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 involved in gastric proton pumping. Am J Physiol Gastrointest Liver Physiol 281: G786–G797, 2001.

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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 (Shiraiashi, Tokyo, Japan) were allowed to feed on staple food for 20 min following 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-Elvehejm). The homogenate was centrifuged at 100,000 × g for 45 min to get the microsomal fraction. The supernatant was centrifuged at 100,000 × g for 2 h. The material on the 18% Ficoll layer was harvested, dilute in 20 vol of the homogenizing buffer, and then centrifuged at 100,000 × g for 45 min. The final material was suspended in the homogenizing buffer 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 KC1, 110 mM choline chloride, 10 mM PIPES-Tris, pH 6.8, 1 μM 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-fifth 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⁺-exchanging pump. 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 revolution 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 promptly 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 (PITPb) 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 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 PITPb 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 Na₂HPO₄, 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.
and incubated with 10 mg/ml phosphatidylinositol at 4°C overnight.

**86Rb** uptake across membrane vesicles. Measurement of 

**86Rb** 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 **86Rb** (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 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. As shown in Fig. 1, addition of valinomycin clearly caused a reduction of **K** uptake attained by valinomycin) as 0. Valinomycin (10 M) was added to the quenching medium. As shown in Fig. 1A, quenching was markedly reduced by this treatment and subsequent addition of valinomycin clearly caused

**RESULTS**

Phosphoprotein phosphatase inhibitors did not prevent reduction of **K** permeability by treatment with high **Mg**. 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, acridine orange quenching was continuously monitored by a spectrofluorometer (CAF 110). Because the extent of quenching was dependent on the stimulatory condition in vivo, the quenching was normalized within each animal. The results were expressed as a relative fluorescence intensity setting the value before the addition of membrane as 1, and the minimal value (the maximal **H** uptake attained by valinomycin) as 0. Valinomycin (10 μM) was added – 10 min after the addition of vesicles (arrowhead). A: gastric vesicles from stimulated stomach were incubated without **Mg** (control) or with 5 mM **Mg** + or 5 mM **Mg** + 20 mM sodium pyrophosphate. B: the vesicles were preincubated with 1 mM ATP alone (control), 5 mM Mg2+ + 1 mM ATP, 5 mM Mg2+ + 1 mM ATP + 1 μM calcineurin A (calyA), or 5 mM Mg2+ + 1 mM ATP + 1 μM calcineurin A + 20 U/ml cAMP-dependent protein kinase catalytic subunit (protein kinase A, PKA). C: the gastric vesicles were preincubated with 5 mM Mg2+, 5 mM Mg2+ + 50 nM calcineurin autoinhibitory peptide (CaN-I), or 5 mM Mg2+ + 10 μM deltamethrin. Each tracing is representative of essentially similar results obtained from at least 3 different samples.

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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 Mg\(^{2+}\) appeared to require some metabolic step(s), because the membrane needed to be pretreated with Mg\(^{2+}\) 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, IIa, and IIC at 1 \(\mu\)M. However, this drug failed to prevent Mg\(^{2+}\)-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 MgSO\(_4\) and 1 mM ATP (Mg\(^{2+}\) treatment), with 5 mM Mg\(^{2+}\), 1 mM ATP, and 1 \(\mu\)M calyculin A (with protein phosphatase inhibitor), and with 5 mM Mg\(^{2+}\), 1 mM ATP, 1 \(\mu\)M 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, IIa, and IIC is excluded and that the putative phosphoprotein does not appear to be the substrate for PKA.

Because there remained the possibility that protein phosphatase IIb (calcineurin) was involved in this process, we examined the effects of 50 nM calcineurin autoinhibitory peptide or 10 \(\mu\)M diltiazem, which was reported (10) to be sufficient to achieve complete inhibition of calcineurin activity. It was found that neither inhibitor could prevent the effect of Mg\(^{2+}\) (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 Mg\(^{2+}\) treatment, we examined another dephosphorylation enzyme, phospholipase. Neither PLAs inhibitor (AA-861, 10 \(\mu\)M) nor phospholipase D inhibitor (propranolol, 100 \(\mu\)M) 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 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 \(\mu\)M neomycin appeared to be equivalent to that of 5 mM Mg\(^{2+}\) (Fig. 2B). The mode of action of neomycin seemed to be similar to that of Mg\(^{2+}\), 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 \(\mu\)M 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 \(\mu\)M. 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).

Because both neomycin and Mg\(^{2+}\) 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 Mg\(^{2+}\) treatment. The threshold concentrations of Mg\(^{2+}\) and neomycin for the reduction of K\(^+\) permeability in preincubation were ~2 mM and 50 \(\mu\)M, respectively. When the membranes were pretreated with a combination of Mg\(^{2+}\) and neomycin, a marked reduction in K\(^+\) permeability was observed (Fig. 2D), suggesting that the sites of action of Mg\(^{2+}\) 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 \(\mu\)M 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 Ca\(^{2+}\) 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 \(\mu\)M streptomycin and observed no effect on acridine quenching (Fig. 2E). It was also confirmed that 100 \(\mu\)M kanamycin had no effect (data not shown). These concentrations were chosen to cause the same degree of inhibition on the cation channels.

**PIP\(_2\) restored attenuated acridine orange quenching by neomycin and Mg\(^{2+}\).** It was reported (9) that the inhibition of neomycin on PLC activity was due to its binding to the substrate PIP\(_2\). We thought that the action of neomycin might be on the phosphoinositide
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. 3, C 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\textsuperscript{2+} and neomycin decreased K\textsuperscript{+} permeability of membrane vesicles. In the above experiments, we estimated the K\textsuperscript{+} permeability by an indirect measure, i.e., valinomycin dependency of the H\textsuperscript{+}-K\textsuperscript{+}-ATPase-operated proton pumping as monitored by acridine orange quenching. As shown in Fig. 5, we measured \textsuperscript{86}Rb\textsuperscript{+} uptake by the vesicle and tested whether the observed effects were related to the K\textsuperscript{+} permeability. Although there was no difference in the \textsuperscript{86}Rb\textsuperscript{+} uptake at 5 min or later, it was significantly inhibited by preincubation with 500 \textmu{}M neomycin at the early time point (15 s after the addition of the ion). This result indicates that inhibition of K\textsuperscript{+} permeability by neomycin was only detectable when the concentration gradient was large.

As the K\textsuperscript{+} movement appeared to be too fast for the filter method, we employed another technique to measure the K\textsuperscript{+} permeability. In the next experiment, we estimated K\textsuperscript{+} permeability by the H\textsuperscript{+}-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-
of the chromatogram suitable for PIP2. It is clearly shown that Mg2+ treatment decreased 32P label in PIP2. Consistent with the results of acridine orange quenching, addition of 20 mM pyrophosphate together with Mg2+ prevented the decrease in 32P label in PIP2. In contrast, treatment with neomycin did not decrease but rather increased the amount of labeled PIP2. With TLC, we could not detect any labeling in the place corresponding to PIP under the present condition. In this system, however, the retardation factor of PIP3 was too small to detect. Figure 7B shows the autoradiography of the chromatogram suitable for PIP3 by extending the labeling time and changing the solvent. It is clearly shown that Mg2+ treatment decreased 32P label also in PIP3. 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.

It was reported (33) that divalent cations inhibited K+ and Cl− conductances in stimulated apical membranes. To investigate if the inhibitory activity was due not to direct blocking on the channels/transporters but to an indirect blocking via the reduction of phosphoinositides, we selected Ni and Zn as the most potent inhibitors. We treated the labeled membranes with Ni (0.7 mM) or Zn (0.3 mM) at the reported concentration and observed no changes in PIP2 or PIP3 (data not shown).

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 H\(^+\)-K\(^+\)-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. From these findings, Im et al. (13) concluded that the gastric membranes contained membrane-bound, Mg\(^{2+}\)-dependent phosphoprotein phosphatase, which desphosphorylated the putative K\(^+\) carrier. 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

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**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 extravascular 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 preincubated with 1 mM neomycin instead of Mg\(^{2+}\); 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 \mu M PIP\(_2\) at the beginning of the pH equilibration (trace c).
phosphorylation in activation of K\textsuperscript{+}-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\textsuperscript{+}-K\textsuperscript{+}-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\textsuperscript{+} permeability manifested by valinomycin-independent proton transport in vesicles from stimulated gastric mucosa was attenuated by treatment with Mg\textsuperscript{2+}, and this effect was prevented by pyrophosphate, a nonspecific phosphatase inhibitor. However, calyculin A at 1 \muM, which was expected to completely suppress the phosphoprotein phosphatases I, IIA, and IIC, failed to prevent the attenuation of K\textsuperscript{+} permeability induced by Mg\textsuperscript{2+}. Moreover, K\textsuperscript{+} permeability was reduced by treatment with Mg\textsuperscript{2+} even in the presence of ATP, PKA, and calyculin A. These results clearly indicated that the effect of Mg\textsuperscript{2+} 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 II\textsubscript{A}, 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-
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 PTP (4). We then prepared a recombinant PTPβ 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, 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 nonspecific 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 $H^+\cdot K^+\cdot$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 acidified 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 both 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 PIP$_2$ does 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 membrane 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 PIP$_2$ but become sensitive to it when the cell is stimulated.

In conclusion, we found in the present study that phosphatidylinositol (possibly PIP$_2$ and/or PIP$_3$) is an essential determinant 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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