min by an ATP regeneration system (see the following section of "Assay of Mg^{2+}-ATPase Activity").

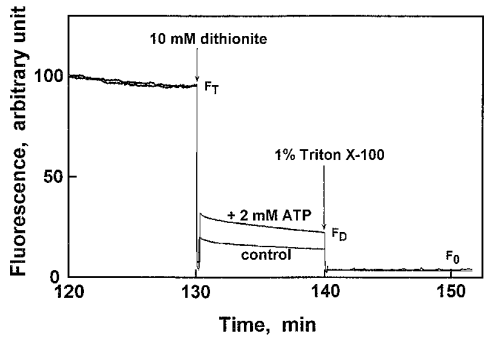

FIG. 1. **Mg^{2+}-ATP-dependent translocation of NBD-C_{12}-HPC in gastric vesicles measured by reduction of the aryl-nitro group of NBD-C_{12}-HPC.** NBD-C_{12}-HPC incorporated gastric vesicles (20 μg/ml) in a solution containing 130 mM NaCl, 4 mM $MgCl_2$, and 40 mM Tris/HCl (pH 7.40) were preincubated at 37 °C for 10 min in a fluorescence cuvette. Then the vesicles were further incubated at 37 °C for 130 min in the presence and the absence (*control*) of 2 mM ATP. Fluorescence emission from NBD-labeled phospholipid of outer and inner leaflets of vesicle membranes was monitored (F_T). Fluorescence was measured at a wavelength of 530 nm with using an excitation wavelength of 470 nm. At the *first arrow*, 10 mM sodium dithionite was added to reduce chemically the fluorescent aryl-nitro group of NBD-C_{12}-HPC in outer leaflet of vesicle membrane and the fluorescence intensity was recorded after 10 min (F_D). At the *second arrow*, the vesicle membrane was solubilized by the addition of 1% (w/v) Triton X-100, and the fluorescence intensity was recorded (F_0).

Assay of H^+ Uptake Rate—The rate of H^+ uptake into gastric vesicles was measured from the quench of fluorescence of acridine orange (19). The reaction mixture contained 10 μg/ml of gastric vesicles, 4 mM $MgCl_2$, 130 mM KCl, 40 mM Tris/HCl (pH 7.40), 5 μM acridine orange, and 10 μg/ml of valinomycin. The rate of H^+ uptake was determined as the initial slope of fluorescence quench after the addition of 2 mM ATP at 37 °C. The fluorescence intensity was recorded at a wavelength of 525 nm with a 3-nm slit width using an excitation wavelength of 495 nm with a 3-nm slit width.

Assay of Mg^{2+}-ATPase Activity—When Mg^{2+}-ATPase activity was measured as a function of ATP concentrations from 2 to 1,000 μM, the ATP concentrations were kept constant during the experiments by use of a pyruvate kinase-lactate dehydrogenase-linked reaction where hydrolysis of ATP is coupled with oxidation of NADH (19, 20). Although pyruvate kinase requires K^+ under normal conditions, we employed the condition that did not require K^+, taking the fact into consideration that high concentrations of Na^+ activate pyruvate kinase without K^+ (21). The reaction mixture contained 20 μg/ml of gastric vesicles, 40 mM Tris/HCl (pH 7.40), 130 mM NaCl, 4 mM $MgCl_2$, 100 μM NADH, 0.8 mM phosphoenolpyruvate, 10 units/ml pyruvate kinase, 15.5 units/ml lactate dehydrogenase, and ATP at the indicated concentrations. The decrease in the amount of NADH was measured at 37 °C with a Beckman DU7500 photodiode array spectrophotometer in a dual wavelength mode at 340 and 500 nm. ATP hydrolyzing activity was calibrated by the addition of a known amount of ADP to a reaction mixture. Mg^{2+}-activated ATP hydrolyzing activity was expressed as μmol of liberated inorganic phosphate/mg protein·h (μmol P_i/mg·h).

When indicated, Mg^{2+}-ATPase activity was measured by the Yoda-Hokin method (22), where the reaction was performed in the presence of 2 mM ATP without the ATP regeneration system.

RESULTS

Translocation of NBD-labeled Phospholipid from the Outer to Inner Leaflet of the Membrane Bilayer of Gastric Vesicles—Fig. 1 shows effects of a 120-min incubation in the presence and the absence of ATP on the fluorescence that comes from both the inner and outer leaflets of the membrane bilayer of gastric vesicles. The absolute fluorescence level of gastric vesicles incubated in the presence of ATP did not differ from that incubated in the absence of ATP. Then 10 mM sodium dithionite was

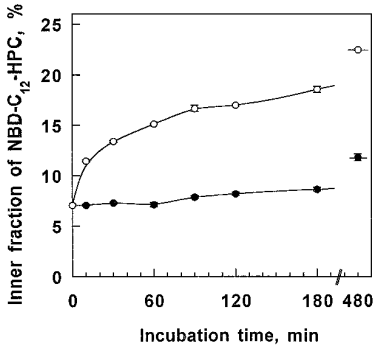

FIG. 2. **Time-dependent translocation of NBD-C_{12}-HPC from the outer (cytosolic) to inner (luminal) leaflet of the gastric vesicle membrane.** The fraction of NBD-C_{12}-HPC in the inner leaflet of gastric vesicle membrane was measured as described in the legend of Fig. 1 at various incubation periods at 37 °C in the presence (○) and the absence of 2 mM ATP (●). Two mM ATP was supplemented to the medium after a 3-h incubation period. The values are the means ± S.E. (n = 3).

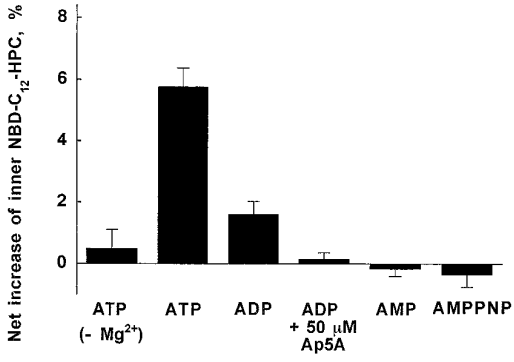

FIG. 3. **Adenine nucleotide specificity of the NBD-C_{12}-HPC flippase activity of gastric vesicles.** Translocation of NBD-C_{12}-HPC from the outer to inner leaflet by several adenosine nucleotides for 1 h at 37 °C was measured in a solution containing 0.2 mM nucleotide, 4 mM $MgCl_2$, 130 mM NaCl, and 40 mM Tris/HCl (pH 7.4). NBD-C_{12}-HPC translocation was expressed as the net increase in the percentage of inner leaflet. When indicated, 50 μM Ap5A was added to inhibit adenylate kinase activity of the gastric vesicle preparation that generates ATP and AMP from 2·ADP. The values are the means ± S.E. (n = 4–5).

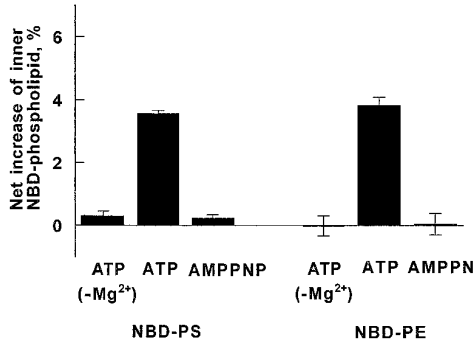

FIG. 4. **The flippase activity of other glycerophospholipids.** Translocations of NBD-PS and NBD-PE from the outer to inner leaflet for 1 h at 37 °C were measured in solutions containing 0.2 mM nucleotide, 4 mM $MgCl_2$, 130 mM NaCl, and 40 mM Tris/HCl (pH 7.4). NBD-labeled phospholipid translocation was expressed as the net increase in the percentage of inner leaflet. The values are the means ± S.E. (n = 3).

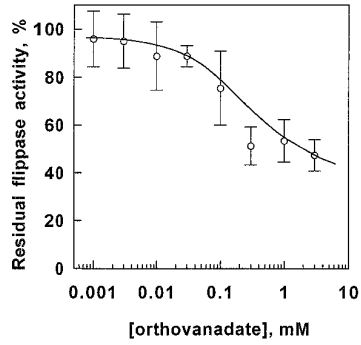

FIG. 5. **Inhibition of the ATP-dependent NBD-C_{12}-HPC flippase activity by orthovanadate.** NBD-C_{12}-HPC-labeled gastric vesicles (20 μg/ml) were incubated in solutions containing 2 mM ATP, 4 mM $MgCl_2$, 130 mM NaCl, 40 mM Tris/HCl (pH 7.4), and various concentrations of sodium orthovanadate for 1 h at 37 °C. Residual flippase activity was expressed as the percentage of the control value obtained in the absence of sodium orthovanadate. The values are the means ± S.E. (n = 3).

added, which induced the decrease in the fluorescence. The fluorescence level depended on the presence and the absence of ATP (Fig. 1). This decrease was attributed to the reduction of the aryl-nitro group of NBD-C_{12}-HPC to the aryl-amino group in the outer leaflet. The subsequent addition of Triton X-100 (its final concentration was 1%) destroyed the vesicle membrane, and sodium dithionite reduced NBD-C_{12}-HPC in the inner leaflet (maybe, also in the intravesicular space). The final level (F_0) did not depend on the presence and the absence of ATP. From this particular experiment shown in Fig. 1, the fraction of NBD-C_{12}-HPC in the inner leaflet after a 130-min incubation was calculated to be 11.5 and 20.9% in the absence and the presence of ATP, respectively. Incubation with 2 mM ATP, therefore, increased the fraction of inner NBD-C_{12}-HPC by about 82%. Fig. 2 shows the time-dependent increase in the fraction of inner NBD-C_{12}-HPC. This result indicates that NBD-C_{12}-HPC in the outer leaflet of the vesicle lipid bilayer was significantly translocated into the inner leaflet and/or the inner space of vesicles. The translocation was saturable.

When AMP or AMPPNP was used in place of ATP, the flippase activity was not found (Fig. 3). In the case of ADP, an apparent flippase activity was observed. However, this activity was completely inhibited by Ap5A, a specific inhibitor of adenylate kinase (23), indicating that part of ADP was changed into ATP and AMP by adenylate kinase which was reported to be present in the gastric vesicle preparation (24). Without Mg^{2+}, no ATP-dependent flippase activity was observed, indicating that this NBD-C_{12}-HPC flippase activity requests not the binding but hydrolysis of ATP.

Fig. 4 shows that transbilayer motion of glycerophospholipids occurs identically for phosphatidylethanolamine and phosphatidylserine in the presence of ATP and Mg^{2+}. AMPPNP was not effective.

We could not find the phosphatidylcholine flippase activity in isolated sarcoplasmic reticulum vesicles that contain Ca^{2+}-ATPase (data not shown), indicating that the flippase activity found in gastric vesicles, therefore, is not commonly associated with P-type ATPases.

Effects of K^+ on the Phospholipid Flippase Activity—Effects of K^+ (0.3–65 mM) on the phospholipid flippase activity were measured in the solution containing 20 μg/ml NBD-C_{12}-HPC-labeled gastric vesicles, 4 mM $MgCl_2$, 2 mM ATP, 40 mM Tris/HCl (pH 7.40), and various concentrations of KCl and NaCl, where the sum of the K^+ and Na^+ concentrations was constant (130 mM). Under the conditions, K^+ gives no effect on the phospholipid flippase activity (data not shown).

Effects of Inhibitors on the Phospholipid Flippase Activity—The concentration-dependent inhibition of the flippase activity by orthovanadate is shown in Fig. 5. Orthovanadate at 10 and 100 μM inhibits the flippase activity by 10 and 25%, respectively. At the same concentrations of orthovanadate, the rate of H^+ uptake into gastric vesicles was inhibited by 20 and 88%, respectively. Table I shows that 100 μM *N,N′*-dicyclohexylcar-

bodiimide, an inhibitor of mitochondrial F_0F_1-ATPase, does not inhibit the flippase activity. Verapamil, which inhibits both activities of the mouse *mdr2* gene product (phosphatidylcholine flippase) and the mouse *mdr3* gene product (multidrug resistance efflux pump) (10), did not inhibit the present flippase activity (Table I). DIDS (10 μM), which is used as an anion channel inhibitor, significantly inhibited the flippase activity, although diphenylamine-2-carboxylic acid (100 μM), which is also an anion channel inhibitor, did not inhibit the flippase activity (Table I). 1 mM *N*-ethylmaleimide, which at 2 mM inhibited the ATP-dependent phosphatidylserine translocation from the luminal to cytosolic leaflet of the bilayer of chromaffin granule membrane (9), did not inhibit the present gastric flippase activity. Progesterone, vinblastine, and tamoxifen, which are substrates of multidrug resistance pump and inhibit multidrug resistance pump, did not inhibit the flippase activity (Table I).

ATP Concentration Dependences of the Flippase and Mg^{2+}-ATPase Activities—Next, we studied effects of ATP concentrations on activities of phosphatidylcholine translocation and Mg^{2+}-ATPase under the same conditions, that is, both solutions contained 20 μg/ml of gastric vesicles, 40 mM Tris/HCl (pH 7.40), 130 mM NaCl, 4 mM $MgCl_2$, 100 μM NADH, 0.8 mM phosphoenolpyruvate, 10 units/ml pyruvate kinase, 15.5 units/ml lactate dehydrogenase, and ATP at the indicated concentrations, which were kept constant during the incubation period. Fig. 6*A* demonstrates that the Mg^{2+}-ATPase activity shows high and low affinities for ATP (K_m = 1.6 and 80 μM determined from the double reciprocal plot), whereas the flippase activity shows one affinity for ATP (K_m = 3.1 μM). Fig. 6*B* shows detailed comparison of the ATP dependences between the flippase and Mg^{2+}-ATPase activities in the range of low ATP concentrations (0–50 μM ATP). In this range, both the flippase activity (the amount of NBD-C_{12}-HPC translocated in 20 min) and the Mg^{2+}-ATPase activity followed single Michaelis-Menten enzyme reaction equations. These results indicate that NBD-C_{12}-HPC was translocated as a substrate by the putative Mg^{2+}-ATP-dependent flippase.

TABLE I

Effects of several agents on the ATP-dependent flippase activity

Phosphatidylcholine flippase activity was measured in a solution containing 20 μg/ml of NBD-C_{12}-HPC-labeled gastric vesicles, 130 mM NaCl, 4 mM $MgCl_2$, 2 mM ATP, and 40 mM Tris/HCl (pH 7.40) with indicated agent for 30 min at 37 °C unless otherwise indicated. The residual activity is expressed as the percentage of control measured in the absence of agent. For *N*-ethylmaleimide, the vesicles were preincubated with *N*-ethylmaleimide for 1 h, and then flippase activity was measured. Values are the means ± S.E. (number of experiments). DCCD, *N,N′*-dicyclohexylcarbodiimide; DPC, diphenylamine-2-carboxylic acid.

Agent	Percentage of control
DCCD (100 μM)	112.7 ± 19.0 (3)
DIDS (10 μM)	16.1 ± 12.1 (3)[a]
DPC (100 μM)	117.0 ± 12.0 (4)
Verapamil (10 μM)	97.1 ± 17.0 (3)
Progesterone (10 μM)	82.7 ± 2.8 (3)
Vinblastine (10 μM)	88.7 ± 17.2 (3)
Tamoxifen (10 μM)	116.5 ± 12.5 (3)
N-Ethylmaleimide (1 mM)	100.7 ± 19.6 (3)

[a] The difference between control and DIDS is significant ($p < 0.01$ by unpaired Student's *t* test).

Effects of SCH 28080 on the Flippase and Mg^{2+}-ATPase Activities—SCH 28080 is a reversible K^+-competitive inhibitor of gastric H^+,K^+-ATPase (25). Effects of SCH 28080 on the flippase and Mg^{2+}-ATPase activities were measured under the same conditions: that is, the both experimental solutions contained 20 μg/ml of gastric vesicles, 130 mM NaCl, 4 mM $MgCl_2$, and 40 mM Tris/HCl (pH 7.40). After a 10-min incubation with various concentration of SCH 28080, 2 mM ATP was added to start the flippase reaction. Here, the amount of NBD-C_{12}-HPC translocated from the outer to inner leaflet of the bilayer for 1 h in the presence of 2 mM ATP was measured. The amount of liberated inorganic phosphate for 1 h was measured by the Yoda-Hokin method (22). Fig. 7 shows that SCH 28080 concentration-dependently inhibits the flippase activity, its IC_{50} value for NBD-C_{12}-HPC being about 0.14 μM. Furthermore, we measured the inhibition of NBD-PS and NBD-PE translocations by SCH 28080 and found the IC_{50} values of 0.25 and 0.20 μM, respectively. These IC_{50} values were not significantly different.

The K^+-ATPase activity of gastric vesicles measured in the presence of 15 mM KCl and 10 μg/ml of valinomycin was inhibited by SCH 28080 (IC_{50} = 0.2 μM) (26). Furthermore, it has been reported that SCH 28080 partially inhibited the Mg^{2+}-ATPase activity of gastric vesicles (27). Fig. 7 shows that about 56% of the Mg^{2+}-ATPase activity is inhibited by 10 μM SCH 28080, whereas 96% of the flippase activity is inhibited by 10 μM SCH 28080, suggesting that only part of the Mg^{2+}-ATPase activity contributed to the flippase activity. From Fig. 7, IC_{50} value for the SCH 28080-inhibitable fraction of the Mg^{2+}-ATPase activity was obtained to be 0.13 μM.

DISCUSSION

We have found that the Mg^{2+}-ATP-dependent phospholipid flippase is present in hog gastric vesicles. Fluorescent ana-

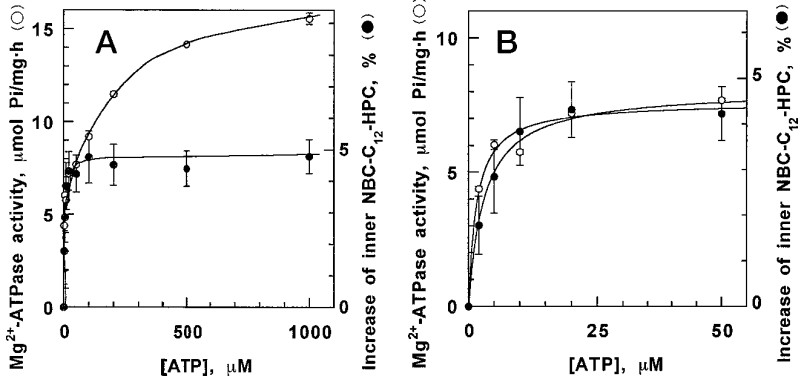

FIG. 6. **Effects of ATP concentrations on the NBD-C_{12}-HPC translocation and Mg^{2+}-ATPase activities.** Translocation of NBD-C_{12}-HPC for 20 min (●) and Mg^{2+}-ATPase activity (○) were measured in the presence of ATP regeneration system: that is, both solutions contained 20 μg/ml of gastric vesicles, 4 mM $MgCl_2$, 130 mM NaCl, 40 mM Tris/HCl (pH 7.40), 100 μM NADH, 0.8 mM phosphoenolpyruvate, 10 units/ml pyruvate kinase, 15.5 units/ml lactate dehydrogenase, and ATP at the indicated concentrations. The ATPase activity was determined as a decrease in the amount of NADH. *A* shows dependences of the flippase and Mg^{2+}-ATPase activities on ATP concentrations (0–1,000 μM). *B* shows the detailed comparison in the range of low ATP concentrations (0–50 μM). The values are the means ± S.E. (n = 3).

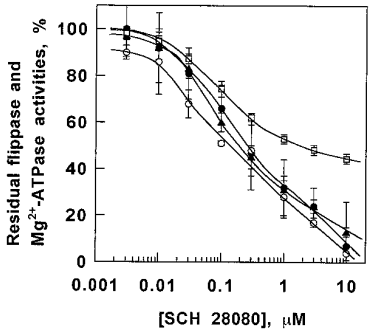

FIG. 7. **Inhibition of flippase and Mg^{2+}-ATPase activities of gastric vesicles by SCH 28080.** Gastric vesicles (20 μg/ml) labeled with NBD-C_{12}-HPC, NBD-PS, or NBD-PE were incubated in solutions containing 2 mM ATP, 4 mM $MgCl_2$, 130 mM NaCl, 40 mM Tris/HCl (pH 7.4), and various concentrations of SCH 28080 for 1 h at 37 °C. Residual flippase activity was expressed as the percentage of the control value obtained in the absence of SCH 28080. ○, NBD-C_{12}-HPC; ●, NBD-PS; ▲, NBD-PE. The Mg^{2+}-ATPase activity (□) was measured by the Yoda-Hokin method (22). The values are the means ± S.E. (n = 3).

logues of phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine, which are distributed more densely in the outer (cytosolic) leaflet of the vesicle membrane bilayer than in the inner (luminal) leaflet (6), were translocated from the outer to inner leaflet of the bilayer in a same manner. It does not require the presence of K^+ and is relatively insensitive to orthovanadate, discriminating this flippase from H^+,K^+-ATPase. The translocation (flippase) activity depends on the ATP concentration. The K_m value of 3.1 μM was obtained from the experiments of a 20-min incubation (Fig. 6). When the incubation period was increased to 1 h, the value of K_m increased to 9 μM (data not shown), indicating that the K_m value would be smaller than 3.1 μM if the initial rate of the flippase activity were plotted against the ATP concentration. Therefore, the K_m value of 3.1 μM may be comparable with that of the high affinity Mg^{2+}-ATPase activity of gastric vesicles (1.6 μM). The low affinity Mg^{2+}-ATPase activity (K_m = 80 μM) seems to have no contribution to the flippase activity (Fig. 6*A*). From these results, we suggest that the phospholipid flippase activity may be attributed mostly to the high affinity Mg^{2+}-ATPase activity of gastric vesicles.

Furthermore, the IC_{50} value obtained from the inhibition of the flippase activity by SCH 28080 (IC_{50} = 0.14–0.25 μM; Fig. 7, 1-h incubation) was comparable with the value obtained from the SCH 28080-sensitive Mg^{2+}-ATPase activity (0.13 μM). This fact may indicate that the flippase activity mostly owes to the SCH 28080-sensitive Mg^{2+}-ATPase activity.

Verapamil is known to inhibit the flippase activity of mouse *mdr2* gene product (10). As shown in Table I, verapamil did not inhibit the present flippase activity in gastric vesicles. Furthermore, substrates of multidrug resistance pump (P-glycoprotein) such as progesterone, tamoxifen, and vinblastine did not inhibit this flippase activity (Table I).

Tamoxifen is also known to inhibit the volume-activated Cl^- channel (28, 29). Similarly, a Cl^- channel inhibitor diphenylamine-2-carboxylic acid did not inhibit this phospholipid flippase, although DIDS, which binds to amino residue and inhibits anion channels, inhibits the flippase.

The direction of the ATP-dependent phospholipid transporting activity of the present gastric flippase and *mdr2* gene product is from the cytosolic to luminal leaflet of the lipid bilayer, whereas that of ATP-dependent aminophospholipid translocase in erythrocyte and chromaffin granules is opposite. Mouse *mdr2* gene product plays an essential role in the secretion of phosphatidylcholine across the canalicular membrane into bile (10). Aminophospholipids such as phosphatidylserine and phosphatidylethanolamine are confined to the inner leaflet of the plasma membrane by aminophospholipid translocase (8), and the appearance of phosphatidylserine on the surface of animal cells triggers phagocytosis (30) and blood coagulation (31). ATPase II of chromaffin granule membrane was assigned to be this aminophospholipid translocase (32), and the gene that codes for this ATPase was cloned (33). The ATPase II is a member of an ancient subfamily of P-type ATPases.

The role of the present flippase is unknown. Several types of flippase functions are considered to be involved in the acid secretory and cytoprotective mechanisms. First, stimulation of the parietal cell by histamine and gastrin causes great morphological changes. One such event is the fusion of tubulovesicles (gastric vesicles) with the apical membrane accompanying increase in the area of the external plasma membrane as evidenced by increase in the external membrane capacitance (34). The fusion process will request the presence of flippase to readjust the balance of phospholipids between the outer and inner leaflets of the lipid bilayer, for example, enabling a curvature change in the membrane.

Second, there are several lines of evidence that gastric mucus is protected against the back diffusion of acid by a hydrophobic barrier comprising of surface-active phospholipids (35). Electron microscopic observations indicated that deeper mucus-free epithelial surfaces of oxyntic ducts and parietal cells were also covered by oligolamellar lining of phospholipids mainly phosphatidylcholine (36), which may be secreted by flippase. Other roles of flippases are also considered. The role of the present flippase in gastric parietal cells is necessary to be clarified in future studies.

The present new gastric phospholipid flippase is an interesting object because only several ATP-dependent flippases are known, and the properties of the present flippase are different from previously known phospholipid flippases.

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