Phosphatidylinositol is essential determinant for K+ permeability involved in gastric proton pumping

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Omi, Noriaiki, Taku Nagao, and Tetsuro Urushidani. Phosphatidylinositol is essential determinant for K+ permeability involved in gastric proton pumping. Am J Physiol Gastrointest Liver Physiol 281: G786–G797, 2001.—Gastric vesicles purified from acid-secreting rabbit stomach display K+ permeability manifested by the valinomycin-independent proton pumping of H+-K+-ATPase as monitored by acridine orange quenching. This apparent K+ permeability is attenuated by the treatment of the membrane with 5 mM Mg2+, and this phenomenon has been attributed to membrane-bound phosphoprotein phosphatase. However, with the exception of the nonspecific inhibitor pyrophosphate, protein phosphatase inhibitors failed to inhibit the loss of K+ permeability. Preincubation of the membrane with neomycin, a phospholipase C inhibitor, surrogated the effect of Mg2+, whereas another inhibitor, U-73122, did not. Phosphatidylinositol 4,5-bisphosphate (PIP2) restored the attenuated K+ permeability by treatment with either Mg2+ or neomycin. Furthermore, either phosphatidylinositol bound to phosphatidylinositol transfer protein or phosphatidylinositol 4,5,6-trisphosphate (PIP3) surrogated the effect of PIP2. Mg2+ and neomycin reduced K+ permeability in the membrane as determined by Rb+ influx and K+-dependent H+ diffusion. Treatment with Mg2+ reduced the contents of PIP2 and PIP3 in the membrane. These results suggest that PIP2 and/or PIP3 maintain K+ permeability, which is essential for proton pumping in the apical membrane of the secreting parietal cell.

GASTRIC ACID SECRETION is conducted by the proton pump H+-K+-ATPase, which exchanges H+ on the cytoplasmic side with K+ on the opposite side using energy supplied by ATP hydrolysis. When the parietal cell is in its resting state, H+-K+-ATPase exists mainly on the intracellular membranous structure called the tubulovesicles. Because the tubulovesicle membrane has poor K+ and Cl− conductance, the exchange cycle cannot continue even though enough ATP is present in the vicinity (for review, see Ref. 29). This has been manifested by vesicular proton transport monitored by the acridine orange quenching technique (15). When the microsomes obtained from resting stomach were used, little proton pumping occurred in the presence of KCl and ATP. For the transport to operate at full activity, valinomycin, a K+ ionophore, is required (7). When the apical membrane fraction purified from the acid-secreting stomach was used, the vesicular proton transport was no longer dependent on valinomycin, indicating that the apical membrane of the stimulated parietal cell acquired K+ conductance (32). Stimulation of acid secretion consists of two steps: the tubulovesicle fuses with the apical membrane of the parietal cell and the secretory membrane acquires K+ and Cl− conductance. Recent studies (1, 6, 20) on the membrane fusion process in the parietal cell have shown that several proteins are involved in membrane recruitment and recycling. However, little is known about the regulation of K+ and Cl− conductance, which is thought to be the direct trigger for the activation of proton pumping. As for the Cl− permeability involved in gastric acid secretion, a candidate channel has been cloned (18) from the cDNA library of gastric parietal cells, activated by cAMP-dependent protein kinase A (PKA). In contrast, the molecular entity required for K+ permeability in acid secretion has not yet been identified. Although secretagogues such as histamine, gastrin, and ACh have been shown to stimulate several intracellular signaling pathways including cAMP, Ca2+, and inositol 1,4,5-trisphosphate/diacylglycerol in the parietal cell (29), PKA is considered to be the essential component for the activation (1, 3). Therefore, it would be conceivable that the putative K+ channel or transporter is expected to be phosphorylated and activated by PKA. This assumption has been supported by the early work of Im et al. (13). They (13) showed that the K+ permeability of gastric vesicles obtained from stimulated rat gastric mucosa was reduced when the vesicles were treated with a high concentration of Mg2+. Im et al. (13) attributed this phenomenon to the putative membrane-bound Mg2+-activated phosphoprotein phosphatase, because the reduction of K+ permeability was prevented by pyrophosphate, a nonspecific phosphatase inhibitor. However, further examination of this phenomenon has not been performed. We examined the results of Im et al. (13) and found that the decrease of K+ permeability by Mg2+ treatment was not due to

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the dephosphorylation of the putative K⁺ channel. Here we report that the phenomenon is related to the metabolism of inositol polyphosphate, which appears to be essential for K⁺ conductance in gastric proton pumping.

MATERIALS AND METHODS

Membrane preparations and ATPase assay. Gastric vesicles enriched in the apical membranes of stimulated parietal cells were purified from the gastric mucosa of rabbit that had been stimulated in vivo according to the method previously described (32) with slight modifications. Japanese White rabbits (Shiraishi, Tokyo, Japan) were allowed to feed on staple food for 20 min followed by a subcutaneous injection of chlorpheniramine maleate (1 mg/kg body wt) and two histamine injections (0.1 mmol/kg each) 10 and 20 min after chlorpheniramine injection. The stomach was taken 5 min after the last injection under anesthesia. The oxyntic glandular region of the mucosa was homogenized in 30 vol of ice-cold homogenizing buffer (125 mM mannitol, 40 mM sucrose, 1 mM EDTA, and 5 mM PIPES, pH 6.7) by 15 passes at 600 rpm through a Teflon piston homogenizer (Potter-Elvehjem). The homogenate was centrifuged at 100,000 × g for 2 h. The material on the 18% Ficoll layer was harvested, diluted in 20 vol of the suspending medium (300 mM sucrose, 5 mM Tris, and 0.2 mM EDTA, pH 7.4), layered underneath the suspension medium, and then centrifuged at 100,000 × g for 2 h. The material on the 18% Ficoll layer was harvested, diluted in 20 vol of the suspending medium, and recentrifuged at 100,000 × g for 45 min. The final material was suspended in the suspending medium and stored at −80°C until use.

The membranes enriched in the tubulovesicles of the resting parietal cell were purified from resting rabbit gastric mucosa as described previously (32). Briefly, a rabbit was treated with 100 mg/kg of cimetidine, and the mucosal homogenate was prepared. The supernatant, after centrifugation at 14,500 × g for 10 min, was further centrifuged at 100,000 × g for 45 min to get the microsomal fraction. The microsomes were suspended in the homogenizing buffer and then layered on top of the discontinuous sucrose gradient (33%, 27%, and 21% sucrose in the homogenizing buffer) and centrifuged at 100,000 × g for 2 h. The material on the top of the 21% sucrose layer was harvested and stored at −80°C until use.

The ATPase assay was performed in a total volume of 1 ml containing 10 mM PIPES, pH 6.5, 1 mM MgSO₄, 0.1 mM ouabain, with or without 20 mM KCl, and 1 mM ATP. Liberated phosphate was quantified as described previously (17).

Measurement of proton accumulation rates by acridine orange quenching. Proton transport into the intravesicular space was monitored by the acridine orange quenching technique as described previously (15). An aliquot of membrane suspension (20 μl of 2.5 mg protein/ml; 50 μg/ml final) was added to 1 ml of the uptake medium (40 mM KCl, 110 mM choline chloride, 10 mM PIPES-Tris, pH 6.8, 1 mM acridine orange, 0.5 mM MgATP, and 2 mM phosphocreatine). Accordingly, when the membrane had been pretreated with ions or drugs, the final concentration of the ions or drugs in the assay medium was one-fiftieth of the pretreated concentration. The mixture was excited with the wavelength of 493 nm under continuous stirring at 37°C, and the emission through a 540 ± 6-nm bandpass filter was recorded by a spectrophotofluorometer (CAF-110; JASCO, Tokyo, Japan).

Acridine orange is a weak base and membrane permeable when it is in the uncharged form in neutral solution. When the intravesicular pH is decreased by the action of H⁺-K⁺-ATPase, acridine orange is protonated and accumulated within the vesicles, and subsequently the fluorescent intensity decreases because of the red shift of the emission (15). This quenching reflects the continuous revolving of the H⁺-K⁺ exchanger in the membrane. This means the membrane has some permeability to K⁺, because there is a continuous supply of K⁺ to the intravesicular site (34). In contrast to the apical membranes from stimulated parietal cells, the tubulovesicle membranes from resting parietal cells show a different feature. Addition of the vesicles to the reaction mixture does not induce acridine orange quenching, because the membrane has little K⁺ permeability, and thus the revolving of the pump does not occur. To continue the pumping by supplying K⁺ to the intravesicular site, the addition of valinomycin, a K⁺-specific ionophore (see Fig. 1A; 7, 32) is necessary. K⁺ permeability of the membrane was therefore estimated by the valinomycin dependency of the acridine orange quenching. The relative fluorescence before and after addition of valinomycin (1 μl of 10 mM stock solution in ethanol was added to make a final concentration of 10 μM). To ensure membrane integrity, membranes once frozen and then thawed were used up in a series of experiments and freezing-thawing was never repeated.

Measurement of passive diffusion of H⁺ from the gastric vesicle. Passive diffusion of H⁺ across the vesicular membrane was measured as previously described (24) with slight modifications. The membrane preparation was incubated for 15 min at room temperature in a medium containing 1.5 μM acridine orange, 10 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP), 10 mM succinate, 300 mM sucrose, and 5.6 mM Tris base (pH 4). The extravesicular pH was then increased to 8 by addition of 25 mM Tris base (25 μl of 1 M solution) with continuous monitoring of the fluorescence as described above. By extravesicular alkalization, the fluorescence of acridine orange abruptly quenched according to the pH gradient made. Thereafter, intravesicular H⁺ ions passively diffused to the external medium as manifested by the recovery of fluorescence. This diffusion was restricted and reached an equilibrium even in the presence of the protonophore CCCP, because of the electrochemical potential created in the absence of any other movable ions. The addition of K⁺ gluconate caused some recovery of fluorescence. This is attributed to the cancellation of the electrical potential by K⁺ influx and thus the recovery rate of fluorescence is taken to be a reflection of the K⁺ influx rate. Inclusion of the proton pump inhibitor, omeprazole or SCH-28080 (10 μM each), showed no effect on K⁺-dependent recovery of fluorescence, suggesting that the apparent K⁺ influx was not through the reversed revolution of the pump but via the putative K⁺ channel or transporter(s).

Preparation of recombinant phosphatidylinositol transfer protein-β. Rattus cloned phosphatidylinositol transfer protein-β (PITPβ) in pET-21a-d(+) vector was kindly given by Dr. H. Arai (Graduate School of Pharmaceutical Sciences, University of Tokyo). The cDNA was cloned into pGEX-4T-1 and transformed XL1-blue. Expression of the PITPβ glutathione-S-transferase fusion protein was induced by isopropyl-β-thiogalactopyranoside (0.1 mM) for 2 h at 25°C, and the bacterial cells were collected and resuspended in 150 mM NaCl, 3 mM K₂HPO₄, 0.64 mM NaH₂PO₄, and 10 mM EDTA (pH 7.0). After freeze-thawing, the sample was centrifuged at 40,000 × g for 30 min at 4°C. Recombinant protein was purified from the supernatant using glutathione-Sepharose 4B resin. Purified fusion protein was cleaved by thrombin treatment.

AJP-Gastrointest Liver Physiol • VOL 281 • SEPTEMBER 2001 • www.ajpgi.org

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and incubated with 10 mg/ml phosphatidylinositol at 4°C overnight.

36Rb+ influx across membrane vesicles. Measurement of 36Rb+ influx across the vesicular membrane was performed as previously described (26) with slight modifications. Vesicles (1 mg/ml protein) were loaded with 150 mM RbCl, 125 mM sucrose, 2 mM PIPES-Tris (pH 7.4), and 36Rb+ (6 μCi/ml) at room temperature. The influx reaction was stopped at 15, 60, 300, and 600 s by a 20-fold dilution of the mixture with 5 ml of ice-cold stop solution (357 mM sucrose plus 2 mM PIPES-Tris; pH = 7.4) and promptly filtered through a Millipore filter (HAWP, 0.45 μm) that had previously been wetted with ice-cold stop solution. The filter was washed twice with 5 ml of ice-cold stop solution, transferred to a scintillation vial, and solubilized with 3 ml liquid scintillation cocktail. 36Rb+ inside the vesicles was analyzed by a liquid scintillation counter. This series of experiments was carried out in the Isotope Center of the University of Tokyo.

Measurement of phosphoinositides in the membrane preparation. Labeling of phosphatidylinositol in the membrane preparation was basically carried out as previously described (19, 28). The sample was added to 0.6 mM MgSO4 and 1 μCi/ml [γ-32P]ATP. The mixture was incubated for 1 or 5 min at 30°C, and then the reaction was stopped by the addition of 0.5 mM EDTA, and centrifuged at 16,000 rpm for 10 min. The pellet was suspended in the suspending medium.

To examine the effects of divalent cations or neomycin on the phosphatidylinositol content, the labeled sample was incubated with or without test agents for 20 min at 37°C and centrifuged. The pellet was suspended in 1 ml of chloroform-methanol-concentrated HCl (200:100:0.75; by volume), and the two-phase separation was induced by the addition of 0.6 M HCl (0.2 ml) followed by vortexing. After centrifugation, the lower phase was collected and washed twice with 0.5 ml of chloroform: methanol: 0.6 M HCl (3:48:47; by volume) by vortexing. The lower phase was dried under a stream of N2, vortexed, and the lipid residue was dissolved in 20 μl of ice-cold chloroform. An aliquot was applied on an oxalate-impregnated silica gel TLC plate, and the lipids were separated by developing the plate in chloroform-methanol-concentrated NH4OH-water (45:45:4:11; by volume) for 3 h [for phosphatidylinositol 4,5,6-trisphosphate (PIP3)]. Lipids were visualized by iodine staining, and the radioactivity was detected by the BAS system (BAS-1500 and BAS-2000, Fuji Film).

RESULTS

Phosphoprotein phosphatase inhibitors did not prevent reduction of K+ permeability by treatment with high Mg2+.

We first examined whether the results reported by Im et al. (13) were reproducible. The apical membrane-rich fraction obtained from acid-secreting rabbit parietal cell was added to a solution containing acridine orange, and quenching of fluorescence due to the accumulation of protons within the vesicles was consistently observed. This was completely independent of addition of valinomycin as shown in Fig. 1A, confirming that this membrane had already acquired enough K+ permeability. The membranes were then treated with 5 mM MgSO4 at 37°C for 10 min and added to the quenching medium. As shown in Fig. 1A, quenching was markedly reduced by this treatment and subsequent addition of valinomycin clearly caused

AJP-Gastrointest Liver Physiol • VOL 281 • SEPTEMBER 2001 • www.ajpgi.org
Further quenching. This indicates that the reduction of the quenching is not due to the decrease in the pumping activity per se but to the decrease of K⁺ permeability of the membrane. This effect of Mg²⁺ appeared to require some metabolic step(s), because the membrane needed to be pretreated with Mg²⁺ at 37°C for 10 min or more and incubation on ice did not develop the valinomycin dependency. Inclusion of 20 mM sodium pyrophosphate, a nonspecific phosphatase inhibitor, during the preincubation time, prevented the development of valinomycin dependency, showing exactly the same trace as the control membrane (Fig. 1A). These results were consistent with observations reported by Im et al. (13) using stimulated rat gastric membranes.

We started an examination of the work by Im et al. (13) using specific inhibitors. Calyculin A was expected to abolish any activities of phosphoprotein phosphatases I, IIα, and IIβ at 1 μM. However, this drug failed to prevent Mg²⁺-induced development of valinomycin dependency (data not shown). To confirm this, we used the following protocol. The stimulated apical membranes were incubated with 5 mM MgSO₄ and 1 mM ATP (Mg²⁺ treatment), with 5 mM Mg²⁺, 1 mM ATP, and 1 μM calyculin A (with protein phosphatase inhibitor), and with 5 mM Mg²⁺, 1 mM ATP, 1 μM calyculin A, and 20 U/ml PKA catalytic subunit (with PKA plus phosphatase inhibitor). As shown in Fig. 1B, all these showed the same degree of reduction in K⁺ permeability. These observations clearly indicate that involvement of protein phosphatases I, IIα, and IIβ is excluded and that the putative phosphoprotein does not appear to be the substrate for PKA.

Because there remained the possibility that protein phosphatase IIβ (calcineurin) was involved in this process, we examined the effects of 50 nM calcineurin autoinhibitory peptide or 10 μM daltamethrin, which was reported (10) to be sufficient to achieve complete inhibition of calcineurin activity (data not shown). It was found that neither inhibitor could prevent the effect of Mg²⁺ (Fig. 1C). Based on the results shown in Fig. 1, we excluded the possibility of involvement of protein phosphatases and PKA from the present system. We then moved on to the other candidates related to phosphorylation.

*Effects of phospholipase C inhibitors and aminoglycoside antibiotics on acridine orange quenching.* As the involvement of phosphoprotein phosphatases appeared to be of minor effect in the decrease of K⁺ permeability by Mg²⁺ treatment, we examined another dephosphorylation enzyme, phospholipase. Neither PLAz inhibitor (AA-861, 10 μM) nor phospholipase D inhibitor (propranolol, 100 μM) was effective (data not shown). We found that neomycin, a phospholipase C (PLC) inhibitor, showed an interesting effect. As shown in Fig. 2A, preincubation of the stimulated membrane vesicles with neomycin at 37°C for 10 min reduced the acridine orange quenching in a dose-dependent manner. This effect of neomycin was considered to be not due to the direct inhibition of proton pumping but due to reduced K⁺ permeability, because the reduced quenching was recovered by the addition of valinomycin, as shown in Fig. 2A. Estimating the K⁺ permeability by the degree of valinomycin-independent acridine orange quenching, we found that the effect of 150 μM neomycin appeared to be equivalent to that of 5 mM Mg²⁺ (Fig. 2B). The mode of action of neomycin seemed to be similar to that of Mg²⁺+, because it was necessary for neomycin to be preincubated with membrane at 37°C for 10 min or more. The effect of neomycin was evident at concentrations higher than 50 μM and reached a maximum as high as 1 mM in the preincubation. The concentration of neomycin in the cuvette during acridine orange quenching assay was reduced to one-fifth of that in preincubation, i.e., 1 mM to 20 μM. Neomycin added just before the assay had little effect on the K⁺ permeability in this concentration range.

When the concentration of neomycin was increased to the millimolar range in the assay condition, the acridine orange quenching was reduced and was not recovered by the addition of valinomycin (data not shown). This might be due to its direct inhibition of the pumping machinery, as we previously reported (24). It was therefore demonstrated that preincubation with 50 to 1,000 μM neomycin specifically reduced the K⁺ permeability in stimulated apical membranes.

Because both neomycin and Mg²⁺ require preincubation for their effect, we considered that the mechanism of their action might share some common step(s). We then examined the effect of pyrophosphate on the neomycin treatment. As shown in Fig. 2C, 20 mM pyrophosphate prevented the development of valinomycin dependency similar to the case of Mg²⁺ treatment. The threshold concentrations of Mg²⁺ and neomycin for the reduction of K⁺ permeability in preincubation were ~2 mM and 50 μM, respectively. When the membranes were pretreated with a combination of Mg²⁺ and neomycin, a marked reduction in K⁺ permeability was observed (Fig. 2D), suggesting that the sites of action of Mg²⁺ and neomycin are different, although their mechanism appears to share a common step.

To examine whether the effect of neomycin was due to the inhibition of PLC, we used 10 μM U-73122, another PLC inhibitor with different mode of action. As shown in Fig. 2E, this inhibitor did not affect K⁺ permeability at all, thus excluding the involvement of PLC in this system. Because aminoglycoside antibiotics were shown to block the P/Q-type Ca²⁺ channel (21), it might be possible that neomycin blocks cation channels by a mechanism common to the antibiotics. We thus examined the effects of 300 μM streptomycin and observed no effect on acridine quenching (Fig. 2E). It was also confirmed that 100 μM kanamycin had no effect (data not shown). These concentrations were chosen to cause the same degree of inhibition on the cation channels.

*PIP₂ restored attenuated acridine orange quenching by neomycin and Mg²⁺.* It was reported (9) that the inhibition of neomycin on PLC activity was due to its binding to the substrate PIP₂. We thought that the action of neomycin might be on the phosphoinositide

AJP-Gastrointest Liver Physiol • VOL 281 • SEPTEMBER 2001 • www.ajpgi.org
metabolism in the membrane by trapping PIP₂. We thus added PIP₂ to the membrane during preincubation with neomycin and found that PIP₂ protected the membrane from neomycin (data not shown). Furthermore, the membrane with reduced K⁺ permeability from neomycin pretreatment restored acridine orange quenching with the addition of 2 mM PIP₂ into the cuvette (Fig. 3A). Although the curve was steep, the effect of PIP₂ was found to be dose dependent in the range of 1–10 μM (Fig. 3B). We also checked the dose-dependent effect of PIP₂ on Mg²⁺-treated vesicles and found a similar recovery of K⁺ permeability (Fig. 3C and D). To elucidate the mode of action of PIP₂, we purified the microsomal fraction containing resting tubulovesicles from cimetidine-treated rabbit stomach. As shown in Fig. 3E, addition of PIP₂ never caused acridine orange quenching in resting tubulovesicles, whereas valinomycin added subsequently induced a marked quenching, demonstrating that the resting tubulovesicles have little K⁺ permeability. From this experiment, it was demonstrated that PIP₂ neither works as a K⁺ ionophore nor activates the putative endogenous K⁺ channel/transporter in this membrane preparation.

To rule out the possible direct effect of PIP₂ on H⁺–K⁺-ATPase, the ATP hydrolyzing activity of permeabilized tubulovesicle preparation was measured in the presence of PIP₂ up to 10 μM. It was found that PIP₂ had practically no effect (<3%) on the K⁺-ATPase activity. In the next experiment, we tested other phosphoinositides using Mg²⁺-treated membranes. PIP₃ also recovered K⁺ permeability, whereas neither phosphatidylinositol 4-monophosphate (PIP) nor phosphatidylinositol induced recovery (data not shown). It is well known that there exists a series of enzymes synthesizing phosphoinositides in the membrane. We postulated that the inactivity of phosphatidylinositol and PIP might be due to their difficulty in being incorporated into the membrane. Therefore, we used PITPβ (4). Although recombinant PITPβ itself had no effect, when this protein had been saturated with phosphatidylinositol at 4°C overnight and added to Mg²⁺-treated membrane, the reduced K⁺ permeability was restored, as evident in Fig. 4.
Mg\(^{2+}\) and neomycin decreased K⁺ permeability of membrane vesicles. In the above experiments, we estimated the K⁺ permeability by an indirect measure, i.e., valinomycin dependency of the H⁺-K⁺-ATPase-operated proton pumping as monitored by acridine orange quenching. As shown in Fig. 5, we measured \(^{86}\)Rb⁺ uptake by the vesicle and tested whether the observed effects were related to the K⁺ permeability. Although there was no difference in the \(^{86}\)Rb⁺ uptake at 5 min or later, it was significantly inhibited by preincubation with 500 \(\mu M\) neomycin at the early time point (15 s after the addition of the ion). This result indicates that inhibition of K⁺ permeability by neomycin was only detectable when the concentration gradient was large. As the K⁺ movement appeared to be too fast for the filter method, we employed another technique to measure the K⁺ permeability.

In the next experiment, we estimated K⁺ permeability by the H⁺-passive diffusion technique. In this experiment, gastric vesicles were equilibrated at pH 4 and the extravesicular pH was brought to 8, which formed a pH gradient, and subsequently acridine orange quenching occurred. As shown in Fig. 6A, the spontaneous recovery from quenching was not promi-
The beginning of the pH equilibration. After the initial stepwise addition of 5 and 20 mM Mg\(^{2+}\) gluconate was accomplished. Addition of 5 \(\mu\)M nigericin further accelerated the recovery of fluorescence. As shown in Fig. 6A, stepwise addition of 5 and 20 mM Na\(^{+}\) gluconate showed a much slower and smaller recovery of fluorescence, and the addition of 20 mM K\(^{+}\) gluconate caused further recovery of fluorescence. This indicates that the pathway for the cation influx seemed to be selective for K\(^{+}\) compared with Na\(^{+}\). Compared with the control, membranes preincubated with 10 mM Mg\(^{2+}\) at 37°C for 10 min showed an attenuated recovery of fluorescence in response to the addition of K\(^{+}\) gluconate (Fig. 6B). The effect of pretreatment with Mg\(^{2+}\) was overcome by the inclusion of valinomycin. Treatment of vesicles with 1 mM neomycin gave essentially the same result in terms of K\(^{+}\) permeability (Fig. 6C). In Fig. 6D, pretreatment with 10 mM MgSO\(_4\) again caused a reduction of the recovery by K\(^{+}\) gluconate in another membrane preparation. This reduction was prevented by the inclusion of 10 \(\mu\)M PIP\(_2\) at the beginning of the pH equilibration.

**Measurement of PIP\(_2\) content in gastric membranes.** To elucidate the mechanism of action of Mg\(^{2+}\) treatment, we tried to measure the contents of phosphoinositides. It was suggested that Mg\(^{2+}\) could deplete PIP\(_2\) from the membrane (12) or absorb PIP\(_2\) (27), although the actual data have not been presented. We pulse labeled the phospholipids in apical membrane vesicles with \(^{32}\)P, and the vesicles were incubated with 10 mM Mg\(^{2+}\) for 20 min at 37°C. As the optimal condition for the detection differed between PIP\(_2\) and PIP\(_3\), slight modifications were made in the labeling time and the solvent system. Figure 7A shows the autoradiography of the chromatogram suitable for PIP\(_2\). It is clearly shown that Mg\(^{2+}\) treatment decreased \(^{32}\)P label in PIP\(_2\). Consistent with the results of acridine orange quenching, addition of 20 mM pyrophosphate together with Mg\(^{2+}\) prevented the decrease in \(^{32}\)P label in PIP\(_2\). In contrast, treatment with neomycin did not decrease but rather increased the amount of labeled PIP\(_2\). With TlCl, we could not detect any labeling in the place corresponding to PIP under the present condition. In this system, however, the retardation factor of PIP\(_3\) was too small to detect. Figure 7B shows the autoradiography of the chromatogram suitable for PIP\(_3\) by extending the labeling time and changing the solvent. It is clearly shown that Mg\(^{2+}\) treatment decreased \(^{32}\)P label also in PIP\(_3\). Although there were several radioactive spots other than these authentic phosphoinositides, and some of them even showed changes in radioactivity by these treatments, we could not identify any of them so far.

**DISCUSSION**

When H\(^{+}\)-K\(^{+}\)-ATPase was first discovered, the mechanism of its activation was an enigma, i.e., the enzyme was activated by K\(^{+}\) but existed on the intra-
cellular membranes where the concentration of $K^+$ was much higher than the Michaelis constant of the enzyme. This mystery was partially solved by the observation that $K^+\text{-}K^+\text{-}ATPase$ required the $K^+$ ionophore valinomycin for its maximal activity (7). Namely, the proton pumping activity was thought to be latent when the membrane had little $K^+$ permeability and thus the $K^+$ site facing the luminal side is not accessible for the cation. Since then, much effort has been made to find out the nature of that $K^+$-transporting activity. Although some candidates have been suggested (23), no conclusion has been obtained yet.

It should be reasonable to postulate that activation of the putative $K^+$ transporter or channel is due to phosphorylation via PKA, because PKA activation has been shown to be essential for acid secretion, at least in rabbit parietal cells (1, 3). As evidence, Im et al. (13) showed that 1) gastric heavy microsomes obtained from acid-secreting rat stomach showed ATP-dependent, valinomycin-independent proton transport; 2) incubation of the membrane with $Mg^{2+}$ and high protein concentration made the vesicle valinomycin dependent; 3) the effect of $Mg^{2+}$ was prevented by a nonspecific phosphatase inhibitor, pyrophosphate; and 4) the microsomal membrane preparation actually showed phosphoprotein phosphatase activity. The role of $Mg^{2+}$ was postulated to activate the enzyme and to facilitate the membrane-membrane contact for the dephosphorylation reaction. However, these results (13) only indirectly suggested the possible involvement of protein

Fig. 6. $Mg^{2+}$ and neomycin inhibit passive diffusion of protons in stimulated gastric vesicles. A: gastric vesicles from stimulated rabbit were equilibrated with a Tris-succinate buffer containing acridine orange and carbonyl cyanide $m$-chlorophenylhydrazone (CCCP; pH 4). The extravesicular pH was promptly increased to 8 by addition of Tris base (first arrowhead). Even in the presence of the protonophore CCCP, the leak of $H^+$ (the recovery of fluorescence) was slow. Addition of 5 and 20 mM $K^+$ gluconate (K-Glc) caused stepwise recovery of fluorescence in the membrane (trace a). On the other hand, 5 and 20 mM $Na^+$ gluconate (Na-Glc) showed a smaller recovery rate and the further addition of 20 mM $K^+$ gluconate caused a recovery to the same extent as trace a (trace b). This indicates that the cation permeability was preferential to $K^+$ rather than $Na^+$; Nig, nigericin. B: the gastric membranes were preincubated with or without 10 mM $MgSO_4$ and showed proton passive diffusion as in A. Treatment with $Mg^{2+}$ reduced the recovery rate of fluorescence by 5 and 20 mM $K^+$ gluconate (trace b) compared with control membranes (trace a). The inclusion of valinomycin from the beginning of the pH equilibration (trace c) restored and even caused faster recovery of fluorescence. C: similar to the experiments in B, except that the membranes were pretreated with 1 mM neomycin instead of $Mg^{2+}$; trace a: control; trace b: neomycin treated; trace c: neomycin treated + valinomycin. A–C: experiments were done with the same membrane preparation and shared the same control tracing. This set of experiments is representative of essentially similar results obtained from at least 3 different samples. D: using different membrane preparations, it was observed that pretreatment with 10 mM $MgSO_4$ again caused a reduction of the recovery by $K^+$ gluconate (trace b vs. a). This reduction was prevented by the inclusion of 10 mM PIP_2 at the beginning of the pH equilibration (trace c).
phosphorylation in activation of K⁺-transporting activity. One disadvantage for the researchers at that time was that no specific inhibitors of phosphoprotein phosphatase were available. As the reaction of H⁺-K⁺-ATPase contains a dephosphorylation step, the usual phosphatase inhibitors also blocked the revolution of the pump and thus analysis utilizing the pump activity (like acridine orange quenching) became difficult. In recent years, calyculin A and okadaic acid have been available as specific inhibitors of phosphoprotein phosphatase type I, IIA, and IIC. Of these, we previously reported (30) that calyculin A stimulated acid secretion in isolated rabbit gastric glands, suggesting that protein phosphorylation plays an important role in acid secretory response.

For the present study, we examined previous reports using rabbits instead of rats and found that similar results were obtained, i.e., the K⁺ permeability manifested by valinomycin-independent proton transport in vesicles from stimulated gastric mucosa was attenuated by treatment with Mg²⁺, and this effect was prevented by pyrophosphate, a nonspecific phosphatase inhibitor. However, calyculin A at 1 μM, which was expected to completely suppress the phosphoprotein phosphatases I, IIA, and IIC, failed to prevent the attenuation of K⁺ permeability induced by Mg²⁺. Moreover, K⁺ permeability was reduced by treatment with Mg²⁺ even in the presence of ATP, PKA, and calyculin A. These results clearly indicated that the effect of Mg²⁺ did not involve dephosphorylation by phosphoprotein phosphatases I, IIA, and IIC and that the dephosphorylation, if any, did not occur on the substrate(s) for PKA. The possible involvement of protein phosphatase IIb, or calcineurin, was excluded by using calcineurin inhibitory peptide and deltamethrin. These results suggest that there was little possibility for the involvement of phosphoprotein phosphatases in the phenomenon. We then decided to search for other possibilities.

Even though protein phosphatases were excluded from the process, it was reasonable to postulate that other phosphorylation events might be involved, be-

Fig. 7. Content of ³²P-labeled phospholipids in gastric membranes. A: condition suitable for the analysis of PIP₂. Gastric vesicles purified from acid-secreting rabbit stomach were incubated with [γ-³²P]ATP at 30°C for 1 min. After various treatments, the extracted lipid fraction was applied onto an oxalate-impregnated silica gel plate and developed by chloroform-methanol-concentrated NH₄OH-water (45:45:4:11), and ³²P incorporation was analyzed by the BAS system. Lane 1: vesicles were incubated at 37°C for 20 min (control); lane 2: vesicles were incubated with 10 mM Mg²⁺; lane 3: incubation was performed with 10 mM Mg²⁺ and 20 mM sodium pyrophosphate; lane 4: incubation was done with 500 μM neomycin. B: condition suitable for the analysis of phosphatidylinositol 4,5,6-trisphosphate (PIP₃). Lipids in the gastric vesicles were labeled for 5 min, and the lipid fraction was developed on the oxalate-impregnated silica gel plate by chloroform-methanol-acetone-acetic acid-water (80:30:26:24:14). Lane 1: vesicles were incubated at 37°C for 20 min (control); lane 2: vesicles were incubated with 10 mM Mg²⁺. The arrows indicate the positions of authentic lipids. A representative of 3 experiments with essentially the same results is shown for A and B. PIP, phosphatidylinositol 4-monophosphate; PC, phosphatidylcholine.
cause the effects of Mg\(^{2+}\) treatment were effectively prevented by pyrophosphate. During screening with phospholipase inhibitors, we found that Mg\(^{2+}\) treatment was surrogated by the treatment with neomycin, a PLC inhibitor. Based on published data, we postulated the following possibilities at that time: 1) PLC plays a pivotal role for K\(^{+}\) permeability, because neomycin was reported to block PIP\(_2\) metabolism and it modulated the open probability of the Ca\(^{2+}\)-sensitive K\(^{+}\) channel (31); 2) neomycin has a direct blocking effect on K\(^{+}\) permeability, because aminoglycoside antibiotics were shown to block the P/Q-type Ca\(^{2+}\) channel (11); and 3) neomycin binds to PIP\(_2\) and changes the functions of some proteins regulated by PIP\(_2\) (9).

The first possibility was excluded by using another PLC inhibitor, U-73122, which failed to surrogate the effect of neomycin. The second possibility was denied by the observation that other aminoglycoside antibiotics, gentamycin and kanamycin, did not mimic neomycin.

Considering the mechanism of neomycin, we postulated that its effect was similar to Mg\(^{2+}\) and related to the metabolism of PIP\(_2\), because the effects of both neomycin and Mg\(^{2+}\) were 1) prevented by pyrophosphate, 2) not prompt and required at least 10 min of pretreatment, and 3) restored by addition of PIP\(_2\). We speculate that the phosphorylation/dephosphorylation cycle is working in the membrane and neomycin traps PIP\(_2\) to prevent its function, whereas Mg\(^{2+}\) affects the cycle by reducing the membrane contents of PIP\(_2\). This assumption is partially supported by the observation that the amount of PIP\(_2\) was reduced by Mg\(^{2+}\) but increased by neomycin.

To identify the key molecule among the phosphoinositides, we checked several compounds and found that PIP\(_3\) also recovered K\(^{+}\) permeability, whereas neither PIP nor phosphatidylinositol did. This result was somewhat surprising because biomembranes usually possess an enzyme complex for phosphoinositide metabolism (8) and the supply of phosphatidylinositol is the rate-limiting step. We thought that the lack of effect of the latter two was due to the difficulty of their incorporation into the membranes. It has been suggested that the supply of PIP or phosphatidylinositol was accelerated in the living cell by the protein PITP (4). We then prepared a recombinant PITP\(_{\beta}\) and found that it restored the reduced K\(^{+}\) permeability by Mg\(^{2+}\) or neomycin treatment after the protein had been preadsorbed with phosphatidylinositol. These results suggest that treatment by Mg\(^{2+}\) or neomycin caused a shortage of some phosphoinositide(s) by affecting the phosphatidylinositol metabolism.

The direct measurement of phosphoinositides in the membrane showed that treatment with Mg\(^{2+}\) actually reduced the labeled PIP\(_2\) as well as PIP\(_3\), and the reduction was prevented by pyrophosphate. A possible explanation is that Mg\(^{2+}\) might activate a phosphatase or inhibit a kinase for the component(s) downstream of PIP\(_2\), and pyrophosphate antagonizes that reaction. It was reported (2) that pyrophosphate affected the contents of phosphoinositides in the neutrophil membranes, although it increased the contents of PIP\(_2\) in this case. However, it is possible that pyrophosphate acts differently in another type of cell with a different phosphoinositide metabolism.

In contrast to Mg\(^{2+}\), neomycin treatment did not decrease but rather increased the phosphate label in PIP\(_2\). Considering the fact that neomycin binds to PIP\(_2\) and inhibits its metabolism (9), we postulate that neomycin intercepted the sequence of phosphorylation/dephosphorylation at the level of PIP\(_2\), and subsequently the labeled compound was accumulated in the membrane. We have not identified the molecules by which the label is decreased from treatment with neomycin.

Wolosin and Forte (33) reported that various cations, including Mg\(^{2+}\), Ni\(^{2+}\), and Zn\(^{2+}\), potently inhibited Cl\(^{-}\) and K\(^{+}\) conductances in the stimulated apical membrane of rabbit parietal cells. They (33) suggested that these cations directly affected the putative channels. Direct effects of Mg\(^{2+}\) on the putative channels could be excluded because that demanded some metabolic process and the Mg\(^{2+}\) concentration during the assay was reduced to one-fiftieth of that in preincubation. On the other hand, the observation of Wolosin and Forte (33) that Ni\(^{2+}\) and Zn\(^{2+}\) inhibited K\(^{+}\) conductance at submillimolar concentration might have been due to the reduction of phosphoinositides in the membrane. However, we did not observe any changes in the contents of PIP\(_2\) and PIP\(_3\) in the membrane preparation by treatment with these cations.

Of the phosphoinositides, PIP\(_2\) has been reported to modulate the activity of various channels or transporters, including inward rectifier K\(^{+}\) channels (ROMK, ROMK2/Kir6.2, GIRK1/4, GIRK2, and IRK1; 12, 16), Na\(^{+}\)-gated nonselective cation channel (34), and Na\(^{+}\)/Ca\(^{2+}\) exchanger (22). It is reasonable to postulate that the putative K\(^{+}\) channel or transporter essential for gastric proton pumping demands PIP\(_2\) for its activity. This information should be quite useful to identify the molecular entity of the putative K\(^{+}\) channel or transporter in a future experiment. However, considering the fact that PIP\(_3\) surrogated PIP\(_2\), the metabolite(s) of PIP\(_2\) would be the key molecule(s). Alternatively, PIP\(_3\) might have been metabolized to be PIP\(_2\) within the membrane. More work is necessary to identify the molecule responsible for K\(^{+}\) permeability by phosphoinositides in the gastric membrane.

In the present study, the K\(^{+}\) permeability of the membrane was mainly estimated by indirect measurements, i.e., valinomycin-dependent proton transport operated by H\(^{+}\)-K\(^{+}\)-ATPase and H\(^{+}\) passive diffusion where K\(^{+}\) alone exists as the permeable ion population. The direct measurement using \(^{86}\)Rb uptake revealed that the K\(^{+}\) (Rb\(^{+}\)) permeability of the membrane was relatively high even though the H\(^{+}\)-K\(^{+}\)-ATPase-operated acridine orange quenching became highly valinomycin dependent from treatment with neomycin. We consider that the intrinsic permeability of the membrane to K\(^{+}\) or Rb\(^{+}\) is relatively high even in the resting state. This possibility has been repeatedly pointed out, and there used to be a hypothesis that K\(^{+}\)
permeability is not necessarily accelerated during the activation of acid secretion (5, 26). However, even though the membrane containing H⁺-K⁺-ATPase has some K⁺ permeability, H⁺ cannot accumulate within the vesicle when permeability to H⁺ in the membrane is higher than that to K⁺. The observed acidic orange quenching was the function of the intrinsic pump activity, and K⁺, Cl⁻, and H⁺ permeability. In this system, we can observe the steady-state level based on these factors and thus the sensitivity to detect the reduction of K⁺ permeability is quite high. In the case of Rb⁺ uptake, the observed measurement is the result of uniflux and is thus much less sensitive. Looking at the traces of the H⁺ passive diffusion experiments, the difference in the influx rate manifested as the proton counterflow, with or without treatment, was only evident within a minute and disappeared when the K⁺ concentration inside the vesicle approached that of the outside, which was consistent with the data of Rb⁺ uptake.

It is noteworthy that the addition of PIP₂ did not induce K⁺ permeability in resting tubulovesicles. This indicates that the molecular entity of the putative K⁺ channels or transporters does not exist on the tubulovesicular membrane but on the other intracellular fuses that fuses with the apical membrane under stimulation (14), or it resides on the apical membrane. Alternatively, the putative K⁺ channels or transporters exist on the tubulovesicles in the resting state and are insensitive to PIP₂ but become sensitive to it when the cell is stimulated.

In conclusion, we found in the present study that phosphatidylinositol (possibly PIP₃ and/or PIP₃) is an essential determinan for the K⁺ permeability involved in gastric proton pumping. In spite of many efforts to identify the molecular entity of the K⁺ transporter that is the direct switch of gastric acid secretion, no conclusive results have been obtained. For example, a study by Supplisson et al. (25) that used a patch-clamp method described K⁺ channels as being possibly located on the apical membrane of the parietal cell. However, it is very difficult to conclude that the observed channel is coupled with the gastric proton pump by electrophysiology alone. To this end, the present study has supplied a powerful tool, phosphoinositides and the drugs affecting their function and metabolism, in the identification of the molecular entity responsible for K⁺ permeability. It is also an interesting question how phosphoinositides participate in the regulation of gastric acid secretion. If it is a direct switch of acid secretion, we have to search for a missing link between PKA and phosphoinositides. If it is indirect, there should be some mechanism that allows the system to be sensitive to this lipid. In any event, we are sure that the present work represents a breakthrough in understanding the molecular basis of gastric proton pumping.

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