A proposed mechanism for the potentiation of cAMP-mediated acid secretion by carbachol

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Muto, Yuko, Taku Nagao, Maki Yamada, Katsuhiro Mikoshiba, and Tetsuro Urushidani. A proposed mechanism for the potentiation of cAMP-mediated acid secretion by carbachol. Am J Physiol Cell Physiol 280: C155–C165, 2001.—Acid secretion in isolated rabbit gastric glands was monitored by the accumulation of [14C]aminopyrine. Stimulation of the glands with carbachol synergistically augmented the response to dibutyryl cAMP. The augmentation persisted even after carbachol was washed out and was resistant to chelated extracellular Ca2+ and to inhibitors of either protein kinase C or calmodulin kinase II. Cytochalasin D at 10 μM preferentially blocked the secretory effect of carbachol and its synergism with cAMP, whereas it had no effect on histamine- or cAMP-stimulated acid secretion within 15 min. Cytochalasin D inhibited the carbachol-stimulated intracellular Ca2+ concentration ([Ca2+]i) increase due to release from the Ca2+ store. Treatment of the glands with cytochalasin D redistributed type 3 inositol 1,4,5-trisphosphate receptor (the major subtype in the parietal cell) from the fraction containing membranes of large size to the microsomal fraction, suggesting a dissociation of the store from the plasma membrane. These findings suggest that intracellular Ca2+ release by cholinergic stimulation is critical for determining synergism with cAMP in parietal cell activation and that functional coupling between the Ca2+ store and the receptor is maintained by actin microfilaments.

inositol 1,4,5-trisphosphate receptor; cytoskeleton; cytochalasin D; calcium; parietal cell; rabbit

IN THE PROCESS OF ACTIVATION of gastric acid secretion, two main pathways exist, i.e., the stimulation of adenylate cyclase via the histamine H2 receptor and the activation of phospholipase C via the muscarinic M3 and cholecystokinin-B (CCK-B) receptors. Intracellular signaling from these pathways is thought to cause synergistic interactions (for review, see Ref. 25). As for the second messengers, it is postulated that a synergistic interaction occurs between cAMP and Ca2+. In rabbit parietal cell, histamine elicits two signals at once, i.e., it increases cAMP as well as intracellular Ca2+ concentration ([Ca2+]i), and synergism occurs in the cell with the agonist alone. This could explain why the synergism between histamine and carbachol is relatively weak (5, 12, 15), whereas a marked synergism is observed between N6,2′-O-dibutyryl cAMP (DBcAMP) and carbachol (1, 15) in this species. In the case of canine parietal cells, in which histamine fails to cause [Ca2+]i elevation (6), the synergism between histamine and carbachol is quite obvious (17). At present, the source as well as the target of Ca2+ utilized for the potentiation is still unclear.

[Ca2+]i increase via M3 and CCK-B receptors is elicited by inositol 1,4,5-trisphosphate (IP3), which is the product of phospholipase C. IP3 has been shown to have three types of specific receptors, type 1, 2, and 3 IP3 receptors, which share 60–70% identity in amino acid sequences with each other (9, 28). Founded on many lines of evidence, all of these are thought to form calcium channels through membranes of intracellular calcium stores and work to raise [Ca2+]i. The intracellular Ca2+ release by IP3 receptor has been shown to be essential for the Ca2+ influx through plasma membranes of B cells (19), and this can hold true for other cell types, including parietal cells (14).

Recently, it was postulated that the intracellular Ca2+ store is not only functionally but also physically coupled with the receptor (23) and that cytoskeletal components are involved in the connection (16). In the present study, it was revealed that release of intracellular Ca2+ plays an important role in the potentiating effect of carbachol on DBcAMP-stimulated acid secretion and that cytochalasin D, the microfilament-disrupting agent, works as an effective inhibitor of synergism by interfering with the functional connection between the muscarinic receptor and the Ca2+ store.

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MATERIALS AND METHODS

Isolation of rabbit gastric glands, measurement of [14C]-aminopyrine accumulation, and [Ca2+]i mobilization. Isolated gastric glands were prepared from Japanese White rabbits (Shiraishi, Tokyo, Japan) by a combination of high-pressure perfusion and collagenase digestion (2). Acid secretion of the glands was monitored by the accumulation of a weak base, [14C]aminopyrine (2). A 1-ml aliquot of isolated glands, suspended in 20× volume of medium, was stimulated in a 1.5-ml Eppendorf tube, and each data point is the mean of the duplicate measurements. Usually, resting control was included every 18 tubes (9 treatments), and the effect of the stimulants was expressed as the aminopyrine ratio above the resting value. The same treatment was never repeated for the same gland preparation from each rabbit. Therefore, the number of experiments (n) appearing in the present study also means the number of rabbits used. Drugs were all dissolved in DMSO so that the final concentration of the drug was always included except for the case of stimulation by endogenous histamine, 100 μM fura 2, 10 μM omeprazole, and 5 μg/ml oligomycin. The sample was excited with dual wavelength, and the fluorescence ratio (F340/F380) at 510-nm emission was recorded by using an intracellular Ca2+ analyzer (CAF-110; JASCO, Tokyo, Japan). At the beginning of the experiment, 2.5 μM of CaCl2 was added, and the decrease of extravesicular Ca2+ concentration was monitored by measuring the Ca2+ uptake by the vesicles. When the ratio approached a constant value, test drugs were added to the cuvette.

Cell staining and immunological analysis. For immunostaining, isolated gastric glands were fixed with 10% Formalin and permeabilized with 0.5% Triton X-100. The glands were incubated with anti-type 1 (18A10, rat monoclonal) or anti-type 3 (KM1082, mouse monoclonal) IP3 receptor antibody (8), anti-H-ATPase α-subunit mouse monoclonal antibody (26), or anti-H-ATPase α-subunit polyclonal antibody (raised in the rat in the present study by using SDS-PAGE-purified rabbit α-subunit as an antigen) at 4°C for 18 h. The glands were washed and then incubated with FITC-anti-rat or -anti-mouse IgG (Sigma) at 4°C for 18 h. F-actin was made visible by staining with FITC-phalloidin (20 μM; Molecular Probes). For double staining (types 1 and 3 or IP3 receptor and H-ATPase), the mouse or rat IgG was visualized by Cy3-anti-mouse IgG, FITC-anti-mouse IgG, tetramethylrhodamine isothiocyanate (TRITC)-anti-rat IgG, or FITC-anti-rat IgG (Sigma). The glands were then examined by microscope (Nikon Eclipse TE300) with a ×60 water-immersion objective (MTB Plan Apo 60×WI) by using a confocal laser scanning system (μRadiance; Bio-Rad). FITC was excited at 488 nm (Argon Ion Laser) and detected with a HQ515/30 filter, and Cy3 and TRITC were excited at 543 nm (Green HeNe Laser) and detected with E570LP. For double staining, a sequential acquisition mode was employed to avoid “bleed through” of the staining. The fractions of gastric glands were separated by 6% SDS-PAGE, blotted on a polyvinylidene difluoride membrane, and then incubated with anti-type 3 IP3 receptor antibody or anti H-ATPase α-subunit antibody. The membrane was visualized by chemiluminescence (Renaissance Western Blot Chemiluminescence Reagent; NEN) for IP3 receptor or by 3,3′-diaminobenzidine for H-ATPase with the use of horseradish peroxidase-anti-mouse or -anti-rabbit IgG as a second antibody. For quantification, the chemiluminescent membranes were exposed to films for at least three different lengths of time and scanned by an Epson GT-8000, and the film darkening of the band was quantified by a computer program, NIH Image 1.61. The values of each band with different exposure time were plotted, and the relative density was expressed as the ratio to the control value.

Statistical analysis. Parametric data are expressed as means ± SE. Multiple comparisons were analyzed by ANOVA and Dunnet’s post hoc test with the use of a computer program (Super ANOVA; Abacus Concepts, Berkeley, CA).
CA). The level of significance was uniformly set at \( P = 0.05 \), and no further calculation of \( P \) value was performed.

**RESULTS**

Potentiating interaction between carbachol and DB-cAMP in aminopyrine accumulation. Isolated rabbit gastric glands were stimulated with 100 \( \mu \text{M} \) CCh, 100 \( \mu \text{M} \) DBcAMP, or their combination, and the aminopyrine ratios were measured 5, 10, and 30 min after stimulation (open symbols). The glands were also stimulated with CCh alone for the first 20 min and then DBcAMP was added, or they were stimulated with DBcAMP alone for the first 20 min and then CCh was added, and the aminopyrine ratios were measured at 30 min (filled symbols). The aminopyrine ratio above resting control (10.0 ± 1.3; mean ± SE, \( n = 4 \)) was calculated and expressed as the mean ± SE of 4 independent experiments performed in duplicate.

To confirm that the potentiating interaction occurred via muscarine receptor, we examined the effects of atropine (1 \( \mu \text{M} \)) on DBcAMP plus carbachol-stimulated aminopyrine accumulation. Summarizing the data for four separate experiments (mean ± SE), stimulation by 100 \( \mu \text{M} \) DBcAMP alone for 30 min gave a ratio of 23.8 ± 0.6 above the resting value, and stimulation by DBcAMP plus carbachol (100 \( \mu \text{M} \) each) showed a ratio of 67.0 ± 13.2 above the resting value. When 1 \( \mu \text{M} \) atropine was included at the beginning of the stimulation by DBcAMP plus carbachol, the aminopyrine ratio was reduced to the same level as that by DBcAMP alone, 21.8 ± 2.0 (\( P < 0.05 \) vs. without atropine). Interestingly, when 1 \( \mu \text{M} \) atropine was added 5 min after the stimulation started, the aminopyrine ratio was 62.5 ± 10.0 above resting; namely, no inhibition was observed.

From the experiments of time course and the effect of atropine, the response to carbachol was determined to be prompt. We then examined whether the augmented response to DBcAMP was maintained even after carbachol was washed out shortly after stimulation. Figure 2 shows the results of this type of experiment. In experiment 1, the glands were incubated with vehicle for 5 min at 37°C and washed three times at room temperature. It was confirmed that this procedure ef-

![Fig. 1. Potentiating interaction between carbachol (CCh) and N\(^{6},2\)-O-dibutyryl cAMP (DBcAMP) in aminopyrine accumulation. Isolated rabbit gastric glands were stimulated with 100 \( \mu \text{M} \) CCh, 100 \( \mu \text{M} \) DBcAMP, or their combination, and the aminopyrine ratios were measured 5, 10, and 30 min after stimulation (open symbols). The glands were also stimulated with CCh alone for the first 20 min and then DBcAMP was added, or they were stimulated with DBcAMP alone for the first 20 min and then CCh was added, and the aminopyrine ratios were measured at 30 min (filled symbols). The aminopyrine ratio above resting control (10.0 ± 1.3; mean ± SE, \( n = 4 \)) was calculated and expressed as the mean ± SE of 4 independent experiments performed in duplicate.](http://ajpcell.physiology.org/)

![Fig. 2. “Priming effect” of CCh on the aminopyrine accumulation in rabbit isolated gastric glands. Isolated rabbit gastric glands were treated for 5 min at 37°C with 100 \( \mu \text{M} \) CCh or indicated agents (pretreatment). The glands were then washed 3 times at room temperature (this procedure took ~20 min) and then stimulated for 30 min at 37°C with 100 \( \mu \text{M} \) DBcAMP or DBcAMP plus 100 \( \mu \text{M} \) CCh as indicated (stimulants). Aminopyrine ratios above resting control were calculated and expressed as a percentage of the control value (obtained from experiment 1). Atr, atropine sulfate; OPZ, omeprazole. Values are means ± SE of 4 independent experiments performed in duplicate. *\( P < 0.05 \) vs. experiment 3.](http://ajpcell.physiology.org/)

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**Table:**

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<th>Pretreatment</th>
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<td>CCh + EGTA 2( \mu \text{M} )</td>
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When carbachol was added 20 min after stimulation with DBcAMP alone, the final value (30 min after the beginning of stimulation) was observed to be about the same as that for the combination of the two agonists from the beginning. However, when DBcAMP was added 20 min after the stimulation with carbachol, the final value (at 30 min) was much less than that obtained by combined stimulation from the beginning (Fig. 1), suggesting that the potentiating effect of carbachol disappears after 20 min at 37°C.
effectively eliminated the effects of several secretagogues and inhibitors (data not shown). The glands were then stimulated for 30 min at 37°C, and the aminopyrine ratio above the resting control value was taken as 100%. In experiment 2, the glands were stimulated with 100 μM carbachol plus 100 μM DBcAMP for 5 min, washed three times, and stimulated again with carbachol plus DBcAMP for 30 min. The aminopyrine ratio in experiment 2 was ~200% of the control value in experiment 1, confirming the potentiating interaction between these agonists. When the glands were treated with 100 μM carbachol for 5 min, washed out, and stimulated with 100 μM DBcAMP alone for 30 min, the aminopyrine ratio was also ~200% of control, showing that the potentiation by carbachol on DBcAMP-stimulated secretion was still active after the washing out (experiment 3). We call this the “priming effect” of carbachol.

The priming effect of carbachol disappeared in the presence of atropine (experiment 4). Omeprazole, included in the first 5 min, did not change the priming effect, indicating that actual acid secretion is not necessary for establishment of priming (experiment 5). To examine the role of Ca²⁺, 5-min stimulation by carbachol was performed in medium containing 2 mM EGTA instead of Ca²⁺, and the potentiation was the same as in the normal medium (experiment 6), suggesting that extracellular Ca²⁺ is not necessary for the priming effect.

Figure 3 shows the effect of various treatments on stimulation with a combination of DBcAMP and carbachol (100 μM each). Even when extracellular Ca²⁺ was eliminated by EGTA throughout stimulation, the potentiating effect was not attenuated. This effect was in contrast to the transient effect of carbachol alone, which was abolished by the elimination of extracellular Ca²⁺ (1, 15). Next, 10 μM bisindolylmaleimide I, a protein kinase C inhibitor, was applied, but no significant effect was observed. We already reported that this concentration of the compound did not affect stimulation by DBcAMP alone (20) but inhibited the stimulatory effect of phorbol ester (1). Therefore, we conclude that the involvement of protein kinase C is minimal in the potentiating interaction between DBcAMP and carbachol. We also checked a calmodulin kinase II inhibitor, KN-62, at 60 μM, which was enough to inhibit the stimulatory effect of carbachol alone (22) but produced no inhibitory effect (Fig. 3). We then screened various drugs to identify specific agents affecting the potentiating interaction and found that cytochalasin D, an actin-depolymerizing agent, abolished the stimulatory effect of DBcAMP plus carbachol (Fig. 3).

Figure 4 shows the effects of cytochalasin D on the acid secretion stimulated by DBcAMP, carbachol, and histamine. When agonist stimulation was performed for 15 min (Fig. 4A), cytochalasin D selectively inhibited carbachol- or carbachol plus DBcAMP-stimulated aminopyrine accumulation, whereas it showed no effect on the stimulation by DBcAMP or histamine. At the point of 30 min of stimulation (Fig. 4B), cytochalasin D tended to inhibit DBcAMP- and histamine-in-
duced aminopyrine accumulation to 60–70% of control, consistent with the report by Forte et al. (7), although these effects were not statistically significant in the present study.

$[\text{Ca}^{2+}]_{i}$ mobilization in the parietal cells. From the experiments under the $\text{Ca}^{2+}$-free condition, it was suggested that release of intracellular $\text{Ca}^{2+}$ was involved in the potentiating interaction between DBCAMP and carbachol. We thus examined intracellular $\text{Ca}^{2+}$ mobilization. As widely observed (6, 13, 15), carbachol elicited a biphasic increase in $[\text{Ca}^{2+}]_{i}$, indicated as a single sharp peak followed by a sustained plateau. As shown in Fig. 5A, cytochalasin D at 10 $\mu$M strongly inhibited the response to carbachol. Dose-response curves were obtained by quantification of the first peak of $\text{Ca}^{2+}$ rise (Fig. 5B), and the increase in $[\text{Ca}^{2+}]_{i}$ elicited by carbachol was demonstrated to be sensitive to cytochalasin D.

It was practically impossible to distinguish whether inhibition occurred on release from the store or on $\text{Ca}^{2+}$ influx on the basis of the shape of the curve. To distinguish these cases, carbachol was added in the $\text{Ca}^{2+}$-free condition. As shown in Fig. 6A, addition of carbachol in the $\text{Ca}^{2+}$-free condition caused a transient $[\text{Ca}^{2+}]_{i}$ rise without plateau phase, although the size of this rise was somewhat smaller than that in normal conditions. When this experiment was performed in the presence of cytochalasin D, the $\text{Ca}^{2+}$ rise was almost abolished. The inhibitory effect of cytochalasin D is evident from the summarized data shown in Fig. 6B. This finding suggested that the site of action of cytochalasin D was the release of $\text{Ca}^{2+}$ from the intracellular store.

To examine whether the inhibitory effect of cytochalasin D on intracellular $\text{Ca}^{2+}$ release was due to its effect on IP$_3$ receptor in the $\text{Ca}^{2+}$-store membrane, we checked the direct effect of cytochalasin D on the $\text{Ca}^{2+}$ store in vitro. Figure 7 shows typical results. IP$_3$ was added to the $\text{Ca}^{2+}$ store isolated from rabbit gastric mucosa, and dose-dependent release of $\text{Ca}^{2+}$ was observed. This effect of IP$_3$ was not affected by 10 $\mu$M cytochalasin D, suggesting that inhibition of cytochalasin D was not on the IP$_3$ receptor.

F-actin staining in the isolated gastric glands. In the previous experiments, cytochalasin D selectively affected some of the physiological functions in the rabbit gastric glands. We then examined the intracellular alignment of actin filaments under several conditions. Figure 8 depicts the observation by a confocal microscope of F-actin staining with FITC-phalloidin in gastric glands. In the resting control glands (Fig. 8A),
phalloidin made visible the fine intracellular canalicu-
lar structure and lining of the basolateral membranes
in the parietal cells. In the glands pretreated with 10
mM cytochalasin D for 10 min at room temperature and
further incubated with carbachol for 5 min at 37°C
(corresponding to the time when Ca\textsuperscript{2+} transient is
observed), the alignment of F-actin was still similar to
that of resting control, although a little decrease in the
staining intensity was noted (Fig. 8B). When the incu-
bation time at 37°C was prolonged to 15 min, fading of
the intracellular structure occurred in some parietal
cells, possibly reflecting the depolymerization of actin
filaments supporting the microvilli of the apical sur-
face (Fig. 8C). After the treatment with cytochalasin D
for 30 min at 37°C, marked disturbance of actin fila-
ment occurred and no structures corresponding to in-
tracellular canaliculi were visible (Fig. 8D). It is note-
worthy that some physiological function, e.g.,
aminopyrine accumulation stimulated by histamine or
DBcAMP, was little affected even in the condition
shown in Fig. 8, C or D.

Localization of IP\textsubscript{3} receptors. It is reasonable to sup-
pose that the inhibitory effect of cytochalasin D on
Ca\textsuperscript{2+} release was due to the depolymerization of actin.
One might expect that there would be a functional
coupling between Ca\textsuperscript{2+} store and drug receptor on the
plasma membrane and that their connection would be
maintained by actin filaments. Although it was re-
ported that both type 1 and 3 receptors of IP\textsubscript{3} are
present and enriched in the murine gastric parietal cell
(11), it was unclear whether this was also the case in
rabbit. We thus stained rabbit gastric glands with
specific antibodies against type 1 and 3 IP\textsubscript{3} receptors.
In the immunohistochemistry analysis performed
using the antibodies, it was revealed that type 3 IP\textsubscript{3}
receptor was relatively rich in parietal cells, as was
reported for murine gastric mucosa (11), whereas type
1 IP\textsubscript{3} receptor was enriched in chief cells within rabbit
gastric gland. Figure 9A shows the staining of type 1
IP\textsubscript{3} receptor in red (TRITC), and Fig. 9B shows H\textsuperscript{+}-K\textsuperscript{+}-
ATPase stained in green (FITC). It is obvious from the
merged image (Fig. 9C) that type 1 IP\textsubscript{3} receptor is

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Fig. 7. Effect of cytoD on Ca\textsuperscript{2+} release from the isolated Ca\textsuperscript{2+} store
by inositol 1,4,5-trisphosphate (IP\textsubscript{3}). Ca\textsuperscript{2+} store isolated from gastric
mucosa was transferred to a cuvette containing 2 mM MgATP, 10
mM creatine phosphate, 5 U/ml creatine kinase, 1.2 \mu M fura 2, 10
\mu M omeprazole, and 5 \mu g/ml oligomycin. The cuvette was excited
with dual wavelength, and the fluorescence ratio (F\textsubscript{340}/F\textsubscript{360}) at
510-nm emission was recorded. The store was loaded with 2.5 \mu M of
CaCl\textsubscript{2}, increasing doses of IP\textsubscript{3} were added as indicated by boxes, and
a dose-dependent Ca\textsuperscript{2+} release was observed. This release was un-
affected by 10 \mu M cytoD. Data are representative of at least 3
experiments with similar results.

Fig. 8. F-actin staining by FITC-phalloidin in isolated gastric glands. Rabbit
gastric glands, incubated with 100 \mu M cimetidine for 10 min at room tempera-
ture and 15 min at 37°C (A), with 10 \mu M cytoD for 10 min at room temperature
and stimulated by 100 \mu M CCh for 5
min (B), 15 min. (C), and 30 min (D) at
37°C, were fixed with Formalin, permea-
bilized with Triton X-100, and in-
ubated with FITC-phalloidin (20 U/ml).
Bars: 10 \mu m.
much more enriched in the small, H\(^+\)-K\(^+\)-ATPase-negative cells (possibly chief cells) than in the larger, H\(^+\)-K\(^+\)-ATPase-positive parietal cells. Within the parietal cell, type 1 IP\(_3\) receptor diffusely distributed to the cytosol without any definite structures. In contrast, type 3 IP\(_3\) receptor is enriched in the cells with large size (Fig. 9D), which could be identified as parietal cell by the positive staining with anti-H\(^+\)-K\(^+\)-ATPase (Fig. 9E) and their merged images (Fig. 9F). Although we were interested in the difference in the intracellular distribution of these proteins, the space resolution was not enough in the present system. This point is clarified in the latter part of the present work.

To estimate nonspecific staining, isolated glands were treated with 50 \(\mu\)g/ml normal rat IgG or 10 \(\mu\)g/ml normal mouse IgG followed by FITC-anti-rat, FITC-anti-mouse, TRITC-anti-rat, and Cy3-anti-mouse as second antibody. Essentially no signal was detected when TRITC- or Cy3-labeled antibody was used and excited at 543 nm (Green HeNe Laser). Although very faint fluorescence was noted in FITC-labeled second antibody was excited at 488 nm (Argon Ion Laser), its intensity was low enough to detect the specific staining under the same condition for the observation. This fluorescence did not appear to be background staining but, rather, autofluorescence, possibly due to the mitochondria in the parietal cell, because it was also observed in the glands without any antibody treatments (data not shown).

It was quite interesting to test whether cytochalasin D affects the intracellular distribution of the IP\(_3\) receptor, especially type 3. However, treatment with 100 \(\mu\)M carbachol and 10 \(\mu\)M cytochalasin D did not cause any detectable changes in the distribution of both types of IP\(_3\) receptor from observation with an optical microscope (data not shown).

We then tried to detect the effect of cytochalasin D on the distribution of the IP\(_3\) receptor by using a biochemical technique. Isolated gastric glands were homogenized and fractionated into pellets P1 (4,000 \(g\), for 10 min), P2 (14,500 \(g\), for 10 min), and P3 (100,000 \(g\), for 45 min) and supernatant. In preliminary Western blotting, type 3 IP\(_3\) receptor was absent in the supernatant and detectable in P2, but the majority of the protein was found in P3. It was hard to detect in P1. Because the plasma membrane was expected to be harvested in the low-speed pellet, P1, this fraction was suspended in 18% Ficoll and centrifuged (100,000 \(g\), for 120 min) to obtain floating membranes. It was then found that type 3 IP\(_3\) receptor became detectable. The microsomal fraction, P3, was further layered on 27% sucrose and centrifuged (100,000 \(g\), for 120 min) to obtain light (top of
the sucrose layer) and heavy (pellet) microsomes. We designated the microsomal fraction as intracellular vesicles and the 18% Ficoll fraction as the plasma membrane. Figure 10 depicts the results of immunoblotting of these fractions. In the control glands, type 3 IP$_3$ receptor was found in the plasma membrane fraction as well as in the microsomes (Fig. 10A). Within the microsomal fraction, the IP$_3$ receptor distributed not to the light but to the heavy membranes (Fig. 10A), whereas the amount of H$^+\cdot$K$^+$-ATPase was inverse, i.e., it was rich in the light and scarce in the heavy microsomes (Fig. 10B). This suggests that the Ca$^{2+}$ store membranes containing IP$_3$ receptors are different from tubulovesicles containing proton pump. When the glands were treated with carbachol plus cytochalasin D, the content of type 3 IP$_3$ receptor in the plasma membrane fraction decreased and redistributed to the heavy microsomes in the microsomal fraction (Fig. 10B). The relative density value for type 3 IP$_3$ receptor in the plasma membrane fraction from cytochalasin D-treated glands was significantly decreased to 53.6 $\pm$ 8.3% of control (mean $\pm$ SE, n = 3; $P < 0.05$), whereas that in the heavy microsomal fraction was significantly increased to 140.0 $\pm$ 6.4% of control (mean $\pm$ SE, n = 3; $P < 0.05$). These changes were not due to a simple breakdown of the structure of intracellular membranes because the content of H$^+\cdot$K$^+$-ATPase in the microsomes or the plasma membrane fraction was not affected by this treatment (Fig. 10B). Similar changes were observed in glands treated with cytochalasin D alone but not with carbachol alone (data not shown).

Type 1 IP$_3$ receptor was barely detectable in both the heavy microsomes and the plasma membrane fraction but was dispersed much more toward the heavy microsomes than was the case for type 3 IP$_3$ receptor. We did not perform further experiments because this type of IP$_3$ receptor is enriched in chief cells rather than parietal cells, as shown in Fig. 9.

**DISCUSSION**

It is well known that a potentiating interaction occurs among histamine, acetylcholine, and gastrin in the activation of parietal cells (17). The potentiation is thought not to be in the area of the drug receptors but in the intracellular signal transduction, although the precise sites are presently unknown. The consensus is that activation of histamine H$_2$ receptor leads to the elevation of intracellular cAMP, and the activation of either acetylcholine or gastrin receptor leads to an increase of intracellular Ca$^{2+}$ induced by the elevation of IP$_3$, as well as activation of protein kinase C. Theoretically, the potentiation should occur between cAMP-dependent protein kinase and Ca$^{2+}$ or between cAMP-dependent protein kinase and protein kinase C. However, there have been many contradictory results so far (for review, see Ref. 25).

Li et al. (12) claim that the augmentation of the cAMP pathway by carbachol or gastrin is due to the elevation of [Ca$^{2+}$]. They showed that secretion stimulated by DBCAMP in the isolated rat parietal cell was augmented by both carbachol and gastrin and that those augmentations were inhibited by the intracellular Ca$^{2+}$ chelator, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid. They also observed that the augmentation by carbachol and gastrin was surrogated by a Ca$^{2+}$ ionophore, A-23187. They considered the effect of Ca$^{2+}$ as the essential component for the morphological changes that are characteristic of the stimulated parietal cell. To support this hypothesis, they showed that carbachol and gastrin caused morphological changes in the parietal cell without large increase in the aminopyrine accumulation, while the addition of DBCAMP increased the accumulation. However, in the rabbit isolated glands, in which normal morphology is better preserved than in isolated cells, only poor morphological changes could be observed in response to
cholinergic stimulation (24). Furthermore, there is little knowledge about the source of Ca$^{2+}$ and the molecular entity responsible for the potentiating interaction.

In the present study, we confirmed that carbachol augmented acid secretion stimulated by DBcAMP in rabbit gastric glands. This effect of carbachol occurred within a short time (5 min) and persisted even after the carbachol was washed out at the room temperature, but it disappeared at 37°C within 20 min. This means that once a parietal cell gets an episode of cholinergic stimulation, it shifts to a condition sensitive to stimulation by DBcAMP for a short period of time and then returns to the preprimed state by a metabolism-dependent pathway. This “priming” effect of carbachol does not involve endogenous histamine but is the result of direct activation of the muscarinic receptor on the parietal cell. Removal of extracellular Ca$^{2+}$ affected neither the priming effect of carbachol nor the augmented secretion by carbachol plus DBcAMP. This strongly suggests that the Ca$^{2+}$ influx is not involved in the potentiating effect of carbachol. Therefore, we deduced that activation of protein kinase C and release of intracellular Ca$^{2+}$ by IP$_3$ were the remaining possibilities.

The dominant opinion concerning the role of protein kinase C in the acid secretion is that it is inhibitory (25). In certain cases, however, protein kinase C is able to work as an accelerator for acid secretion. For example, when intracellular cAMP is elevated (1, 4), or when extracellular K$^+$ is elevated (1), phorbol esters apparently increase aminopyrine accumulation in rabbit gastric glands, effects that are suppressed by protein kinase C inhibitors. This means that protein kinase C could also be a candidate for the mediator for potentiation. We screened several agents that inhibited potentiation by DBcAMP. This “priming” effect of carbachol involves the release of intracellular Ca$^{2+}$ and not protein kinase C.

The importance of the role of intracellular Ca$^{2+}$ release was also supported by another significant fact. We screened several agents that inhibited potentiation between cAMP and carbachol and finally found that cytochalasin D fit this purpose. The inhibitory effect of cytochalasin D has been considered to operate by disrupting the microvilli, which are essential for acid secretion (7). Cytochalasin D tended to inhibit both histamine- and DBcAMP-stimulated acid secretion by ~40% at the end of stimulation for 30 min, whereas inhibition was negligible at 15 min of stimulation. In the case of secretion stimulated by carbachol alone (15 min of stimulation) and carbachol plus DBcAMP (15 and 30 min of stimulation), the inhibition was almost 100%. This clearly indicated that the inhibitory effect of cytochalasin D was more specifically directed to the effects of carbachol alone and its augmentation on DBcAMP.

Because the action of intracellular Ca$^{2+}$ release was considered to be important in the stimulatory effect of carbachol, we examined the effect of cytochalasin D on [Ca$^{2+}$]$_i$ mobilization in parietal cells. It was clearly shown that cytochalasin D suppressed the increase in [Ca$^{2+}$]$_i$ by carbachol. On the basis of analysis of the Ca$^{2+}$-free condition, its inhibition was considered to be on the intracellular Ca$^{2+}$ release rather than Ca$^{2+}$ influx. Cytochalasin D did not change the effect of IP$_3$ on the isolated Ca$^{2+}$-stores from the gastric mucosa. Therefore, we conclude that the inhibitory effect of cytochalasin D on intracellular Ca$^{2+}$ release is indirect, possibly due to the uncoupling between the receptor and the effector. On the basis of these observations, we consider it reasonable to suppose that the M$_3$ receptor and the Ca$^{2+}$ store are functionally connected via actin microfilaments in the parietal cell, that depolymerization of F-actin by cytochalasin D disrupts this functional connection, and thus that it inhibits the potentiating interaction between carbachol and cAMP. Alternatively, cytochalasin D may block the putative actin-binding site on the M$_3$ and/or IP$_3$ receptors, considering the observation that uncoupling of M$_3$ receptor to Ca$^{2+}$ release occurred even when F-actin staining on the apical membrane of parietal cell was apparently not disturbed.

Tsunoda (21) reported that intracellular Ca$^{2+}$ release and acid secretion induced by gastrin were inhibited not only by microtubule- but also microfilament-disrupting agents. More recently, receptors producing IP$_3$ and Ca$^{2+}$ stores were shown to be physically and functionally coupled via cytoskeletal components in the fibroblast cell line NIH/3T3 (16). In their case, however, treatment with cytochalasin D was so intense that most of the microfilament in the cell was completely disrupted and many physiological functions might be distorted. In the present study, we were able to show that cytochalasin D selectively abolished the physiological function mediated by receptor-coupled intracellular Ca$^{2+}$ release when other functions, e.g., cAMP-mediated acid secretion, as well as the morphology of the cell were normal. This is thus the first example that Ca$^{2+}$ signaling involved in the physiological function is maintained by microfilaments in the normal cell.

We have no idea at present about the target for the released Ca$^{2+}$. Li et al. (12) discussed a possible involvement of calmodulin kinase II. In the present study, however, 60 μM KN-62, a calmodulin kinase II inhibitor, failed to inhibit the augmented secretion by DBcAMP plus carbachol, suggesting that the priming effect of carbachol mediated by intracellular Ca$^{2+}$ release does not involve this enzyme activation. Further work is clearly necessary to find out the target for Ca$^{2+}$ focusing on the calmodulin-independent pathway as well.

Recently, the subtypes of the IP$_3$ receptor in the gastric parietal cell were identified, and they were
mainly types 1 and 3, but not type 2 (11), in the murine stomach. It is interesting that there might be subtype-specific regulation in the acid secretion, e.g., type 1 has calmodulin-binding domain but type 3 does not (27). In the present study, staining of each receptor with the corresponding antibody revealed the characteristic distribution in rabbit gastric gland. In contrast to findings in the murine stomach, type 1 IP$_3$ receptor was scarce in rabbit parietal cell. Within the parietal cell, type 3 IP$_3$ receptor was diffusely distributed in the cytosol. Because IP$_3$ receptor is definitely membrane protein, the cytosolic staining reflects its presence in the intracellular membranes, which was confirmed by Western blotting showing that the type 3 IP$_3$ receptor was found not in the cytosol but in the microsomal fraction. The tubulovesicles of the parietal cell were harvested from the light microsomes, and the amount of H$^+\cdot$K$^+$-ATPase was inversely proportional to the density of the microsome, consistent with a previous report (10). In contrast, the type 3 IP$_3$ receptor was harvested from the heavy membranes in the microsome, suggesting that the membrane population of these two proteins is clearly different. In the endocrine or neuroendocrine cells, an interesting hypothesis has been proposed stating that the secretory granule itself has type 3 IP$_3$ receptors on its membrane and utilizes them for the exocytosis or membrane traffic (3). However, this type of regulation might not be working in the case of the parietal cell, because tubulovesicles do not appear to contain IP$_3$ receptors.

A considerable amount of type 3 IP$_3$ receptor was found in the plasma membrane fraction in addition to the heavy microsomal fraction. This suggests that the Ca$^{2+}$ store-containing type 3 IP$_3$ receptor either consists of both large and small membrane vesicles or consists of only small vesicles but has a physical connection to the large plasma membrane. After the cytochalasin D treatment, the content of the IP$_3$ receptor in the larger size membranes drastically decreased and that in the heavy microsomes increased, as judged by Western blotting. This suggests that type 3 IP$_3$ receptor resides not on the larger membranes but on the small vesicles, which are physically connected to the larger membrane, possibly via cytoskeletal components. This strongly supports our hypothesis mentioned above that cytochalasin D preferentially inhibits the priming effect of carbachol by disrupting the physical and functional connection between M$_3$ receptor and Ca$^{2+}$ store via actin filaments. We could not find any detectable changes in the immunostaining of type 3 IP$_3$ receptor in the cytochalasin D-treated cells. This seems to be a limitation of observation by a light microscope, and study using an electron microscope is necessary in future work.

As for the physical connection between M$_3$ receptor and IP$_3$ receptor, the present data only show that the connection was maintained by the actin microfilament. It was reported that group 1 metabotropic glutamate receptor and IP$_3$ receptor coimmunoprecipitated as a complex with Homer protein. It was also shown that the Ca$^{2+}$ response via the glutamate receptor was modulated by the peptide fragments of Homer (23). The NH$_2$-terminal region of Homer has homology to EVH [enabled/vasodilator-stimulated phosphoprotein (VASP) homology] family proteins, which have been postulated to interact with cytoskeletal components. Although this mechanism is proposed in the neuronal cells, it is possible that a similar mechanism also exists in the parietal cell.

Judging from the distribution of the IP$_3$ receptor within rabbit gastric gland, type 1 IP$_3$ receptor might have a role in pepsinogen secretion rather than acid secretion, at least in this species. It was observed that the type 1 IP$_3$ receptor knockout mouse showed a normal acid secretory response to carbachol (Yamada M, Horie S, Watanabe K, and Mikoshiba T, unpublished observation), possibly because the type 3 IP$_3$ receptor surrogated the specific physiological role, if any, of the type 1 IP$_3$ receptor. It was also suggested that type 1 and 3 may form a heterotetrameric structure (11) in the parietal cell. The possible typespecific function of IP$_3$ receptor is thus left for future study.

In conclusion, cholinergic stimulation in the parietal cell works synergistically with the cAMP pathway and is mediated by the release of intracellular Ca$^{2+}$ from the store, physically and functionally connected to the muscarinic receptor. The coupling between type 3 IP$_3$ receptor and the store requires a cytoskeletal connection consisting of F-actin.

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