Reconstitution of Acid Secretion in Digitonin-permeabilized Rabbit Gastric Glands

IDENTIFICATION OF CYTOSOLIC REGULATORY FACTORS*

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When isolated rabbit gastric glands were permeabilized with digitonin, they lost their ability to secrete acid, as monitored by [14C]aminopyrine accumulation, and they never recovered by supplement with cytosol prepared from gastric mucosa. However, the permeabilized glands elicited acid secretion when brain cytosol was supplemented. Fractionation of gastric cytosol by gel filtration revealed that the fraction at 30 kDa stimulated permeabilized glands by itself, whereas the 200-kDa fraction potently inhibited brain cytosol-stimulated acid secretion. Brain cytosol contained only the former stimulatory factor. With further gel filtration, the 30-kDa activator was separated into two components, 20 kDa (peak 1) and 1.8 kDa (peak 2), both of which are necessary for full activity. We purified peak 1 from bovine brain, and phosphatidylinositol transfer protein (PITP) was identified as the main component of the activity. The stimulating activity in brain and gastric mucosa correlated with the contents of PITP, and recombinant PITP mimicked the effect of peak 1, suggesting that PITP is one of the essential components in gastric acid secretion. When gastric glands were stimulated, the inhibitory activity, but not stimulatory activity, in the cytosol was increased. This suggests a regulatory mechanism such as stimulation translocates proteins involved in the ATP-dependent process (9, 10) or the Ca2+-dependent process (11, 12) have been successfully identified.

In the present study, we report that the secretory responsiveness of digitonin-permeabilized rabbit gastric glands can be reconstituted by cytosolic factors and that this model is a powerful tool for searching for the cytosolic factors involved in the activation of acid secretion.

EXPERIMENTAL PROCEDURES

Materials—Rat cloned β isoform of phosphatidylinositol transfer protein (PITP) (13) in pET-21a-d(+) vector was kindly gifted by Dr. H. Arai (Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan). The cDNA was cloned into pGEX-4T-1, and transformed XL1-blue. Expression of the fusion protein was induced by isopropyl-β-thiogalactopyranoside (0.1 mM) for 2 h at 25 °C, and the recombinant protein was purified from the bacterial cells by glutathione-Sepharose 4B resin, and then resuspended in 150 mM NaCl, 3 mM K2HPO4, 0.64 mM NaH2PO4, 10 mM EDTA (pH 7.0). After freezing 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, and then the fusion protein was cleaved by thrombin treatment.

All the chemicals were reagent grade and obtained from Sigma or Nacalai Tesque except otherwise noted.

Preparation of Isolated Glands, Cells, and Permeabilized Glands—Gastric glands were isolated from Japanese white rabbits (Shiraishi amide gel electrophoresis; GST, glutathione S-transferase; HPLC, high performance liquid chromatography; PIPES, 1,4-piperazinediethanesulfonic acid; PIP₅₋, phosphatidylinositol 4,5-bisphosphate; GTPγS, guanosine 5’-O-(thiotriphosphate); PI, phosphatidylinositol.
Co., Tokyo, Japan) essentially by the method of Berglindh (14). Isolated glands, suspended in the normal medium containing 132.6 mM NaCl, 5 mM NaHPO₄, 1 mM NaH₂PO₄, 5.4 mM KCl, 1.2 mM MgSO₄, 1.0 mM CaCl₂, 25 mM HEPES-Na (pH = 7.4), 11.1 mM glucose, and 1 mg/ml bovine serum albumin, were washed three times with a permeabilizing medium containing 200 mM NaCl, 100 mM KCl, to make fractions as pellets, the cytosolic fraction without dialysis was concentrated to 50–60 mg of protein/ml by lyophilization and loaded on a 75 cm × 2-cm² Superdex 200- pg column (Amersham Pharmacia Biotech), which had been equilibrated with the high K⁺ medium. Elution was performed at 0.4 ml/min, and the fractions were collected every 15 min. As described under "Results," the activity of this crude cytosol was separated by this procedure into two peaks, i.e., peaks 1, subsequently designated as p35, and peak 2, with an apparent molecular mass of 1.8 kDa. Since activity was only evident when both fractions were present, the bioassay procedure beyond this point was performed as follows.

Cytosol was prepared from rabbit whole brain, concentrated, and loaded on the Superdex 200- pg as described above, and the fractions corresponding to ~1.8 kDa (20–25 ml) were collected and designated as peak 2. For the aminopyrine accumulation assay, 50 μl of peak 2 was included such that the final composition of the medium was equivalent to that of the high K⁺ medium.

RESULTS

Brain Cytosol Stimulates Acid Secretion in Digitonin-permeabilized Glands—Acid secretion in digitonin-permeabilized rabbit gastric glands was monitored by the accumulation of [¹⁴C]aminopyrine and is summarized in Fig. 1A. In contrast to the model using α-toxin (7, 8) or β-escin (3), digitonin-permeabilized rabbit glands were not stimulated by 100 μM CAMP, and this is consistent with previously reported results (6). To make up for the possible loss of PKA (the target of cAMP), from...
Digitonin-permeabilized Gastric Glands

A. cytotoxic fractions (1.5 mg/ml final) were prepared from stomach (STM), brain (BRN), skeletal muscle (SKM), and pancreas (PNC) of rabbit, and added to digitonin-permeabilized glands without (control; open column) or with (filled column) 100 μM cAMP to see if they reconstituted the secretory activity. In the case of the catalytic subunit of PKA (1000 units/ml), the experiment with cAMP was omitted. B. isolated gastric cells were purified into parietal cell-rich (~80%) and poor (~20%, mainly chief cells) fractions by Percoll gradient. Digitonin-permeabilized glands were incubated without (R) or with 1 mg/ml brain cytosol (control) to measure the aminopyrine ratio. Cytosol obtained from either parietal cell-rich or poor fraction (0.8 mg/ml) was added to brain cytosol. The aminopyrine ratio was measured in duplicate and means ± S.E. of three to five independent experiments are shown (except skeletal muscle and pancreas, which were both two experiments apiece).

This case, the cytosol (0.8 mg/ml) was added to 1 mg/ml brain cytosol, which showed about half the maximal stimulation (see Fig. 2), in order to pick up either stimulatory or inhibitory activity. As shown in Fig. 2B, the cytosol from highly purified parietal cell still showed inhibitory activity on brain cytosol-stimulated aminopyrine accumulation; furthermore, its activity was more potent than that from the parietal cell-poor fraction. This observation also excluded the possible involvement of pepsin activity remaining, if any, in the presence of protease inhibitors.

FIG. 1. Effects of cytosol from various tissues on digitonin-permeabilized isolated gastric glands. A, concentration-response relationships of brain cytosol in the absence (control; open circles) or presence (closed circles) of 100 μM cAMP. B, effects of various agents on the brain cytosol (1.5 mg/ml)-stimulated aminopyrine accumulation in the permeabilized glands. PKI, heat-stable cAMP-dependent protein kinase inhibitor peptide (30 μM); Ca, calcium chloride (100 μM); OPZ, omeprazole (30 μM); SM-1, myosin light chain kinase pseudo substrate (50 μM); GTP[S], 100 μM. Aminopyrine ratio was measured in duplicate, and means ± S.E. of three or four independent experiments are shown. The inhibitory effects of calcium chloride, omeprazole, myosin light chain kinase pseudo substrate, and GTP[S] were all statistically significant (p < 0.05 by Dunnet’s post hoc test).
In order to examine this hypothesis, we fractionated gastric cytosol by gel filtration. The cytosolic fraction was prepared from rabbit gastric mucosa and separated on a Sepharose CL4B gel filtration column. The protein amount in each fraction (6 ml) was estimated by $A_{280}$ (closed circles; the data are in common for A and B). The positions of the molecular size markers are shown at the top of the panel. a, blue dextran (2000 kDa); b, catalase (232 kDa); c, ovalbumin (48 kDa); d, trypsin inhibitor (22 kDa). A, fractions 10–26 were tested for stimulatory activity on aminopyrine accumulation in digitonin-permeabilized glands. Note that the peak of stimulation was 19–21. B, fractions 10–18 (300 μl each) were also tested for inhibitory activity on aminopyrine accumulation stimulated by brain cytosol in digitonin-permeabilized glands. Note that the peak of inhibition was 13–15. As for fractions 1–9, we confirmed in other purification experiments that no obviously active peaks were evident.

observed (data not shown). The stimulatory effect of brain cytosol was also inhibited by 100 μM GTPγS, a non-hydrolyzable analogue of GTP, and by 50 μM SM-1, a pseudo substrate of myosin light chain kinase.

Cytosol of Gastric Mucosa Contains Both Stimulatory and Inhibitory Factors—In the previous section, it was suggested that gastric mucosal cytosol contains inhibitory factors, with the essential factors for the activation of acid secretion, if any, which result in inhibitory activity with respect to brain cytosol. In order to examine this hypothesis, we fractionated gastric cytosol by gel filtration. The cytosolic fraction was separated by Sepharose CL4B column chromatography, which works well for separation of components with relatively high molecular weights. Each fraction was assayed for stimulatory activity by itself as well as for inhibitory activity by using brain cytosol-stimulated aminopyrine accumulation in digitonin-permeabilized gastric glands. As shown in Fig. 3A, gastric cytosol obviously contained the activity to stimulate digitonin-permeabilized glands in the relatively small molecular mass range (~30 kDa). An assay for inhibitory activity revealed that gastric cytosol also contained inhibitory activity at a much higher molecular mass (~200 kDa) as evident in Fig. 3B.

When brain cytosol was fractionated in the same way as above, the stimulatory activity eluted in the same position as above (~30 kDa), whereas no inhibitory activity was observed in any position (data not shown). We then concluded that the gastric cytosol contains both stimulatory and inhibitory factors, whereas brain cytosol only contains the former.

Purification of Stimulatory Factors from Brain Cytosol—We first tried to purify the stimulatory factor(s) from gastric cytosol. However, we encountered too many difficulties and had to switch the source to brain cytosol, supposing that the stimulatory factor(s) in both tissues were in common.

The stimulatory activity in the brain cytosol, which eluted at approximately 30 kDa with a Sepharose CL4B column, was separated by another gel filtration column, Superdex 200, good for the smaller molecules. As shown by open bars in Fig. 4, the stimulating activity split into two broad peaks (designated as peak 1 for the larger and peak 2 for the smaller) and the total as well as the specific activity drastically decreased. We postulated that the activity was due to multiple components. To examine this hypothesis, aminopyrine accumulation assay was repeated in the presence of peak 2 at the concentration that showed little stimulating activity by itself. As evident from the filled bars in Fig. 4, peak 1, with an apparent molecular mass of 20 kDa, had potent acid stimulating activity in the presence of peak 2. Although not shown in the figure, we confirmed in other purification experiments that the other fractions showed no such activity. As described later, peak 2 did not appear to be peptide, so we decided to purify peak 1 first, employing the bioassay in the presence of peak 2.

Purification of Peak 1 and Identification of p35—In order to increase the scale of the purification, we employed bovine brain instead of rabbit as a source. To ensure the full activity, the assay of purified material using digitonin-permeabilized glands was performed in the presence of a threshold amount of peak 2. In the pilot experiments, it was found that bovine brain
was recovered in the fraction corresponding to the apparent molecular mass of 1.8 kDa on a Superdex 200 gel filtration column. Its activity was unchanged after dialysis against 0.1 M potassium phosphate at pH 7.3 (but not at 6.7) and was retained in red-agarose.

We thus applied bovine brain cytosol to a DEAE-Sepharose column and eluted by decreasing pH, and then applied on red-agarose and eluted by high salt, as described under “Experimental Procedures.” This partially purified material was then separated on a Superdex 200 gel filtration column, and it was found that the fraction corresponding to the apparent molecular mass of 20 kDa showed acid stimulating activity as in the case of rabbit brain (data not shown). This active fraction was further separated on HPLC Diol-150, and the activity (aminopyrine accumulation in digitonin-permeabilized glands in the presence of peak 2; open column) and protein concentration (A$_{280}$, closed circles) was measured. The positions of the molecular size markers are shown at the top of the panel. A, blue dextran (2,000 kDa); B, bovine serum albumin (68 kDa); c, ovalbumin (45 kDa); d, cytochrome c (10 kDa). The stimulating activity was recovered in the fraction corresponding to the apparent molecular mass of 10 kDa (fraction numbers 18 and 19; asterisk). B, fractions 14–21 in panel A were analyzed on 15% SDS-PAGE and stained with Coomassie Blue. Note that the active fraction was correlated with the bands near 35 kDa assigned by the Coomassie Blue. Note that the active fraction was correlated with the bands near 35 kDa assigned by the Coomassie Blue. 

Using p35 purified from bovine brain as antigen, anti-p35 antiserum was raised in rats and it recognized recombinant rat PITP as well as bovine p35. Using this antiserum, the presence of anti-p35 positive bands were screened in rabbit brain fractions separated on a Superdex 200 column. As shown in Fig. 7A, it was evident that PITP existed in fractions 10 and 11, where peak 1 activity was also found (see also Fig. 4). It was confirmed that the active peak contained PITP in gastric cytosol as well (Fig. 7B). In the latter case, for cytosol fractionated by Sepharose CL4B, there was some discrepancy between the biological activity and the contents of p35. The activity in the early fractions (approximately fraction 18 in Fig. 3A) showed low stimulatory activity, whereas those in the late fractions (approximately fraction 22 in Fig. 3A) showed high activity as compared with the p35 contents. This could be explained by the presence of the inhibitory factor and peak 2. The early fractions have been completely separated from the low molecular weight activator peak 2, but not from the inhibitory factor, and subsequently the biological activity in the early fractions would have been underestimated. On the other hand, the late fractions have been completely separated from the high molecular weight inhibitor peak but not from activator peak 2. Subsequently, the biological activity in the later fractions was overestimated in terms of their p35 contents.

**Characterization of Peak 2**—To date, the active component in peak 2 from the rabbit brain cytosol has not been identified. To find a clue for its identification, we examined its features, and this is summarized in Table II. Peak 2 was harvested as a fraction with apparent molecular mass of 1.8 kDa on a Superdex 200 gel filtration column. Its activity was unchanged after 15 min of boiling, and was also resistant to treatment with 0.05% chymotrypsin or 0.05% carboxypeptidase at 30 °C for 1 h. These indicated that the active component was non-peptide. It did not adsorb to an ODS reverse-phase column in methanol/chloroform (2:1), and did not bind to an ODS reverse-phase column. The activity was also resistant to treatment with 50 μM PKI, as was that of peak 1 (data not shown). In separate experiments, it was shown that neither 10 μM phosphatidylinositol nor 10 μM phosphatidylinositol 4,5-bisphosphate (PIP$_2$) augmented the stimulatory effect of β-PITP (data not shown), suggesting that peak 1 is different from these phosphoinositides.

Using p35 purified from bovine brain as antigen, anti-p35 antiserum was raised in rats and it recognized recombinant rat PITP as well as bovine p35. Using this antiserum, the presence of anti-p35 positive bands were screened in rabbit brain fractions separated on a Superdex 200 column. As shown in Fig. 7A, it was evident that PITP existed in fractions 10 and 11, where peak 1 activity was also found (see also Fig. 4). It was confirmed that the active peak contained PITP in gastric cytosol as well (Fig. 7B). In the latter case, for cytosol fractionated by Sepharose CL4B, there was some discrepancy between the biological activity and the contents of p35. The activity in the early fractions (approximately fraction 18 in Fig. 3A) showed low stimulatory activity, whereas those in the late fractions (approximately fraction 22 in Fig. 3A) showed high activity as compared with the p35 contents. This could be explained by the presence of the inhibitory factor and peak 2. The early fractions have been completely separated from the low molecular weight activator peak 2, but not from the inhibitory factor, and subsequently the biological activity in the early fractions would have been underestimated. On the other hand, the late fractions have been completely separated from the high molecular weight inhibitor peak but not from activator peak 2. Subsequently, the biological activity in the later fractions was overestimated in terms of their p35 contents.

**Identification of p35 as an active component of peak 1.** Bovine brain cytosol was sequentially purified by DEAE-Sepharose, red-agarose, and Superdex 200 gel filtration, and an active fraction was seen corresponding to the apparent molecular mass of 20 kDa, as in the case of rabbit brain (see Fig. 4). A, this active fraction was further separated on HPLC Diol-150, and the activity (aminopyrine accumulation in digitonin-permeabilized glands in the presence of peak 2; open column) and protein concentration (A$_{280}$, closed circles) was measured. The positions of the molecular size markers are shown at the top of the panel, a, blue dextran (2,000 kDa); b, bovine serum albumin (68 kDa); c, ovalbumin (45 kDa); d, cytochrome c (10 kDa). The stimulating activity was recovered in the fraction corresponding to the apparent molecular mass of 10 kDa (fraction numbers 18 and 19; asterisk). B, fractions 14–21 in panel A were analyzed on 15% SDS-PAGE and stained with Coomassie Blue. Note that the active fraction was correlated with the bands near 35 kDa assigned by the Coomassie Blue (fractons 18 and 19).
but it disappeared within a few days as an aqueous solution even in the frozen state.

Changes in the Stimulatory and Inhibitory Components in the Gastric Glands by Activation of Acid Secretion—We examined the possible changes in activity of both the stimulatory and inhibitory components with activation of acid secretion in gastric glands. A suspension of isolated rabbit gastric glands was separated into two aliquots; one was maximally stimulated with 100 \( \mu \text{M} \) histamine plus 50 \( \mu \text{M} \) isobutylmethylxanthine at 37 °C for 30 min, whereas the other was kept in resting state by the addition of 100 \( \mu \text{M} \) cimetidine. After the treatments, the glands were harvested and homogenized and the cytosol was prepared. Each cytosol was separated on a gel filtration column, Sepharose CL-4B, and both stimulatory and inhibitory components were assayed using digitonin-permeabilized glands.

Unexpectedly, the activity of the stimulatory component was not changed at all by stimulation of the gastric glands (Fig. 8A). Moreover, the activity of the inhibitory component was markedly increased in the stimulated, not resting, glands (Fig. 8B).

We have tried to identify the inhibitory components, but have not yet succeeded. The main difficulty is that the inhibitory activity is unstable and always spreads into multiple peaks with little activity after the separation of any type of column chromatography. This could indicate that the active material consists of multiple components functioning synergistically.

**DISCUSSION**

Reconstitution of the Acid Secretory Response of Digitonin-permeabilized Glands with Brain Cytosol—It has been known that permeabilization of gastric glands with digitonin ruins...
meabilized (6) or β-escin-permeabilized (3) glands, where cAMP-stimulated acid secretion was not augmented, but rather inhibited, by Ca^{2+}. Myosin light chain kinase inhibitory peptide, SM-1, which potently inhibited cAMP-dependent acid secretion in β-escin-permeabilized glands (3), was also effective in the present model. This observation again supports our opinion that myosin light chain kinase-like, or SM-1-sensitive kinase, is involved in the activation step downstream of PKA. GTPγS, a non-hydrolyzable analogue of GTP, is known to be a potent inhibitor of acid secretion in glands permeabilized by a-toxin (6) or β-escin (3), possibly due to the disturbance of the normal cycle of the small GTP-binding protein. This compound also inhibited brain cytosol-stimulated acid secretion in the digitonin-permeabilized model, although the extent of inhibition was weaker than that observed in the β-escin model. This might reflect the leakage or the disturbance of the normal distribution of target GTP-binding proteins, which could not be supplemented by the addition of brain cytosol. From these observations, it could be concluded that acid secretion stimulated by brain cytosol in digitonin-permeabilized glands has a similar property to that observed in our previous model, β-escin-permeabilized glands, except that the activation occurs at a point downstream of PKA.

Identification of Stimulatory Factors in Brain Cytosol—Using the digitonin-permeabilized gland model as an assay system, we purified the active components in the brain cytosol and finally identified PTP as one of the activation factors of acid secretion. It would be needless to mention, but we cannot conclude that the active components in the brain are completely the same as that in the parietal cell. We started the purification of the assumption that there might be common substances, and in this consequence we have presently identified PTP and peak 2, which could explain the stimulatory activity present in the gastric mucosal cytosol, at least in part.

PTP's function in transporting phosphatidylinositol and phosphatidylcholine between intracellular membranes and subsequently regulate the activity of phospholipase C via the synthesis of PIP_{2} (19). Using the permeabilized cell model, this protein was also identified as the factor involved in the ATP-dependent priming step in chromaffin cell (9) as well as in the budding of the vesicles in PC12 cells (20). It has been clarified that phosphatidylinositolides play important roles in vesicular transport (21). It would be reasonable to postulate that PTPs play an important role in gastric acid secretion, since vesicular traffic is considered to be the crucial step in the activation of parietal cell (1, 22). In the chromaffin cell, PTP is essential for the priming step by ATP, but it does not directly regulate its Ca^{2+}-dependent secretion step. In the present digitonin-permeabilized glands, the effect of PTP was independent of cAMP. This might indicate that PTP is not the regulatory switch of acid secretion, but is indeed an essential component of the vesicular transport system common to all the secretory cell types. It is necessary in future study to elucidate the possible involvement of PTP in the intracellular membrane traffic.

Interestingly, our recent work (23) revealed that the K^{+}-per-

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**TABLE II**

**Characteristics of peak 2 activity**

| Treatment | Activity |
|-----------|----------|
| Gel filtration (Superdex 200) | Apparently 1.8 kDa |
| 15-min boil | Resistant |
| 0.05% chymotrypsin (30 °C, 1 h) | Resistant |
| 0.05% carboxypeptidase (30 °C, 1 h) | Resistant |
| MeOH:CHCl3 extraction | Recovered in aqueous phase |
| ODS reverse phase column (pH 2.6) | Recovered in the flow through fraction |

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![Graph A](image1.png)

![Graph B](image2.png)

**FIG. 8.** Effects of stimulation of gastric glands on their contents of stimulatory and inhibitory activities in the cytosol. Resting (incubated with 100 μM cimetidine for 30 min) or stimulated (100 μM histamine plus 50 μM isobutylmethylxanthine) rabbit isolated gastric glands were homogenized to prepare cytosol, which was separated on a Sepharose CL4B pre-equilibrated with the assay buffer. A, the fraction near 30 kDa was tested for stimulating activity on aminopyrine accumulation in digitonin-permeabilized glands and expressed as the percentage of maximum obtained by crude brain cytosol (1.5 mg/ml). B, the fraction near 200 kDa was tested for inhibitory activity on aminopyrine accumulation stimulated by crude brain cytosol (1.5 mg/ml) in digitonin-permeabilized glands and expressed as percentage of inhibition. Mean ± S.E. of four pairs of experiments are shown. The difference between resting and stimulated group in B was statistically significant (Student’s t test, p < 0.05).

Their responsiveness to any secretagogues, although their ability to maintain the proton gradient preformed in the luminal space was preserved (6). This phenomenon has been attributed to the leakage of the proteins essential for the activation process of acid secretion. Taking advantage of this, one might postulate that these essential proteins could be identified by “reconstitution” of secretion. In fact, several proteins involved in catecholamine secretion in the chromaffin cell have been identified employing this strategy (9–12). However, no such success has been reported in the case of parietal cell. We have started the purification of the assumption that there might be common substances, and in this consequence we have presently identified PTP and peak 2, which could explain the stimulatory activity present in the gastric mucosal cytosol, at least in part.

PITPs function in transporting phosphatidylinositol and phosphatidylcholine between intracellular membranes and subsequently regulate the activity of phospholipase C via the synthesis of PIP_{2} (19). Using the permeabilized cell model, this protein was also identified as the factor involved in the ATP-dependent priming step in chromaffin cell (9) as well as in the budding of the vesicles in PC12 cells (20). It has been clarified that phosphatidylinositolides play important roles in vesicular transport (21). It would be reasonable to postulate that PTPs play an important role in gastric acid secretion, since vesicular traffic is considered to be the crucial step in the activation of parietal cell (1, 22). In the chromaffin cell, PTP is essential for the priming step by ATP, but it does not directly regulate its Ca^{2+}-dependent secretion step. In the present digitonin-permeabilized glands, the effect of PTP was independent of cAMP. This might indicate that PTP is not the regulatory switch of acid secretion, but is indeed an essential component of the vesicular transport system common to all the secretory cell types. It is necessary in future study to elucidate the possible involvement of PTP in the intracellular membrane traffic.

Interestingly, our recent work (23) revealed that the K^{+}-per-
meability, essential for the activity of H⁺,K⁺-ATPase on the apical membrane of parietal cell, was dependent on PIP₂. This observation indicates the critical role of phosphoinositides in acid secretion and supports the potential involvement of PITP in parietal cell function.

**Peak 2, the Low Molecular Weight Active Component**—By using separation with a Superdex 200 column, it was revealed that the active component in brain cytosol contained a factor with apparent molecular mass of 1.8 kDa (peak 2). As peak 2 was resistant to treatments with proteases and heat, it seems not to be peptide. Following our observation that peak 2 potently augmented the PITP-stimulated acid secretion, we then examined the possibility that peak 2 was either PI or PIP₂, but neither was the case. In general, PITP does not require exogenous PI in the cell level since PI is synthesized within endoplasmic reticulum, and PITP is able to transport it to the target membrane (17).

We have tried to purify peak 2, but to no avail. Looking at its physical properties, it appears to be similar to phosphoinositides, although any of derivatives presently available fail to mimic the effect of peak 2. Further work is obviously necessary to identify this interesting factor.

**Inhibitory Factor in the Gastric Glands**—In the present study, we revealed that gastric mucosal cytosol contains an acid-stimulatory factor similar to that in brain cytosol in the low molecular weight range, and it also contains an acid-inhibitory factor in the high molecular weight range. This indicates that gastric acid secretion may be regulated by a balance between these positive and negative factors. Walent et al. (2) found an inhibitory factor of catecholamine secretion in the flow-through fraction of a Matrix Green column when they purified CAPS (calcium-dependent activator protein for secretion), which activates catecholamine secretion in chromaffin cell. Similar factors might exist in various secretory cell types.

Based on the fact that the stimulatory factor is independent of cAMP, it might be possible that the inhibitory factor, and not the stimulatory factor, is involved in the switching of activation. To examine this hypothesis, we compared the activity of these factors in resting and stimulated gastric glands, and we found that the inhibitory activity in the cytosol was markedly increased by stimulation, whereas the stimulatory activity was not. This result was somewhat puzzling because it appeared that stimulation increased the inhibitory activity. However, it would be reasonable to interpret this in such a way that the inhibitory factor, which exists and inhibits acid secretion on the membrane of parietal cell, translocates to the cytosol in association with stimulation and subsequently the inhibition is canceled. In fact, it has been reported that Rab 3 translocates with stimulation from the membrane fraction to cytosol in the neuronal cell (24), and Rab 3 on the membrane inhibits catecholamine secretion in the resting stage (25). Another example is syncollin, which has been identified as a Ca²⁺-sensitive inhibitory factor in the fusion process of rat pancreatic zymogen granule (26). Recombinant syncollin inhibits the fusion of zymogen granule by binding to syntaxin. When it dissociates from syntaxin with addition of Ca²⁺, the inhibition of the fusion is released. In case of rat parotid gland, where CAMP-dependent secretion occurs, there exists a protein factor, which dissociates from VAMP2 (vesicle associated membrane protein 2) depending on activation by PKA (27). In analogy, it would be possible in the parietal cell that inhibitory factor(s), regulated by PKA, can be translocated from the membrane to cytosol during the activation process.

In conclusion, we established a new system useful for assaying essential factors of regulation of gastric acid secretion with reconstituted digitonin-permeabilized rabbit gastric glands. Using this system, we identified PITP as an activation factor of acid secretion. Furthermore, we found an inhibitory component in gastric cytosol and postulated a hypothesis that the acid secretion was regulated via this inhibitory component. The present system is considered to be a powerful tool to identify unknown factors and to analyze their mode of action in the regulation of gastric acid secretion.

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